[CANCER RESEARCH 55, 5283–5287, November 15, 1995]

Adenovirus-mediated Gene Therapy of Hepatocellular Carcinoma Using Cancer-specific Gene Expression

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ABSTRACT

Most patients with hepatocellular carcinoma have an elevated α-fetoprotein (AFP) level. This high level of AFP expression is transcriptionally controlled by the 5′-flanking sequence of the *AFP* gene. Using the 5′-flanking sequence as a promoter for the herpes simplex virus thymidine kinase (*HSV-TK*) gene in an adenoviral vector (Av1AFPTK1), the therapeutic efficacy of adenovirus-mediated *HSV-TK* gene transduction, followed by ganciclovir (GCV) administration, was studied in tumors in athymic nude mice. Av1AFPTK1 transduction of two cell lines demonstrated *HSV-TK* enzyme activity only in the AFP-producing cells (HuH7) and not in the AFP nonproducing cells (SK-Hep-1). As expected, only transduced HuH7 cells were killed by GCV treatment. Transduction by an adenoviral vector harboring a Rous sarcoma virus promoter and *HSV-TK* gene (Av1TK1) showed enzymatic activity and GCV killing in both cell lines. All HuH7 tumors that were transduced with either Av1AFPTK1 or Av1TK1 completely regressed after GCV treatment. On the other hand, there was complete regression of SK-Hep-1 tumors only when treated with Av1TK1 and GCV and not when treated with Av1AFPTK1 and GCV. Thus, cell-specific killing was achieved by adenoviral vector containing AFP promoter for the *HSV-TK* gene and GCV treatment.

INTRODUCTION

Hepatocellular carcinoma is one of the most common cancers in the world. Only a minority of patients are cured by removal of the tumor either by resection or transplantation (1–3). For the majority of patients, the current treatments remain unsatisfactory, and the prognosis is poor. One characteristic of hepatocellular carcinoma is that most of patients have an elevated concentration of serum AFP[2] concentration (4). The AFP concentration in the serum generally tends to stabilize or gradually increase with progression of disease, and high levels of AFP are frequently found in patients with advanced hepatocellular carcinoma. The serum AFP levels in the patients appear to be regulated by AFP expression in hepatocellular carcinoma but not in surrounding normal liver (5, 6).

Retrovirus-mediated transfer of the *HSV-TK* gene has been used to confer cytotoxic sensitivity to a nucleoside analogue, GCV, in a variety of tumor cells *in vitro* and *in vivo* (7–11). *HSV-TK* converts GCV into a phosphorylated compound that acts as a chain terminator in DNA synthesis, killing *HSV-TK*-expressing cells (12). However, this vector system is limited by relatively low viral titer and low target cell transduction frequency.

Recombinant adenoviruses can be produced in high titers and efficiently infect a variety of cells, allowing high transduction frequency in a solid tumor (13–17). Therefore, the adenoviral vector encoding the *TK* gene has been used in gene therapy for the treatment of solid tumors in the past few years. One of the limits of adenoviral vectors is that they can infect nontumorous cells as well as tumor cells. This would potentially cause toxicity in nontumorous tissues. However, if the *HSV-TK* expression can be limited to tumor cells by utilizing a tumor-specific promoter, this problem might be overcome. To attain specific killing of hepatocellular carcinoma by adenovirus-mediated gene therapy, transcriptional targeting was achieved by the use of the 5′-flanking sequences of the *AFP* gene to restrict *HSV-TK* gene expression to hepatocellular carcinoma only. The efficacy of the adenoviral vector, along with a control adenoviral vector that expresses *HSV-TK* from ubiquitously utilized RSV promoter, was evaluated using AFP-producing and AFP-nonproducing hepatocellular carcinoma transplanted in athymic nude mice.

MATERIALS AND METHODS

Cell Lines. Four human hepatoma cell lines [Hep3B (18), HepG2 (18), SK-Hep-1 (19), and HuH7 (20)] were studied, as well as human cervical cancer cell line HeLa. SK-Hep-1 and HeLa cells do not express AFP, whereas HuH7 cells do express AFP (21–23). The cell lines were maintained in culture in DMEM (BioWhittaker, Walkersville, MD) with 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT). Cells (293) were maintained in improved MEM (BioWhittaker) containing 10% fetal bovine serum.

