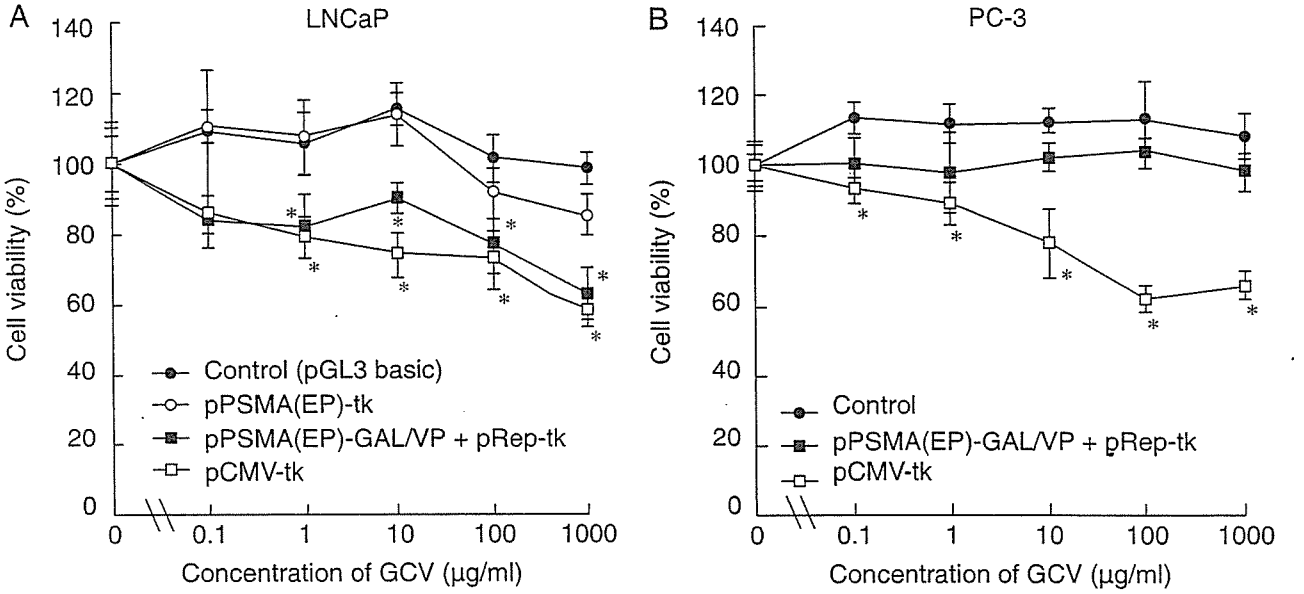

Fig. 6. *In vitro* suicide gene therapy using the two-step transcriptional amplification (TSTA) system with a prostate-specific membrane antigen (PSMA) promoter. (A) LNCaP and (B) PC-3 cells were transfected with various plasmids (0.2 μg). After 12 h incubation, the medium was changed to one containing ganciclovir (GCV). Data points indicate the mean ± SD ($n = 4$). Statistical significance of the data was evaluated using the Bonferroni/Dunn test. *$P < 0.05$, compared with control plasmid.

The metastatic prostate cancer within an individual is composed heterogeneously of clones of both androgen-dependent and androgen-independent cancer cells. At present, the androgen-dependent and androgen-independent prostate cancer cells available are LNCaP and PC-3, respectively.[36] PSMA mRNA was expressed in LNCaP cells, but not in PC-3 cells (Fig. 2B). Mk mRNA was expressed strongly in PC-3 cells, but not in LNCaP cells (Fig. 2B). These findings suggest that the PSMA promoter could be utilized with androgen-dependent prostate cancer (e.g. LNCaP cells), and the Mk promoter with androgen-independent prostate cancer (e.g. PC-3 cells) for tumor-specific gene therapy. The PSMA and Mk promoters have already been cloned and used for suicide gene therapy with the one-step transcription system.[13,37,38] However, usage of the TSTA system without the PSA promoter has not been reported in prostate cancer.

