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In vivo Adenovirus-mediated Prodrug Gene Therapy for Carcinoembryonic Antigen-producing Pancreatic Cancer

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In gene therapy for malignancy, the herpes simplex virus thymidine kinase (HSVtk)-ganciclovir (GCV) system has been widely used. For pancreatic cancer targeting, we estimated the therapeutic efficacy of gene transduction by an adenovirus-carrying *HSVtk* gene under the control of a carcinoembryonic antigen (CEA) promoter (AdCEAtk) followed by systemic administration of GCV. Four cell lines, CEA-producing Su.86.86, BxPC-3 (pancreatic cancer cells), MKN45 (gastric cancer cells) and CEA-nonproducing HeLa, were used for analysis of GCV sensitivity induced by adenoviral gene transduction. To evaluate the therapeutic efficacy of AdCEAtk and GCV administration in human CEA-positive pancreatic cancer *in vivo*, a subcutaneously implanted tumor-bearing nude mouse model was used. When the *HSVtk* gene was transduced with a ubiquitous promoter into these cells, increase of the GCV sensitivity was independent of CEA-production. In contrast, when the cells were transduced with a CEA promoter, the cell-killing effect of GCV was increased in only CEA-producing cells. For *in vivo* analysis, AdCEAtk was delivered into subcutaneously established tumors of Su.86.86 cells. Immunohistochemical staining of the tumor showed that HSVtk protein was expressed only in tumor cells, and tumor growth was markedly suppressed by administration of GCV. These results suggest that the adenovirus-mediated transfer of *HSVtk* gene with CEA promoter specifically increases the GCV sensitivity of CEA-producing pancreatic cancer cells *in vitro* and *in vivo*. This strategy may provide a useful tool for treating pancreatic cancer, especially CEA-producing tumor cells.

Key words: Gene therapy — Pancreatic carcinoma — Adenovirus — Carcinoembryonic antigen — Herpes simplex virus thymidine kinase

Pancreatic cancer is one of the most difficult cancers to treat today; it has a poor prognosis with a 5-year survival of less than 3% after diagnosis.[1)] The reasons for the poor prognosis include: (a) difficulty in early-stage diagnosis due to lack of specific early symptoms and the anatomical location of the pancreas; (b) tendency of the tumor rapidly to invade surrounding organs; (c) frequent occurrence of metastasis from a small primary tumor of less than 2 cm in diameter; and (d) poor response to existing chemotherapy or radiotherapy.[1)] Pancreatic cancer ranks fifth as a cause of cancer-related mortality in Japan, as well as in the United States.[2, 3)] In Japan, the death rate for pancreatic cancer has gradually increased from 2.1/100,000 in 1960 to 12.1/100,000 in 1994.[3)] Thus, the development of a new treatment modality for pancreatic cancer is urgently required.

Gene therapy is one novel approach to cancer treatment.[4)] The herpes simplex virus thymidine kinase (HSVtk)-ganciclovir (GCV) system[5)] is an established one which has often been used as the basis for a "prodrug" strategy. However, its clinical efficacy may depend on the properties of the gene delivery systems. Adenovirus-mediated gene transfer is a promising delivery system *in vivo* with its high titer and high transduction rate, but it transduces therapeutic genes non-specifically into target and non-target cells. One strategy to circumvent this limitation is to use a tumor-tissue specific/selective promoter or enhancer to identify target cells.[6, 7)]

Tissue-specific gene expression may be achieved by using the promoter elements of tumor marker genes which are mainly transcribed by tumors. Carcinoembryonic antigen (CEA) is an oncofetal protein which is often expressed at high levels in gastrointestinal malignancies including colon, stomach and pancreas, and its transcriptional regulation has been studied mainly in colon cancer cell lines.[8)] However, CEA is also important in pancreatic cancer because it is known that serum levels of CEA are greater than 2.5 ng/ml in 79–92% of patients with proven

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disease.[9] We have already developed an adenovirus-containing *HSVtk* gene under the control of the CEA promoter (AdCEAtk) and reported that AdCEAtk with GCV treatment specifically suppresses CEA-producing gastric cancer *in vitro* and *in vivo*.[10, 11]

In the present study, we evaluated the ability of the adenoviral vector carrying the CEA promoter and *HSVtk* gene to confer GCV sensitivity upon pancreatic cancer cells *in vitro* and *in vivo*. Our results suggest that an adenoviral vector system containing CEA promoter is capable of directing efficient and selective expression of heterologous genes in CEA-producing pancreatic cancers.