Recombinant Adenovirus. Recombinant, replication-deficient adenoviral vectors were constructed by the homologous recombination method using pAvS6, an adenoviral vector construction "shuttle" plasmid and *Cla*I fragment derived from Ad-dl327 as described (24, 25). Av1TK1, an E1-deleted (1.18–9.2 map units), E3-deleted (78.5–84.7 map units) adeno type 5-based viral vector was constructed by inserting a *HSV-TK* fragment under the control of RSV promoter and the major late mRNA tripartite leader of pAvS6. Av1AFPTK1 contains the 4.9-kb *Hind*III-*Hind*III fragment of the 5′-flanking sequences of the *AFP* gene instead of the RSV promoter and tripartite leader upstream of the *HSV-TK* gene (26). Av1LacZ4 was constructed by inserting *Escherichia coli* β-galactosidase gene into pAvS6 (25). Viral stocks were propagated in 293 cells, and titers were quantified by 293 plaque assays (24).

LacZ Expression in Av1LacZ4-infected Cells. Transduction efficiency of adenoviral vectors in hepatocellular carcinoma cell lines were tested using Av1LacZ4. Exponentially growing cells were seeded onto duplicate of 12-well tissue culture dishes at a concentration of 2×10^5 cells/well. The next day, serial dilutions of different MOIs of Av1LacZ4 were added: 2×10^8 (MOI = 1000); 2×10^7 (MOI = 100); and 2×10^6 (MOI = 10) PFUs/well. The following day, the cells were fixed with 0.5% glutaraldehyde and then stained with X-gal. The number of lacZ-positive and lacZ-negative cells in three high-power fields from each well was recorded, and the percentage of the positive cells is presented as mean ± SD.

Enzymatic Assay for *HSV-TK*. Cells were seeded onto T12.5 tissue culture flasks at a concentration of 8×10^5 cells/flask. The next day, different MOIs of adenoviral vectors were added. Sixteen h after infection, the cells were harvested using a cell lifter. After washing twice with 2 ml of lysis buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 mM EDTA, and 20% glycerol, the cell pellet was suspended in 0.2 ml of lysis buffer containing 200 μg/ml of pefabloc SC (Boehringer Mannheim, Indianapolis, IN), 40 μg/ml of aprotinin (Boehringer Mannheim), and 5 μg/ml of leupeptin (Boehringer Mannheim). The cell lysate was obtained by centrifugation after 5 cycles of

Received 4/19/95; accepted 9/13/95.

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[2] The abbreviations used are: AFP, α-fetoprotein; HSV-TK, herpes simplex virus thymidine kinase; GCV, ganciclovir; RSV, Rous sarcoma virus; MOI, multiplicity of infection; PFU, plaque-forming unit; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; i.a., intra-arterially; i.t., intratumoral.

Table 1 *Adenovirus-mediated gene transfer of hepatocellular carcinoma cell lines*[a]

MOI	HuH7 (%)	SK-Hep-1 (%)	Hep3B (%)	HepG2 (%)	HeLa (%)
1	<1	<1	<1	<1	<1
10	8.9 ± 2.2	2.9 ± 0.5	3.8 ± 1.4	12.0 ± 1.8	8.2 ± 2.1
100	90.4 ± 2.9	35.0 ± 6.5	70.7 ± 5.9	77.9 ± 6.2	71.3 ± 7.7
1000	96.5 ± 2.5	99.0 ± 1.2	96.0 ± 1.8	98.5 ± 1.3	97.3 ± 2.1

[a] Four human hepatocellular carcinoma and HeLa cell lines were infected with different MOIs of Av1LacZ4 adenoviral vector. One day later, the cells were stained with X-gal, and the percentage of blue cells was determined by counting three fields.

freezing and thawing the cells. *HSV-TK* activity was determined as described previously with a slight modification (26). All experiments were carried out in duplicate, and the mean cpm is presented.

***In Vitro* GCV Sensitivity.** The sensitivity to GCV of infected cells was measured with a nonradioactive cell proliferation assay according to the manufacture's protocol (CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay; Promega, Madison, WI). The day after plating of 4×10^3 cells in triplicate wells into a 96-well plate, different MOIs of adenoviral vectors were infected. Sixteen h after infection, increasing concentrations (0, 1, 10, and 50 μg/ml) of GCV (Syntex Laboratories, Inc., Palo Alto, CA) were added. The percentage survival of cells is presented as a percentage of the absorbance found in the GCV-treated cells divided by that in the cells without GCV treatment (mean ± SD).

