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Polyethylenimine-Mediated Suicide Gene Transfer Induces a Therapeutic Effect for Hepatocellular Carcinoma *in Vivo* by Using an Epstein–Barr Virus-Based Plasmid Vector

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The present study aimed to establish a novel efficient nonviral strategy for suicide gene transfer in hepatocellular carcinoma (HCC) *in vivo*. We employed branched polyethylenimine (PEI) and combined it with Epstein–Barr virus (EBV)-based plasmid vectors. The HCC cells transfected with an EBV-based plasmid carrying the herpes simplex virus-1 thymidine kinase (HSV-1 Tk) gene (pSES.Tk) showed up to 30-fold higher susceptibilities to ganciclovir (GCV) than those transfected with a conventional plasmid vector carrying the HSV-1 Tk gene (pS.Tk). The therapeutic effect *in vivo* was tested by intratumoral injection of the plasmids into HuH-7 hepatomas transplanted into C.B-17 scid/scid mutant (SCID) mice and subsequent GCV administrations. Treatment with pSES.Tk, but not pS.Tk, markedly suppressed growth of hepatomas *in vivo*, resulting in a significantly prolonged survival period of the mice. These findings suggest that PEI-mediated gene transfer system can confer efficient expression of the suicide gene in HCC cells *in vivo* by using EBV-based plasmid vectors.

Key Words: **polyethylenimine; herpes simplex virus-1 thymidine kinase gene; Epstein–Barr virus-based plasmid vector.**

Hepatocellular carcinoma (HCC) is one of the most frequent malignancies in areas endemic for hepatitis virus infections. There is still no satisfactory treatment that markedly improves the overall survival rate of patients with HCCs, in spite of recent advances in HCC therapy (1, 2) including percutaneous ethanol injection (3) and radiofrequency ablation (4). Gene therapy is one developing approach for the treatment of HCC. We turned our attention to a non-viral vector system to transduce genes for the treatment of this malignancy, because they may have potential advantages in terms of safety and low immunogenecity compared with viral vectors (5–7).

Although many non-viral vector systems are known to be efficient in transfecting cultured cells *in vitro* (8–10), only few have been applied to treat HCC animal models. Polyethylenimine (PEI) is a synthetic polymer, and has been widely used for the purpose of cell transfection to both *in vitro* and *in vivo*. However, the intensity of gene expression obtained by PEI has been relatively low, as long as conventional plasmid vectors are used. We designed Epstein–Barr virus (EBV)-based plasmid vectors to improve the transfection efficiency (11–15) and recently demonstrated that highly efficient transfection can be achieved *in vitro* with EBV-based plasmid vectors even in HCC which is an EBV-negative cancer (16). So, we applied the EBV-based plasmid vectors in the HCC gene therapy of an animal model as a component of a nonviral vector system.

The EBV-based plasmid vector contains the Epstein–Barr virus nuclear antigen 1 (EBNA1) gene and the oriP element from the EBV genome. EBNA1 facilitates nuclear localization, replication and binding to the nuclear matrix of the plasmid DNA and makes this vector highly efficient (11, 17–21). Due to a lack of other EBV genes, no infectious virus particle can be produced.

Abbreviations used: HCC, hepatocellular carcinoma; EBV, Epstein–Barr virus; PEI, polyethylenimine; β-gal, β-galactosidase; HSV-1 Tk, herpes simplex virus-1 thymidine kinase; GCV, ganciclovir; EBNA1, Epstein–Barr virus nuclear antigen 1; PBS, phosphate-buffered saline; ip, intraperitoneally; sc, subcutaneously; CEA, carcinoembryonic antigen.

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In this study, we transduced the HSV-1 Tk gene (22, 23) into HCC cells using PEI combined with an EBV-based plasmid vector (24, 25), and showed high expression of the suicide gene both *in vitro* and *in vivo*. Importantly, an efficient therapeutic effect was observed *in vivo*, indicating the possible application of this non-viral vector system to the gene therapy of HCC.