MATERIALS AND METHODS

Cell cultures Human pancreatic cancer cell line Su.86.86, BxPC-3, gastric cancer cell line MKN45, uterine cervical cancer cell line HeLa and human embryonal kidney cell line 293 were obtained from American Type Culture Collection (Rockville, MD). Su.86.86, BxPC-3 and MKN45 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin-25 μg/ml streptomycin. HeLa and 293 cells were grown in Dulbecco's modified Eagle's medium with penicillin-streptomycin containing 10% and 5% FBS, respectively.

***In vitro* CEA production by the cells** Su.86.86, BxPC-3, MKN45 and HeLa cells were cultured in 10 cm culture dishes (Iwaki Glass, Tokyo) at 37°C in a 95% air/5% CO_2 humidified atmosphere. Cells were washed and suspended in phosphate-buffered saline and homogenized with a sonicator at 130 W for 2 min. The CEA content in the cell lysate was measured by radioimmunoassay (RIA beads kit, Special Reference Laboratory, Tokyo), with which the minimal detectable level of CEA was 0.5 ng/ml. Protein concentration of the lysate was assayed using Micro BCA Protein Assay Reagent (Pierce, Rockford, IL). Each value (ng/mg protein) represents the mean±SD.

Recombinant adenovirus preparation Three types of recombinant replication-defective adenovirus lacking the viral E1 and E3 regions were used in this study. We used the −424 to −2 fragment of the 5′-flanking sequence of the *CEA* gene as the CEA promoter.[8] AdCEAtk is the adenovirus which contains the promoter region of *CEA* gene and *HSVtk* gene.[10] AdCEAlacZ has the *Escherichia coli* β-galactosidase gene driven by the same CEA promoter region as AdCEAtk. AdPGKtk has the *HSVtk* gene driven by the phosphoglycerokinase (PGK) promoter.[10]

To prepare recombinant adenoviruses, the expression cosmid and adenovirus DNA terminal protein complex were co-transfected into the 293 cells by calcium phosphate precipitation. Recombinant adenovirus was isolated from a single plaque, then expanded in the 293 cells, and the viral solution was stored at −80°C. The virus titer was determined by plaque assay on the 293 cells as described.[12, 13] None of the virus stocks used in the experiments contained detectable replication-competent viruses as evaluated by polymerase chain reaction (PCR) assay, using two pairs of primers in the same reaction to detect adenoviral E1A DNA with co-amplification of E2B DNA as an internal control.[14]

***In vitro* GCV sensitivity of the cells infected with AdCEAtk or AdPGKtk** Su.86.86, BxPC-3, MKN45 and HeLa cells were seeded in 96-well plates (Iwaki Glass) at a cell density of 6×10^3 cells in 100 μl of medium and were infected with AdCEAtk or AdPGKtk at a multiplicity of infection (moi) ranging from 0 to 100. After incubation for 24 h, the medium containing virus was removed and the cells were washed and incubated with medium containing various concentrations of GCV (0 to 100 μM) for 6 days. Viability of the cells was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, as described previously.[15] Cell proliferation was proportional to the absorbance at the test wavelength (570 nm) from which that at the reference wavelength (620 nm) was subtracted. The 50% growth-inhibitory concentration (IC_{50}) of GCV was calculated using a curve-fitting parameter, and the results are represented as mean±SD from three independent experiments.

Animal models Subcutaneous tumors were established by injecting 1×10^6 Su.86.86 cells into the flanks of male athymic BALB/AJcl-nu mice at 6 to 8 weeks of age (Clea Japan, Inc., Tokyo). In brief, the cells were suspended in 70 μl of normal saline and injected subcutaneously with a 27-gauge needle. Tumor size was measured with calipers at three-day intervals. Animal experiments were performed in accordance with institutional guidelines and approved by the University Committee on the Use and Care of Animals.