Hepatocellular Carcinoma Models in Nude Mice and Treatment of the Tumor. Sixty adult (7-week-old) athymic nude mice (Harlan-Sprague-Dawley, Indianapolis, IN) were given s.c. injections in the right flank with hepatocellular carcinoma cells: 30 mice were given 1×10^7 HuH7 cells, and 30 mice were given 5×10^6 SK-Hep-1 cells.

The mice given injections of each cell line were divided into 6 groups according to the treatment schedules: Av1AFPTK1 injection and GCV treatment ($n = 5$); Av1AFPTK1 injection without GCV treatment ($n = 5$); Av1TK1 injection and GCV treatment ($n = 5$); Av1TK1 injection without GCV treatment ($n = 5$); Av1LacZ4 injection with GCV treatment ($n = 5$); and injection of dialysis buffer used for adenovirus preparation with GCV treatment ($n = 5$). Five days after injection of HuH7 cells and 9 days after SK-Hep-1 cells, 1×10^9 PFUs of adenoviral vectors in a total volume of 100 μl of dialysis buffer were directly injected into the growing tumor from three directions for 2 successive days. The needle was retracted over 10 s. The following day, GCV was administered i.p. at 100 mg/kg once daily for 10 days. Size of the tumor was measured twice weekly with calipers in three dimensions. Tumor size is presented as the mean ± SE mm^3.

RESULTS

Transduction Efficiency of Av1LacZ4 Adenoviral Vector in Hepatocellular Carcinoma Cells. We determined the efficiency of adenoviral vector-mediated gene transfer to human hepatocellular carcinoma cells in 4 cell lines by transducing with Av1LacZ4. The efficiency was assessed by counting the number of blue cells after X-gal staining (Table 1). For all 5 cell lines, at a MOI of 1000, 96–100% of attached cells were transduced, and the cytopathic effect of the vector was evident. Lower MOIs of the vector resulted in proportionally reduced transduction efficiencies. The efficiency in SK-Hep-1 was lower at a MOI of 100 compared with those in the other 3 hepatocellular carcinoma cell lines.

Adenoviral Vector-mediated *HSV-TK* Expression *in Vitro*. *HSV-TK* expression *in vitro* was tested by an enzymatic assay for phosphorylated GCV after infection with recombinant adenoviral vectors Av1AFPTK1 and Av1TK1 (Table 2). Two hepatocellular carcinoma cell lines and a cervical cancer cell line were infected with different MOIs of the vectors. At MOIs of 1000 and 5000, the cells transduced with Av1AFPTK1 demonstrated a cytopathic effect by the vector. In the AFP-producing cell line HuH7, there appeared to be a relationship between the enzymatic activity and the number of Av1AFPTK1 PFU used (MOI). However, the activity in the AFP-nonproducing cell line SK-Hep-1 was significantly lower, even at a MOI of 5000. The AFP-negative nonhepatocellular carcinoma HeLa cells also demonstrated low activity of the enzyme, as expected. Higher *HSV-TK* activity was found in all three cell lines that were infected with Av1TK1, although the activity in SK-Hep-1 was low compared with that found for HuH7 and HeLa. The *HSV-TK* activity by Av1AFPTK1 transduction into HuH7 was clearly lower than that by Av1TK1 transduction. Nevertheless, tumor cell-specific expression of *HSV-TK* gene was demonstrated in hepatocellular carcinoma cells.

Adenoviral Vector-mediated *HSV-TK* Gene Transfer to Hepatocellular Carcinoma Cell Lines and GCV Sensitivity. After infection with adenoviral vectors Av1AFPTK1 or Av1TK1, cells were treated with varying doses of GCV for 4 days, and the number of viable cells was determined by a cell proliferation assay (Figs. 1, *a–f*). Cytopathic effect by the adenoviral vectors was clear when infected at high MOIs. HuH7 cells were more sensitive to the effect than were SK-Hep-1 cells. HuH7 cells infected with either Av1TK1 or Av1AFPTK1 at a MOI of 1000 were killed after 4 days in the absence of GCV treatment (Fig. 1, *a* and *c*). HuH7 cells infected with Av1AFPTK1 exhibited GCV sensitivity as low as 1 μg/ml of concentration at a MOI of 100 (Fig. 1*a*). SK-Hep-1 cells infected with Av1AFPTK1, however, did not show any GCV sensitivity, even at high concentrations of GCV at a MOI of 100, although there was a slight decrease in the percent survival at 10 and 50 μg/ml when infected at a MOI of 1000 (Fig. 1*b*). On the other hand, both cell lines infected with Av1TK1 similarly became sensitive to GCV (Fig. 1, *c* and *d*). Infection with a control adenoviral vector, Av1LacZ4, did not demonstrate GCV sensitivity in both cell lines (Fig. 1, *e* and *f*). These experiments indicate that Av1AFPTK1-mediated transfer of the *HSV-TK* gene resulted in GCV killing of only AFP-producing cells.