MATERIALS AND METHODS

Plasmid vectors. pSES.β (Fig. 1A), pSES.Tk (Fig. 2A), pS.β (Fig. 1A), and pS.Tk (Fig. 2A) were constructed using standard molecular biology techniques (11, 12, 26). Briefly, the pSES.β and pSES.Tk are composed of the *E. coli* β-gal (pSES.β) or HSV-1 Tk (pSES.Tk) gene located downstream of the SR α promoter, the EBV oriP and EBV EBNA1 gene under the control of SR α promoter. pS.β and pS.Tk contain expression units for β-gal and HSV1-Tk genes, respectively, but lack the SR α-EBNA1 and oriP.

Cells. Human HCC cell lines, HLE and HuH-7 (Health Science Research Resources Bank, Osaka, Japan), were passaged in Dulbecco's modified Eagle medium (DMEM) (Gibco-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL), 100 units/ml of ampicillin and 100 mg/ml of streptomycin (complete medium).

Transfection by PEI. For PEI-mediated *in vitro* transfection, cells were seeded at 5×10^4 cells per well in 24-well dishes (Becton–Dickinson Laboware, Franklin Lakes, NJ) 18 h before transfection. Shortly before transfection, cells were rinsed and supplemented with fresh serum-free culture medium (1 ml). Two milligrams of plasmid DNA and various amounts of branched PEI polymer (average molecular weight 25 kDa) (Aldrich Chemical Co. Inc., Milwaukee, WI) were each diluted into 50 ml of 150 mM NaCl and vortexed. The two solutions were mixed, and the resulting mixture was vortexed. Ten minutes later, the PEI/DNA complex was added to the cells. After 3 h incubation at 37°C in 5% CO_2/95% humidified air, the medium was supplemented with 10% FBS. The cells were further cultured until they were subjected to the β-gal assay and Alamar blue assay.

β-Gal assay. Cells were scraped off dishes, washed twice with phosphate-buffered saline (PBS) and resuspended in 50 ml of Tris–HCl (pH 7.8). After 2 cycles of freezing and thawing, the lysate was centrifuged at 15,000 rpm for 5 min. The β-gal activity in the cell supernatant fraction was assayed with a β-gal assay kit (Invitrogen, San Diego, CA) according to the manufacturer's protocol. The optical density (OD) was measured at 420 nm. The activities were calculated using the following formula: β-gal units = $[(OD_{420\times380})/30]$/mg protein, where 380 is a conversion factor and 30 is the incubation time in minutes. The protein concentration of the supernatant was assessed according to the method of Bradford (27).

Alamar blue assay. The susceptibility of cells to GCV was examined with an Alamar blue assay (28, 29). Briefly, quadruplicate aliquots of cells were seeded in 96-well flat-bottom microtiter plates (FALCON, Lincoln Park, NJ) (5×10^3 cells in 200 ml of complete medium per well). Twenty-four hours later, GCV (Tanabe Co., Tokyo, Japan) was added at various concentrations ranging from 0 to 10,000 mM. After further incubation in 5% CO_2/95% humidified air at 37°C for 96 h, Alamar blue (Alamar Biosciences Inc., Sacramento, CA) was added according to the manufacturer's protocol. The cells were further cultured for 4 h, and the OD of each well was measured with a microplate reader at test and reference wave lengths of 570 and 600 nm, respectively. The percentages of viable cells were calculated according to the following formula: % viable cells = $(OD_{570} - OD_{600})$ of GCV-treated cells/$(OD_{570} - OD_{600})$ of untreated cells.

In vivo therapeutic experiments. C.B-17 scid/scid mutant (SCID) mice were purchased from CLEA Japan (Osaka, Japan). Mice were used at 6 to 10 weeks of age. Twenty four hours before tumor cell inoculation, anti-asialo-GM1 antibody (Wako, Osaka, Japan) was intra-peritoneally (ip) administered to the mice. To establish tumors, 5×10^6 HuH-7 cells were injected subcutaneously (sc) into the flank of the SCID mice. Mice were examined daily until palpable tumors developed. Each group consisted of eight animals. Mice received intratumoral injections of 50 mg of plasmid coupled with 1.5 mmol of PEI on days 1 and 2, followed by daily ip administrations of GCV (100 mg/kg body wt) from day 3 to 16. The plasmid/PEI injections were repeated on days 9 and 10. Mice in the control group were injected ip with PEI alone plus GCV administrations. The diameter of tumors was measured in two dimensions two to three times a week using callipers. The tumor volume was calculated as follows: Volume = $a^2 \times b/2$ (mm^3), where a is long diameter and b is short diameter. All of the experiments were conducted following the Guidelines for Animal Experimentation (Japanese Association for Laboratory Animal Science, 1987).