Immunohistochemistry of CEA or HSVtk protein in subcutaneous tumors: Twenty-four hours after subcutaneous injection of tumor cells, 50 μl of AdCEAtk or AdCEAlacZ (2.6×10^8 pfu) was injected intratumorally via a 27-gauge needle. At ten days after injection of adenoviruses, tumor masses were removed for immunohistochemical staining of CEA or HSVtk protein using a biotin-streptoavidin complex method. Tumors were fixed in 4% paraformaldehyde for 24 h at 4°C, embedded in paraffin and serially sectioned at 6 μm. The sections were sequentially reacted with (1) 5% normal goat serum, (2) polyclonal rabbit anti-CEA antibody at 1/5000 dilution (Dako Corp., Carpenteria, CA) or polyclonal rabbit anti-HSVtk antibody at 1/100 dilution (courtesy of W. C. Summers, Yale University, New Haven, CT) (optimal dilutions) and (3) biotinylated goat anti-rabbit immunoglobulin G antibody (Nichirei Co., Tokyo) at 1/500 dilution. The bound primary antibody was detected with the Histofine SAB-PO kit (Nichirei Co.) according to the manufacturer's

instructions. Normal rabbit serum was used as a negative control. One of the sections was also stained with hematoxylin and eosin for histological examination.

In vivo evaluation of GCV sensitivity by tumor regression: To evaluate the ability of AdCEAtk with systemic GCV administration to suppress tumor growth *in vivo*, seven groups of tumors were compared; (1) AdCEAtk (2.6×10^8 pfu) treatment followed by GCV (n=14), or (2) AdCEAtk treatment without GCV (n=12), (3) treatment with medium without adenovirus followed by GCV (n=9), or (4) treatment with medium without adenovirus without GCV (n=8), (5) AdCEAlacZ (2.6×10^8 pfu) treatment followed by GCV (n=6), (6) AdPGKtk (2.6×10^8 pfu) treatment followed by GCV (n=3) and (7) AdPGKtk treatment without GCV (n=3). Twenty-four hours after subcutaneous injection of tumor cells, virus or medium without virus was delivered intratumorally in a volume of 50 μl via a 1 ml syringe with a 27-gauge needle. After 24 h (day 2), the animals received once daily intraperitoneal injections of GCV every other day until day 8 (50 mg/kg body weight intraperitoneally). Tumor volume was calculated by using the formula:

$$\text{volume}=(\text{length (mm)}\times\text{width (mm)}^2)/2.$$

Statistical analysis Results were expressed as the mean±SD. Statistical analysis was performed by the use of Scheffe's F test and StatView J-4.51.2 software (Abacus Concepts Inc., Berkeley, CA). A P value <0.05 was taken as the criterion statistical significance.

RESULTS

CEA production by four cancer cell lines Su.86.86, BxPC-3 and MKN45 cells produced CEA (Su.86.86 2.94±0.18, BxPC-3 111.2±7.2 and MKN45 2295±267 ng/mg protein). In contrast, CEA production of HeLa cells was below the detection threshold.

CEA-production-dependent GCV sensitivity *in vitro* To test the ability of CEA promoter-induced *HSVtk* gene expression to confer sensitivity of GCV, cell lines were infected with AdCEAtk or AdPGKtk at moi ranging from 0 to 100 and then exposed to GCV at various concentrations for 6 days. Fig. 1 shows the IC_{50} of each cell line at moi 30. When the four cell lines were infected with AdPGKtk, the IC_{50} values were below 2 μM. When the cells were infected with AdCEAtk, the IC_{50} values of Su.86.86, BxPC-3 and MKN45 cells were 14.7, 0.88 and 1.62 μM, respectively, while that of HeLa cells was over 1000 μM (P<0.01). These data suggest that AdCEAtk induces GCV sensitivity only in CEA-producing cells, although AdPGKtk induces GCV sensitivity in both CEA-producing and -nonproducing cells.