Antitumor Effect Mediated by Direct Injection of Adenoviral Vectors and GCV Treatment into Preestablished Tumor. Two hepatocellular carcinoma models were obtained by s.c. injection of either HuH7 or SK-Hep-1 cells into athymic nude mice. Adenoviral vectors were directly injected into the growing tumors, and the mice were treated with GCV (Fig. 2, *a* and *b*, respectively). Tumor growth in each treatment group was similar in size when the adenoviral vectors were injected into each hepatocellular carcinoma model (HuH7, 83.2 ± 3.5; SK-Hep-1; 10.9 ± 0.3 mm^3). All 10 HuH7 tumors that received either Av1AFPTK1 or Av1TK1 demonstrated complete regression after GCV treatment, whereas all 5 tumors without vector injection markedly increased their size, even after GCV treatment. It was of interest that injection of 2×10^9 PFUs of Av1LacZ4 vector inhibited tumor growth. However, the combination of *HSV-TK* expression with GCV treatment was clearly necessary to obtain complete regression of the tumor, as demonstrated in the tumors that were given injections of either Av1AFPTK1 or Av1TK1 in the absence of GCV administration.

There was no complete regression in SK-Hep-1 tumors that re-

Table 2 *HSV-TK gene expression by recombinant adenoviral vectors*[a]

Adeno virus	MOI	HuH7 (cpm)	SK-Hep-1 (cpm)	HeLa (cpm)
AV1AFP.TK1	10	24	0	
AV1AFP.TK1	100	128	0	
AV1AFP.TK1	1000	361	15	35
AV1AFP.TK1	5000	1757	43	
AV1.TK1	1	103	65	
AV1.TK1	10	849	110	
AV1.TK1	100	17472	5270	32579
AV1.LacZ4	10	0	0	

[a] AFP-producing (HuH7) and AFP-nonproducing hepatocellular carcinoma cells (SK-Hep-1) were infected with either Av1AFPTK1, Av1TK1, or Av1LacZ4. HSV-TK activity in the infected cells was determined by TK enzyme assay using ^{3}H-labeled GCV. The amount of phosphorylated GCV was measured by scintillation counting.