RESULTS

PEI/EBV-Based Vector Complex-Mediated Transfection Is Highly Efficient for HCC Cells in Vitro

First, we estimated the efficiency of the transfection/expression into HCC cell lines by the PEI/EBV. The optimal preparation and transfection conditions were determined for HuH-7 cells. Several different ratios of PEI to DNA were tested. At every ratio, the transfection with the EBV-based plasmid vector (pSES.β) resulted in significantly higher β-gal activity compared to that with the conventional plasmid vector (pS.β) 60 h after transfection (Fig. 1B). The highest β-gal activity was observed when 60 to 100 nmol of PEI was combined with 2 μg of pSES.β. At this optimal preparation, the PEI/pSES.β yielded 6-fold higher β-gal activity compared with the optimal preparation of PEI/pS.β. Second, we estimated the time course of β-gal expression in the HCC cell lines by the PEI/EBV. The β-gal activity was assayed following 4, 8, 14, and 21 days of *in vitro* culture. The activity was about 25-fold (HLE), and 5.4-fold (HuH-7) stronger in pSES.β-transfected cells than in pS.β-transfected cells on day 4 posttransfection (Fig. 1C). Moreover, gene expression after pSES.β transfection could be detected for as long as 3 weeks.

High Susceptibility to GCV of HCC Cells Transfected with PEI/pSES.Tk in Vitro

Next, to examine the efficiency of the EBV-based plasmid vector for suicide gene transfer, pSES.Tk (an EBV-based plasmid vector carrying the HSV1-Tk gene) or pS.Tk (a conventional plasmid vector with the HSV1-Tk gene) was transfected into HCC cells and the susceptibility to GCV of the cells was assessed. Seventy-two hours after transfection, the cells were cultured in the presence of various concentrations of