In the present study, we modified the TSTA system for use with the SV40 enhancer in reporter and effector plasmids to amplify the transcriptional activity, and tried to apply the PSMA or Mk promoter to the TSTA system. We confirmed that our cloned PSA or PSMA promoter and enhancer regions had prostate-specific transcriptional activity based on findings reported previously in which androgen-responsive LNCaP cells showed increasing PSMA promoter activity and decreasing PSA promoter activity when DHT was not added into the culture medium (Fig. 2).[28,29] Thus, the PSMA promoter system seems to offer an advantage under conditions of androgen ablation.

In PSMA promoter-related plasmids, the TSTA system with the paired plasmid, pPSMA(EP)-GAL/VP and pRep-luc, amplified the expression of the luciferase gene 640-fold and 85-fold more than one-step transcription with pPSMA(P)-luc and pPSMA(EP)-luc, respectively, in LNCaP cells. However, the paired plasmids showed weak promoter activity in PC-3 cells (Fig. 4B), whereas PSMA mRNA could not be detected by RT-PCR analysis (Fig. 2). Recently, Laidler *et al.* reported that basic fibroblast growth factor (bFGF) and estradiol (E2) induce weak expression of PSMA in PC-3 cells.[39] A growth factor such as bFGF is included in the serum for the culture medium. Therefore, in our study, the PSMA promoter activity in PC-3 cells may be induced weakly by bFGF or E2 in the culture medium and be amplified by the TSTA system. In Mk promoter-based plasmids, TSTA with the paired plasmids pMk(P)-GAL/VP and pRep-luc resulted in 5.0-fold and 2.6-fold higher luciferase activity than one-step transcription with pMk(P)-luc in PC-3 and HeLa cells (Fig. 5). The difference in the degree of amplification by the TSTA system between the PSMA and Mk promoters might be attributed to different transcriptional activity by the promoters.

In cancer gene therapy, the TSTA system with the PSA promoter used to drive the expression of expanded polyglutamine (ex-polyQ) for induction of apoptosis selectively eliminated PSA-positive LNCaP to an extent comparable with that of CMV promoter-driven ex-polyQ.[15] The TSTA system with the CEA promoter used to control the expression of Bax for induction of apoptosis can suppress *in vitro* and *in vivo* tumor growth in lung cancer and colon adenocarcinoma.[21] In the present study, we applied the TSTA system to suicide therapy with HSV-tk and demonstrated that the paired plasmids, pPSMA(EP)-GAL/VP and pRep-tk, and pMk(P)-GAL/VP and pRep-tk, enhanced cytotoxic activity in LNCaP cells, and in PC-3 and HeLa cells, respectively. In PSMA-positive LNCaP cells, the inhibitory effect was dependent on the concentration of GCV in the cells transfected with the paired plasmids, pPSMA(EP)-GAL/VP and pRep-tk (Fig. 6A). In PC-3 cells, the paired plasmids showed comparatively less promoter activity (Fig. 4B); however, the activity was apparently non-toxic as long as the level of HSV-tk expression by