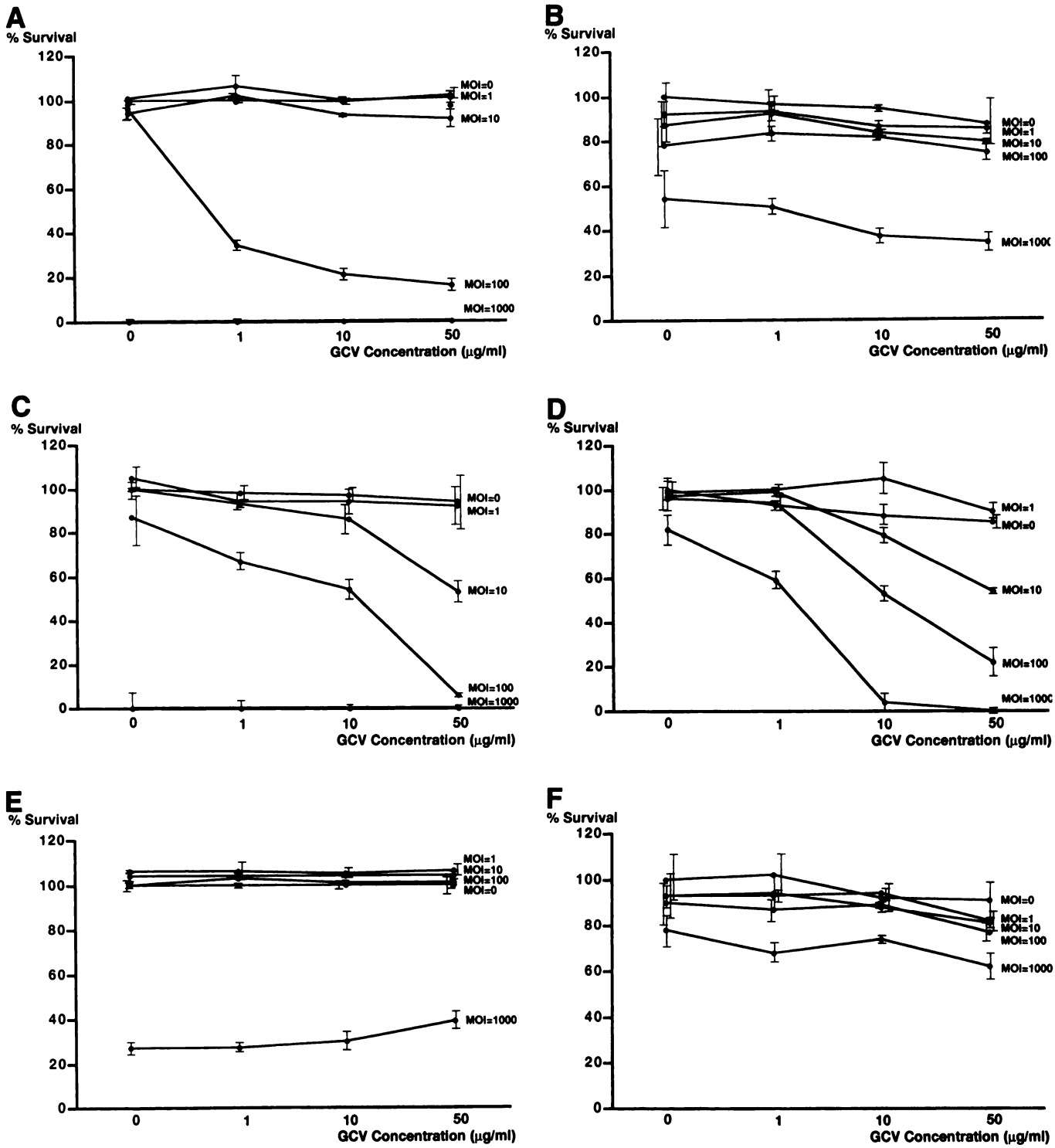

Fig. 1. *In vitro* sensitivity of adenoviral vector-transduced hepatocellular carcinoma cells to GCV. After infection of cell lines at different MOIs of adenoviral vectors, AFP-producing (HuH7) and AFP-nonproducing (SK-Hep-1) hepatocellular carcinoma cells were cultured in increasing concentrations of GCV. Four days later, viable cells were measured by a cell proliferation assay. Absorbance at 490 nm in cells treated with GCV was expressed as a percentage of the absorbance found in the cells without GCV treatment. *Points*, mean; *bars*, SD. *A*, HuH7 cells infected with Av1AFPTK1; *B*, SK-Hep-1 cells infected with Av1AFPTK1; *C*, HuH7 cells infected with Av1TK1; *D*, SK-Hep-1 cells infected with Av1TK1; *E*, HuH7 cells infected with Av1LacZ4; *F*, SK-Hep-1 cells infected with Av1LacZ4.

ceived Av1AFPTK1 and GCV treatments. However, all five SK-Hep-1 tumors that had Av1TK1 injection and GCV administration showed complete regression similar to the HuH7 tumors. Thus, the Av1TK1/GCV system could kill tumors derived from both tumor models, whereas the Av1AFPTK1/GCV system was only effective in the AFP-producing tumors.

DISCUSSION

Elevated levels of serum AFP have been observed in the majority of patients with advanced hepatocellular carcinoma, making serum AFP a reliable diagnostic marker (4). The detailed mechanism of the elevation has been studied using the 5′-flanking region of *AFP* gene,