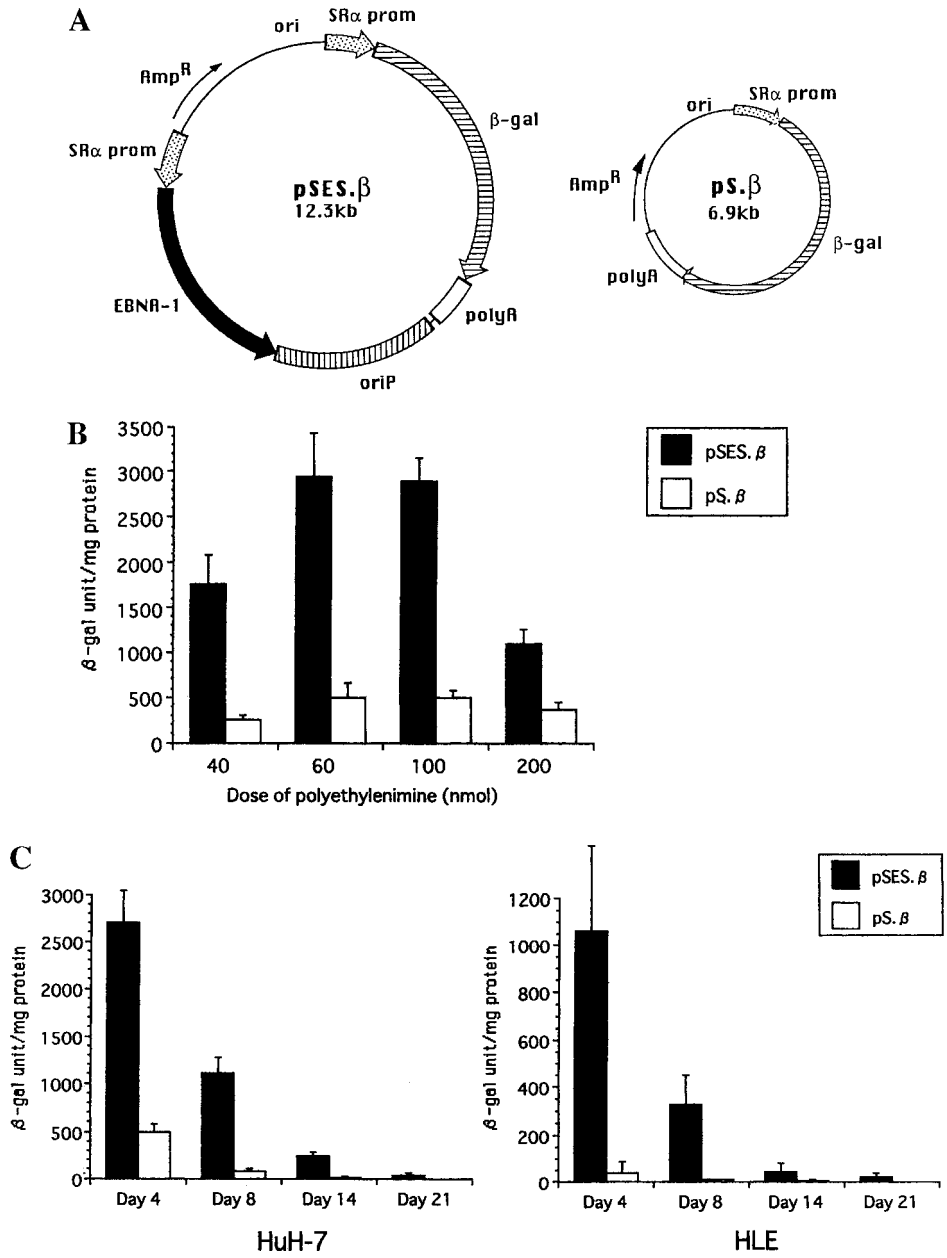

FIG. 1. (A) Schematic representation of the pSES.β and pS.β. Prom, promoter; polyA, SV40 polyA additional signal. (B) Optimal transfection conditions for HuH-7 cells by EBV-based vector/PEI *in vitro*. HuH-7 cells (5×10^4 cells) were plated in 24-well plates, and 18 h later, incubated for 2 h with 2 μg of pSES.β (closed bars) or pS.β (hatched bars) combined with the indicated amount of PEI. Sixty hours after transfection, β-gal activities were measured and normalized to 1 mg of protein. Mean ± SE of quadruplicate samples are shown. (C) Time course of β-gal expression in HCC cells transfected with PEI/EBV-based plasmid. HuH-7 and HLE cells were transfected with PEI/pSES.β (closed bars) or pS.β (open bars) under optimal conditions (2 μg of DNA and 60 nmol of PEI per 5×10^4 cells/well of 24-well plates). β-Gal activities were determined on the indicated days after transfection. Means ± SE of quadruplicate samples are shown.

GCV for 4 days, and cell viability was measured with an Alamar blue assay (Fig. 2B). The transfer of pS.Tk moderately affected the susceptibilities of the HLE and HuH-7 cells to GCV. In contrast, the transfection of pSES.Tk rendered the cells more susceptible to GCV. In both cell lines, the GCV-susceptibilities of cells transfected with PEI/pSES.Tk were 10- to 30-fold higher than those of PEI/pS.Tk-transfected cells.

Marked in Vivo Anti-HCC Effect Induced by PEI/pSES.Tk Complex Treatment

We also evaluated the ability of PEI/pSES.Tk plus systemic GCV to suppress tumor growth *in vivo*. Consistent with the *in vitro* studies, administration of PEI/pSES.Tk into tumor foci of HuH-7, along with systemic GCV administration, resulted in a marked suppression