***In vivo* HSVtk gene expression in CEA-producing tumor** To evaluate the effectiveness of adenovirus-mediated gene transfer to CEA-producing pancreatic cancer *in vivo*, tumor foci were established by injecting 1×10^6 Su.86.86 cells subcutaneously in the flanks of athymic mice, followed by intratumoral injection of AdCEAtk or AdCEAlacZ (2.6×10^8 pfu) 24 h after tumor establishment. Ten days after injection of adenoviruses, subcutaneous tumors were removed and immunohistochemical staining of tumor tissue specimens was performed. As shown in Fig. 2, almost all Su.86.86 cells in subcutaneous tumors exhibited positive staining in the cytoplasm with anti-CEA antibody (B, D). With anti-HSVtk antibody, HSVtk staining was positive only in CEA-positive cells of AdCEAtk-injected tumors (A). CEA-positive cells of AdCEAlacZ-injected tumors did not reveal any positive HSVtk staining (C). These results suggest that the *HSVtk* gene expression is specifically seen in CEA-producing cancer cells when mediated by AdCEAtk.

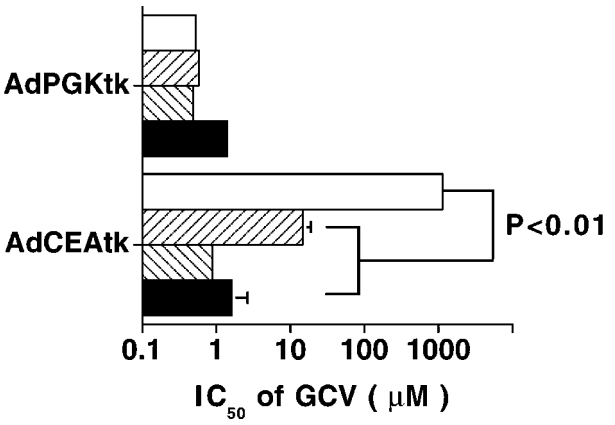

Fig. 1. The concentration of GCV yielding 50% inhibition of cell growth (IC_{50}) at moi 30. Su.86.86 (▧), BxPC-3 (▨), MKN45 (■) and HeLa (□) cells (6×10^3) were infected with AdPGKtk or AdCEAtk at moi ranging from 0 to 100. After 24 h, cells were incubated in medium containing various concentration of GCV (0 to 100 μM) for 6 days, and the viability of cells was assessed by MTT assay. The IC_{50} values of GCV are represented as mean±SD from three independent experiments.

Growth suppression of tumors infected with AdCEAtk followed by GCV administration In order to evaluate the therapeutic efficacy of AdCEAtk treatment and GCV administration in human CEA-positive pancreatic cancer *in vivo*, a subcutaneously implanted tumor-bearing nude mouse model was used. The delivery of AdCEAtk into tumors followed by GCV administration resulted in the suppression of tumor growth compared to the control groups (Fig. 3). Tumors not treated with adenovirus and GCV grew the largest, while those treated with either adenovirus or GCV showed a slightly decreased growth curve at day 19. However, the decrease was not statisti-