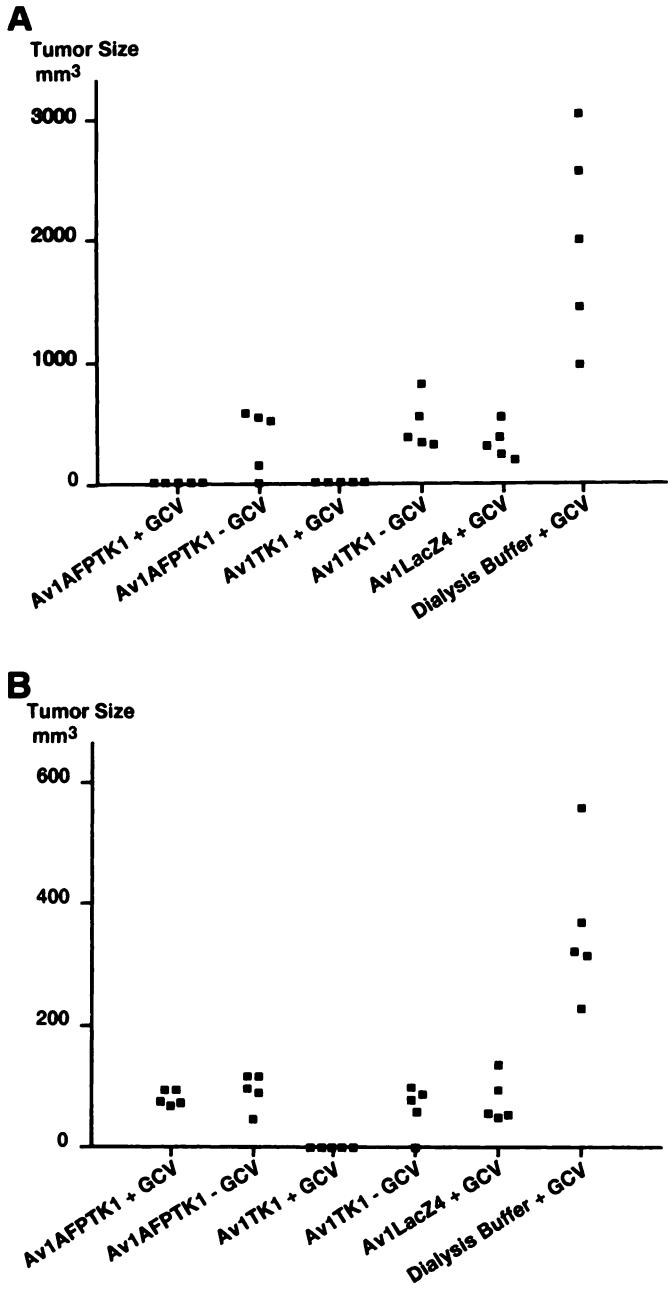

Fig. 2. Effect of injection of adenoviral vectors into preestablished hepatocellular carcinoma, followed by GCV administration. Tumor size in the 6 groups (Av1AFPTK1 and GCV treatment, Av1AFPTK1 without GCV treatment, Av1TK1 and GCV treatment, Av1TK1 without GCV treatment, Av1LacZ4 and GCV treatment, and dialysis buffer used for adenovirus preparation and GCV treatment) is shown. The growing tumors derived from AFP-producing HuH7 cells (*A*) and AFP-nonproducing SK-Hep-1 cells (*B*) received an i.t. injection of adenoviral vectors, followed by GCV administration for 10 days. Tumor size was measured 10 days after cessation of GCV treatment.

which has enhancer, silencer, and promoter sequences (22, 23). The results indicated that the 5′-flanking sequences is responsible for the control of *AFP* gene expression. This sequence has been shown to direct a specific expression of reporter genes in AFP-producing cells. In this study, we used the 4.9-kb 5′-flanking sequence to express the *HSV-TK* gene specifically in AFP-producing cells. Indeed, *TK* enzymatic assays demonstrated that the *HSV-TK* gene transferred by the Av1AFPTK1 adenoviral vector was efficiently expressed in only in the AFP-producing cell line and not in the AFP-nonproducing cell lines. The difference of *HSV-TK* expression could contribute to the different numbers of the adenovirus receptors and the different rate of internalization of the vector or transgene expression in each cell line, rather than different AFP production. However, *TK* enzyme activity in HuH7 was 10–20-fold higher than that in SK-Hep-1 or HeLa at MOI 1000 and 40-fold higher at MOI 5000, whereas transduction efficiency of Av1LacZ4 adenoviral vector was similar at MOI 1000 in these cell lines (Table 1). Therefore, the difference of *HSV-TK* gene expression would be directed by the difference of AFP production in each cell line.