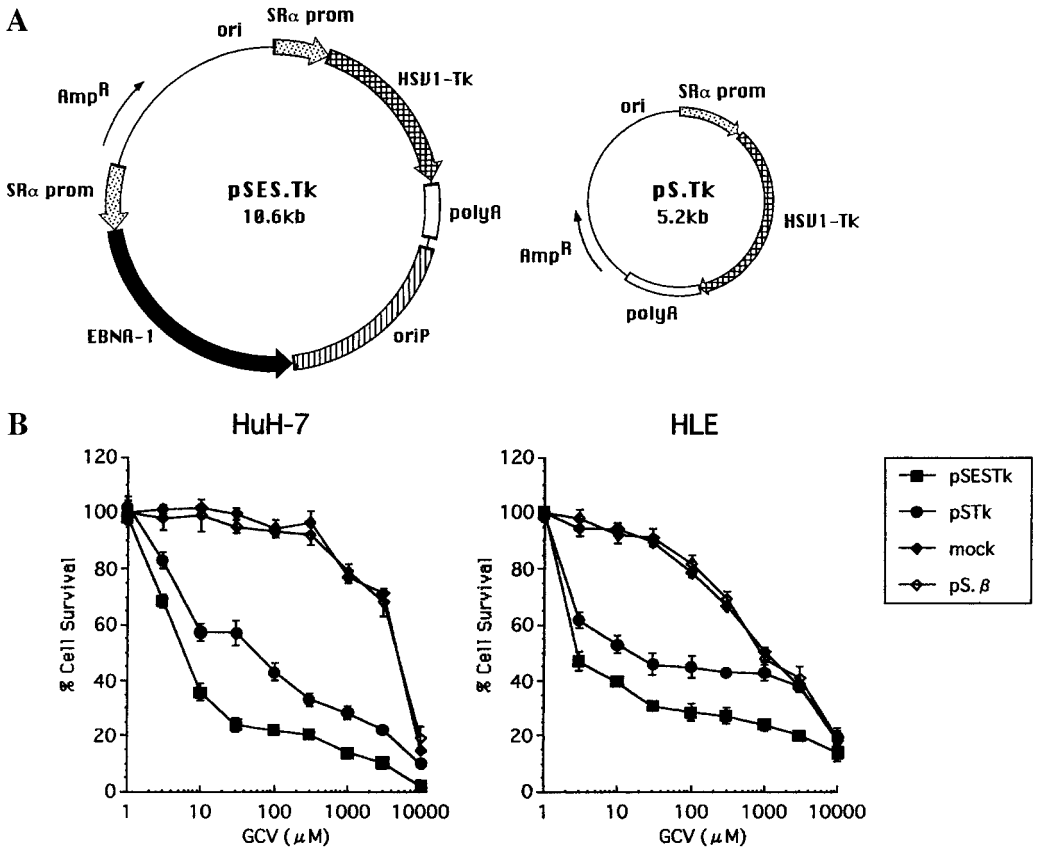

FIG. 2. (A) Schematic representation of the pSES.Tk and pS.Tk. (B) Susceptibilities to GCV of HCC cells transfected with the HSV1-Tk gene *in vitro.* HuH-7 (left) or HLE (right) cells were transfected with PEI/pSES.Tk (squares), pS.Tk (circles), or pS.β (open diamonds) as described in the legend to Fig. 1A. Cells treated with PEI alone were also prepared as a control (closed diamonds). Three days later, 5×10^3 of the cells were seeded into 96-well plates, and cultured in the presence of various concentrations of GCV for 4 days. The viabilities of cells were determined via Alamar blue assay. The points on the ordinate correspond to the relative cell viability compared with the mock-transfected cells without GCV. Each point represents the mean ± SE of quadruplicate determinations.

of tumor growth (Fig. 3A). Tumors treated with pS.Tk showed almost similar growth curves with those treated with PEI/control plasmid or PEI alone.

The PEI/pSES.Tk-treated mice survived significantly longer than those treated with PEI alone or PEI/pS.β, while PEI/pS.Tk treatment did not significantly affect survival (Fig. 3B).

Histological examination of tumors injected with PEI/pSES.Tk or PEI alone was performed. Four days after the GCV treatment, tumors were excised and stained with hematoxylin–eosin. A number of damaged tumor cells, and acidophilic glass-like cells with condensed nuclei were observed in the tumors injected with PEI/pSES.Tk (Fig. 3C). Tumors injected with PEI alone showed a monotonous pattern of irregularly shaped cells and massive tumor cell damage was not observed (Fig. 3D).

DISCUSSION

The present study demonstrated the ability of a PEI/EBV-based plasmid vector complex to efficiently transfer a suicide gene not only into human HCC cells *in vitro,* but also into tumors *in vivo* in a therapeutic animal model of HCC. As far as we know, there has been no report of PEI-mediated suicide gene therapy, and the present study is the first report showing that PEI-mediated transfection is effective in suicide gene transfer *in vivo* by using EBV-based vector. Furthermore, this is the first study showing that an EBV-based plasmid vector is successfully combined with PEI.