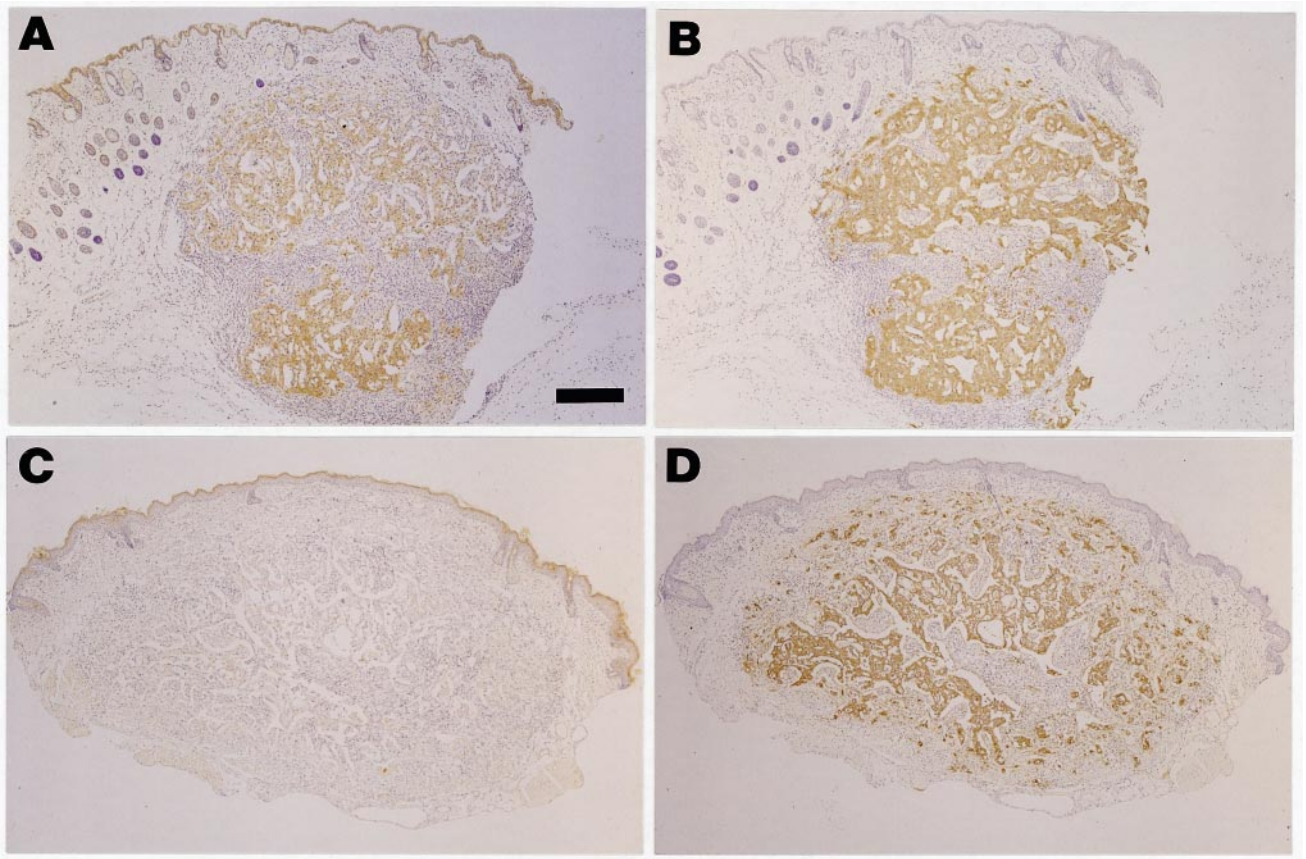

Fig. 2. *In vivo* adenovirus-mediated HSVtk protein expression in CEA-positive Su.86.86 cells of AdCEAtk-injected tumor. Subcutaneous tumors were established by injecting 1×10^6 Su.86.86 cells and 2.6×10^8 pfu of AdCEAtk or AdCEAlacZ was injected intratumorally 24 h after tumor establishment. Ten days after injection of the adenovirus, tumor masses (A, B, AdCEAtk-injected tumor; C, D, AdCEAlacZ-injected tumor) were removed for immunohistochemical staining of HSVtk (A, C) and CEA protein (B, D). Bar indicates 300 μm.

cally significant (P=0.117). In contrast, tumors treated with both AdCEAtk and GCV showed an 84.6% reduction in tumor volume compared to control groups (all controls averaged) at day 10 and a 94.1% reduction at day 19 (P<0.05).

DISCUSSION

Pancreatic cancer is a malignancy with an extremely poor prognosis. Since treatment with chemotherapeutic agents or irradiation has shown limited success, an effective strategy is still needed. Recently there have been many reports on *in vitro* and *in vivo* tumor gene therapy using replication-deficient recombinant adenoviruses. Adenoviral gene transfer is characterized by high titer and high efficiency of gene transduction, and is therefore considered to be a promising system for *in vivo* gene therapy. However, adenovirus-mediated gene transduction has some limitations; one is the non-specific delivery of therapeutic genes to cells. To circumvent this problem, the use of a tumor-specific promoter is one approach for avoiding normal cell damage. Some reports have stated that target cell-specific suicide gene expression can be achieved by linking the enzyme gene to transcriptional control elements selective for the tumors.[6, 7, 16] Further, adenovirus vectors containing a tumor-marker promoter upstream to a suicide gene have recently been used for tumor-specific gene therapy *in vivo* by several groups, including ours.[10, 11, 17]