Both the amount of phosphorylated GCV (cpm) and percent survival of infected cells after treatment of GCV correlated with the amount of adenoviral vector infected. The vector dose used in the animal studies has not yet been optimized. Although 2×10^9 PFUs of either vector was sufficient to cause complete regression of AFP-positive HuH7 hepatocellular carcinoma after GCV treatment when injected into 83.2 ± 3.5 mm^3 tumors, it is not yet clear whether lower viral doses will be equally efficacious. HuH7 cells may be more sensitive to cytotoxic effect of adenoviral vector injections than are SK-Hep-1 cells (Fig. 1). However, 14 of 15 tumors that received adenoviral injections (Av1AFPTK1-GCV, Av1TK1-GCV, Av1LacZ4+GCV) demonstrated significant tumor growth. Low levels (15–43 cpm) of *HSV-TK* enzyme activity in SK-Hep-1 cells were found at higher MOIs. However, the level was not high enough to kill the transduced cells *in vitro*.

The same amount of the adenoviral vector (2×10^9 PFUs) was used for the treatment of AFP-nonproducing hepatocellular carcinoma Sk-Hep-1 (10.9 ± 0.3 mm^3). Cytotoxic effect of adenoviral vectors alone was apparent in tumors that received adenoviral vector injections compared with tumors without injection, and tumor sizes among the four groups of tumors (Av1AFPTK1+GCV, Av1AFPTK1-GCV, Av1TK1-GCV, and Av1LacZ4+GCV) were similar, irrespective of which adenoviral vector was utilized (Fig. 2*b*). In contrast to HuH7 tumors, complete regression was not obtained by Av1AFPTK1 injection and GCV treatment in SK-Hep-1 tumors. Therefore, adenoviral vector-mediated AFP-producing hepatocellular carcinoma-specific gene therapy was achieved using cell-specific gene expression.

Higher expression of the *HSV-TK* gene by a RSV promoter of Av1TK1 was found in all three cell lines. The enzymatic levels of Av1TK1-transduced HuH7 cells was 35–137-fold higher than those transduced with Av1AFPTK1 at MOIs of 10 and 100, respectively. GCV killing of Av1TK1-transduced cells was clearly demonstrated *in vitro* and *in vivo*. Therefore, Av1TK1-mediated gene therapy also has the potential for the treatment of hepatocellular carcinoma. In this case, however, the toxicity of the procedure against nontumorous tissue must be considered. Different from retroviral vector system, adenoviral vectors can infect a variety of cells, including nondividing normal cells, as well as dividing cancer cells. Efficient expression of target genes has shown in normal cells, including hepatocytes using the RSV promoter in adenoviral vectors (27–29). Therefore, Av1TK1 will infect and express the *HSV-TK* gene in normal cells if the virus is delivered to nontumorous tissues. Because only dividing cells are damaged by GCV in principle (12), quiescent hepatocytes may not be damaged. However, localized delivery to the tumor such as a direct injection of Av1TK1 into hepatocellular carcinoma would be preferable to minimize GCV toxicity to normal cells.

Tumor-specific expression of the *HSV-TK* gene by Av1AFPTK1 will help to decrease potential damage of nontumorous surrounding tissue when the vectors are directly injected into tumors. In addition, other delivery routes for hepatocellular carcinoma may be possible by utilizing Av1AFPTK1. Patients in the late stage of hepatocellular carcinoma often have different sizes of multiple tumors in the liver and sometimes in extrahepatic lesions. Direct injection of adenoviral vectors into all the tumors would be difficult in many cases. However, the majority of such patients have an elevated serum AFP concentra-

tion. The serum AFP levels in the patients appear to be regulated by AFP mRNA expression in hepatocellular carcinoma but not in surrounding liver, although weaker expression of the gene has been reported in cirrhotic or surrounding dysplastic cells (5, 6, 30, 31). Further studies will be necessary; however, Av1AFPTK1 can be infused into liver via the hepatic artery. The *HSV-TK* gene will be expressed only in the multiple tumors and not in nontumorous tissues, and it will possibly kill all the tumors without a toxic effect to nontumorous tissues. Thus, adenovirus-mediated gene therapy using the *HSV-TK*/GCV system appears to have the potential for the treatment of human hepatocellular carcinoma. Nevertheless, a large amount of adenoviral vector injections into tumor clearly shows significant toxicity, and safety studies of these vectors directly injected into liver or i.a. must be performed to consider a clinical trial for hepatocellular carcinoma in the future.

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