Recently, the effectiveness of the HSV1-Tk/GCV system in the treatment of cancer has been demonstrated in animal models with various types of cancers, and viral vector-mediated gene transfer protocols have been applied to clinical trials for the treatment of cancer (30). Adenoviral and retroviral vectors have commonly been used to deliver the HSV1-Tk gene into HCC (30–33), while there have been few reports showing that a suicide gene was effectively transferred *in vivo* with a nonviral vector system. In this study, we demonstrated that even a nonviral vector system may be applicable to *in vivo* suicide gene therapy of HCC.

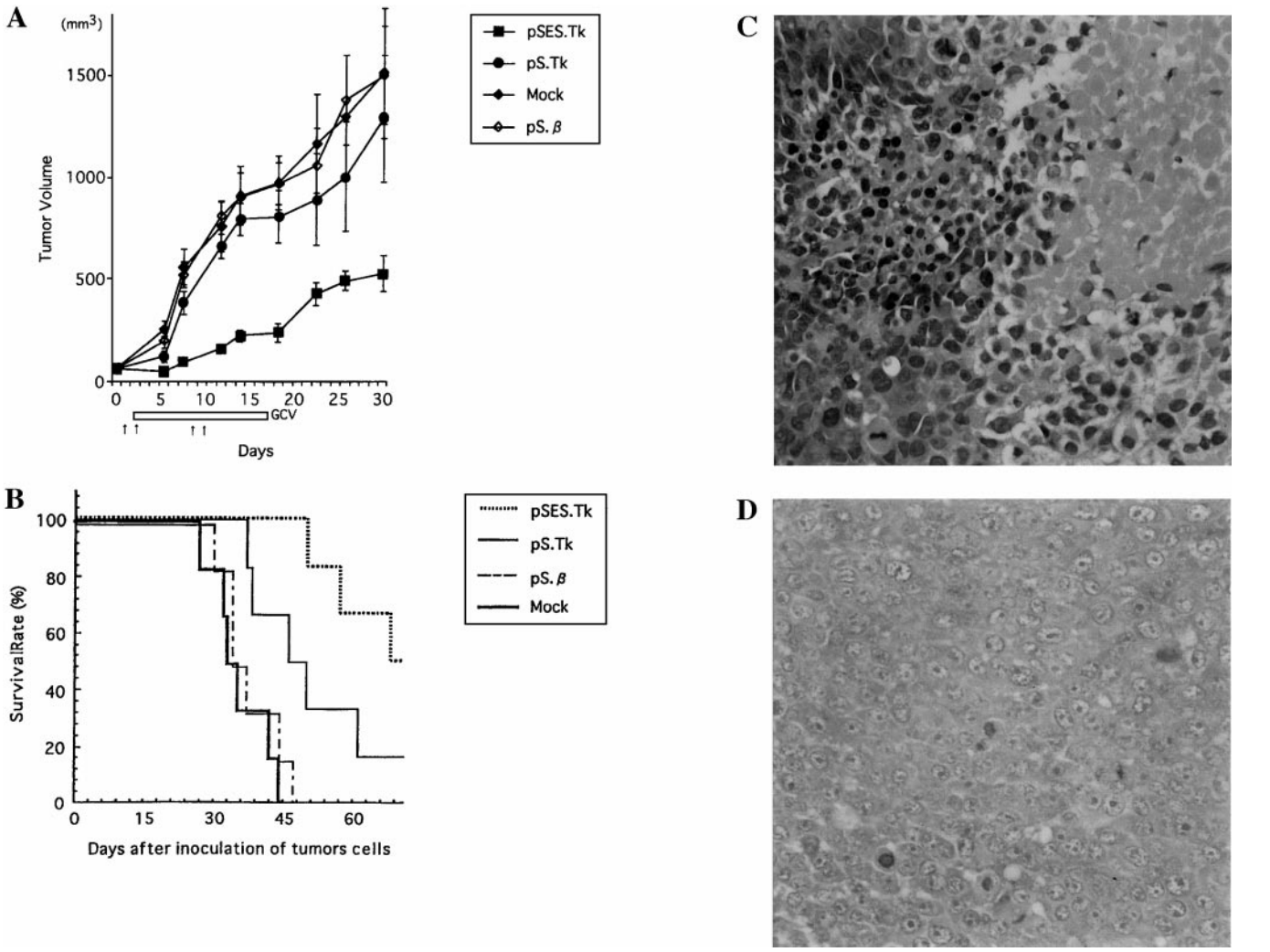

FIG. 3. *In vivo* therapeutic efficacy of PEI/pSES.Tk against HuH-7-derived sc tumors in SCID mice. (A and B) Suppression of tumor growth *in vivo* by PEI/pSES.Tk administration plus GCV. Five million HuH-7 cells were inoculated sc into the flanks of SCID mice pretreated with an anti-asialo-GM1 antibody. Established tumors of 5 mm in diameter were injected with PEI/pSES.Tk (squares), PEI/pS.Tk (circles), PEI/pS.β (open diamonds), or PEI alone (closed diamonds) (50 mg of plasmid and 1.5 mmol of PEI) on days 1 and 2 (arrows). From the second day, 100 mg/kg/day of GCV was ip administered for 15 consecutive days (open bars). The injections with PEI/plasmid and GCV treatment were repeated twice. The tumor diameter was measured on the indicated days and tumor volume was calculated. Each point represents the mean $\pm$ SE ($n = 8$ in each group) (A). Kaplan–Meyer's analysis is shown in B. The survival rate of mice injected with PEI/pSES.Tk was significantly different compared with that of PEI/pS.Tk. (C and D) Histological analysis of tumors injected with PEI/pSES.Tk complex. The mice bearing established tumors with a diameter of 5 mm were given an intratumoral injection of PEI/pSES.Tk (C) or PEI alone (D), followed by ip administrations of GCV (100 mg/kg/day) for consecutive 7 days. Two days after the termination of the treatment, tumors were excised and stained with hematoxylin–eosin (original magnification, $\times$250).