In this study, we constructed an adenovirus vector containing *HSVtk* gene for prodrug therapy under the control of the CEA promoter (AdCEAtk) to achieve tumor specificity. *In vitro*, the IC_{50} of GCV in CEA-nonproducing HeLa cells at moi 30 was over 1000 μM. In contrast, that of GCV in CEA-producing Su.86.86, BxPC-3 and MKN45 cells at the same moi was below 15 μM, which is

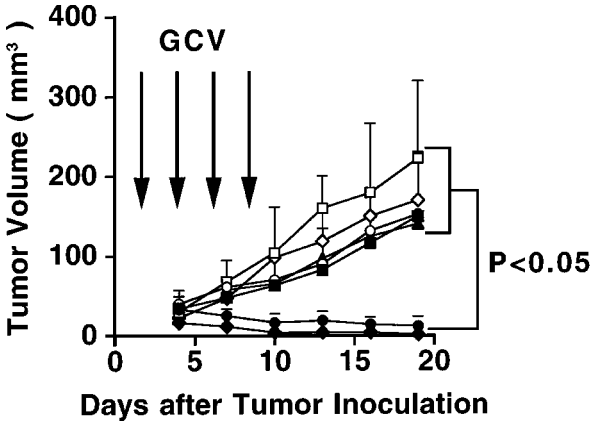

Fig. 3. The delivery of AdCEAtk into tumors followed by GCV administration resulted in suppression of tumor growth. Tumors were treated with 2.6×10^8 pfu of AdCEAtk followed by GCV (●) or no GCV (○), with medium alone followed by GCV (■) or no GCV (□), with 2.6×10^8 pfu of AdCEAlacZ followed by GCV (▲) and with 2.6×10^8 pfu of AdPGKtk followed by GCV (◆) or no GCV (◇). After 24 h, the animals received intraperitoneal injections of GCV (total 200 mg/kg body weight). Tumor volume was calculated by using the formula: volume=(length (mm)×width $(mm)^2$)/2.

almost within the range of clinical administration of GCV, free from toxic effects.[18] In addition, after direct injection of AdCEAtk into subcutaneous tumors *in vivo*, expression of thymidine kinase protein was observed only in CEA-producing tumor cells by immunohistochemical staining. From these results, it seems reasonable to suppose that the *HSVtk* gene with the CEA promoter was expressed only in CEA-producing cells. Tumors treated with AdCEAtk showed marked growth suppression by GCV. Though the growth of tumors treated with either adenovirus or GCV also seemed slightly suppressed, this was not statistically significant.

There have heen some reports on *in vivo* gene therapy for pancreatic cancer[19–21] but none has dealt with adenovirus-mediated suicide gene transfer using a tumor-specific promoter as therapy for pancreatic cancer. The fact that over 80% of proven pancreatic cancer patients show an increased level of serum CEA indicates that the CEA promoter is operating in their cancer cells and our strategy may be effective in clinical application. Further, the suicide gene system shows a "bystander effect," in which a considerable number of GCV-resistant cells adjacent to infected cells are killed by GCV administration.[22] This effect makes it unnecessary to achieve transfer of genes into all tumor cells *in vivo*. The Su.86.86 cell survival after administration of 40 μM GCV was reduced to 40 to 50% when infected cells accounted for only 10% of the total cells (data not shown).

The effectiveness of tissue-specific gene expression may be improved by the application of recent findings on transcriptional regulation of the *CEA* gene.[23] It appears that two upstream regions of CEA, −13.6 to −10.7 kb and −6.1 to −4.0 kb, lead to a high-level, selective expression and a "quadrupled CEA promoter" having four repeats of −89 to −40 bp, shows a two- to four-fold higher expression than the SV40 enhancer/promoter in CEA-producing cells. We may be able to achieve more efficient and specific expression of the transduced gene by using an adenovirus vector containing these CEA promoter regions.

In conclusion, our study has indicated that recombinant adenovirus-mediated transfer of the *HSVtk* gene under the control of CEA promoter, followed by GCV administration can efficiently inhibit the growth of CEA-producing pancreatic cancer. We are in the process of performing further studies to apply this strategy to the treatment of a clinically more relevant model.

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