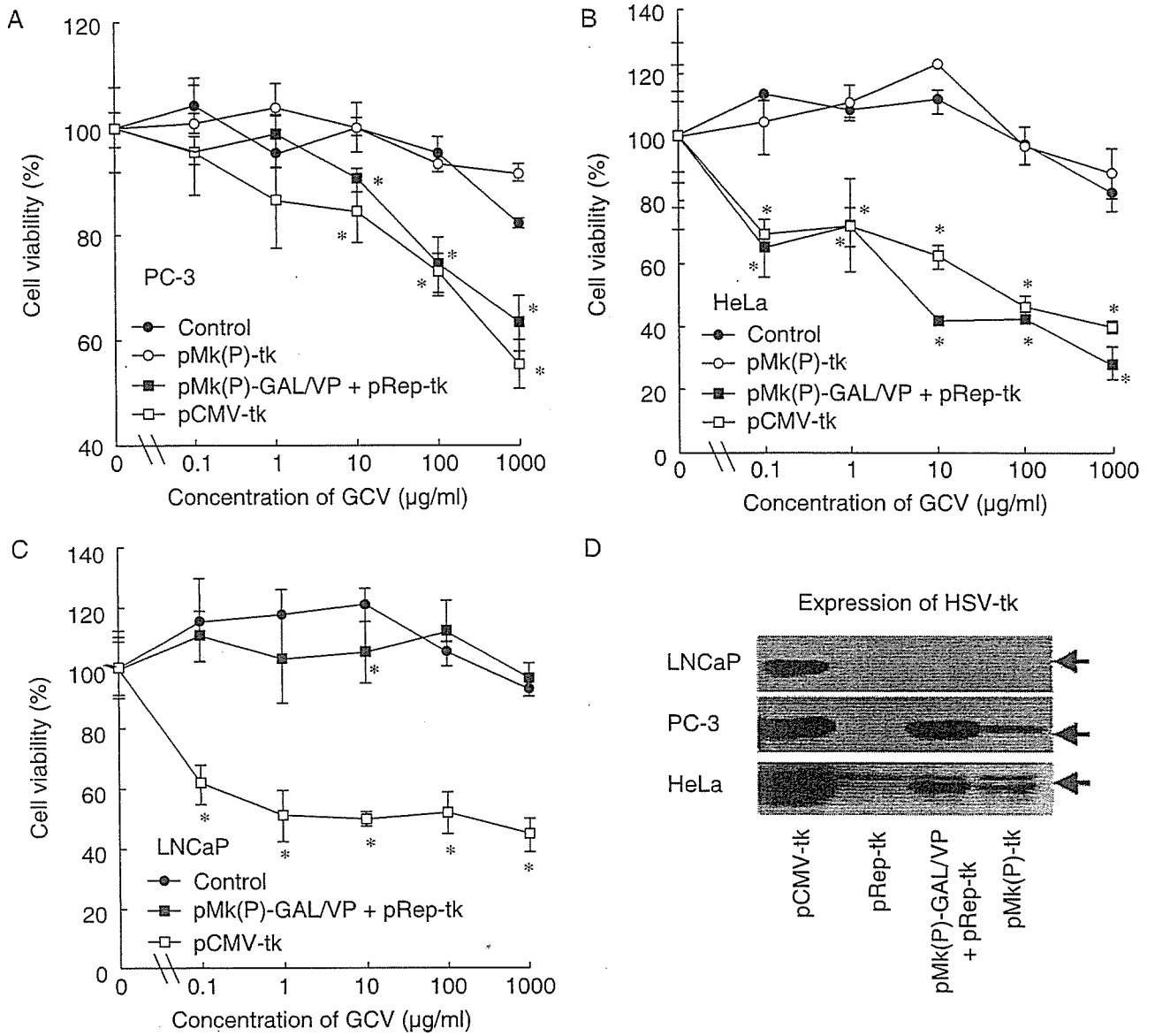

Fig. 7. (A–C) *In vitro* suicide gene therapy and (D) herpes simplex virus thymidine kinase (HSV-tk) expression using the two-step transcriptional amplification (TSTA) system with a midkine (Mk) promoter. (A) PC-3, (B) HeLa and (C) LNCaP cells were transfected with various plasmids (0.2 μg). After 12 h incubation, the medium was changed to one containing ganciclovir (GCV). Data points indicate the mean ± SD ($n = 4$). Statistical significance of the data was evaluated using the Bonferroni/Dunn test. *$P < 0.05$, compared with control plasmid. (D) Specific detection of HSV-tk protein expressed by one-step transcription and the TSTA system with the Mk promoter by western blotting.

the paired plasmid remained low (Fig. 6B). In PC-3 and HeLa cells, inhibitory effects were observed with paired plasmids, pMk(P)-GAL/VP and pRep-tk (Fig. 7A,B), but not in Mk-negative LNCaP cells (Fig. 7C). HSV-tk expression with the TSTA system with the Mk promoter was stronger than that with one-step transcription in PC-3 and HeLa cells, being equivalent to that with one-step transcription with the CMV promoter in PC-3 cells (Fig. 7D). Furthermore, in *in vivo* suicide gene therapy, the TSTA system with the Mk promoter suppressed the growth of PC-3 tumor xenografts greatly, compared with one-step transcription (Fig. 8). These characteristics can be used most powerfully in prostate cancer when combining the TSTA system with the PSMA or Mk promoter, in which strong expression is restricted to target cells, and would minimize the side-effects in non-targeted cells.