We previously demonstrated the advantages of nonviral vector systems in which EBV-based plasmid vectors are employed instead of conventional plasmid vectors (11–16). Highly efficient transfection of reporter and suicide genes was achieved with an EBV-based plasmid vectors/polyamidoamine dendrimer in HCC cells *in vitro* (16). The polyamidoamine dendrimer is an attractive gene delivery vehicle (34, 35), but its safety problems *in vivo* have not been established. So we focused on branched PEI for experimental suicide gene therapy of HCC. PEI is a synthetic macromolecule with high cationic-charge-density potential, and reportedly an efficient vector for gene transfer into cells not only *in vitro* but also *in vivo* (24, 25). No sign of acute or chronic toxicity has been documented, when introduced by oral or sc routes into rats and rabbits (36). However, the transfection/expression efficiency by PEI is relatively low, as far as conventional plasmid vectors are employed. By means of the PEI/EBV, more intensive repetitive therapy could be possible leading to more satisfactory results, then PEI/EBV can be one of non-surgical therapy for small HCC without major complication as well as ethanol injection (37) and radiofrequency ablation (38).

Although nontargeted gene transfer may be applied *in vivo* for nodular HCCs into which PEI/EBV-based vector complexes are stereotactically injected, specific targeting of HCC cells would be required for practical HCC gene therapy. One of the most promising strategies for HCC targeting is receptor-mediated gene transfer system (39, 40). For specific targeting of α-fetoprotein (AFP)-producing HCC, the AFP gene regulatory element has been effectively used in combination with retroviral or adenoviral vectors (31, 33, 41). We have recently demonstrated that EBV-based plasmid vectors equipped with the carcinoembryonic antigen (CEA) gene promoter could specifically transfect CEA-positive cholangiocellular carcinoma cells (42). We are planning to employ the AFP promoter in EBV-based vectors to drive the EBNA1 and/or HSV-1 Tk genes.

In conclusions, PEI/EBV-based vector complex is superior to PEI/conventional plasmid with regard to gene transfer efficiency, as well as therapeutic effects *in vivo*. PEI-mediated transfection of the EBV-based vector may contribute to nonviral gene therapy for HCC, and it may be applied for local control of HCC.

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