The TSTA system has been applied to gene therapy mediated by adenovirus,[20,21,40,41] lentivirus[42] and cationic liposomes.[15,16] Adenoviruses and lentiviruses can evoke non-specific inflammation, and readily induce strong antivirus immune responses. The mixing of cationic liposomes with DNA often results in large aggregated lipoplexes, which cannot be injected into blood vessels and yield very low levels of transfection efficiency *in vivo*. Recently, we developed a lipid-based nanoparticle that formed injectable-sized nanoplexes (200–300 nm).[25] When

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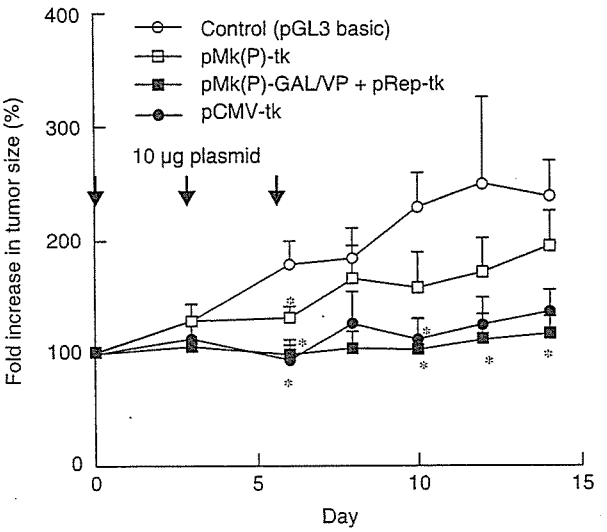

Fig. 8. *In vivo* suicide gene therapy of PC-3 tumor xenografts with ganciclovir (GCV) in mice. When the average volume of PC-3 xenograft tumors reached 150 mm^3 (day 0), mice were divided into four groups: group I, pGL3-basic (10 μg) as a control; group II, pMk(P)-tk (10 μg); group III, pMk(P)-GAL/VP (5 μg) plus pRep-tk (5 μg); group IV, pCMV-tk (10 μg). The nanoplexes of the plasmids were injected directly into the tumor three times (days 0, 3 and 6). GCV (25 mg/kg) was administered intraperitoneally at 12, 24 and 36 h after the injection of nanoplexes. The results indicate the mean volume ± SE ($n = 4$). Statistical significance of the data was evaluated using the Fisher's exact test. $^*P < 0.05$, compared with control.

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the nanoplexes were injected directly into PC-3 xenografts, they showed approximately 6.5-fold higher luciferase activity than *in vivo*-Jet PEI-Gal, a commercially available cationic polymer transfection reagent (PolyPlus-transfection; ILLKIRCH, Illkirch, France; data not shown). Therefore, in the present study, we used this nanoparticle to induce strong expression in the TSTA system *in vitro* and *in vivo*. Furthermore, we reported recently that a folate-linked nanoparticle could deliver DNA with high transfection efficiency and selectivity into human nasopharyngeal and prostate cancer cells.[22,25] The combination of this folate-linked nanoparticle with the TSTA system has great potential as a tumor-specific vector for *in vivo* cancer gene therapy.

In conclusion, we developed an ideal gene expression amplification system with the PSMA and Mk promoters. This system is a promising tool with which to create targeted gene-based therapeutic applications.

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RESEARCH ARTICLE

The role of a HSV thymidine kinase stimulating substance, scopadulciol, in improving the efficacy of cancer gene therapy

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Abstract

Background The most extensively investigated strategy of suicide gene therapy for treatment of cancer is the transfer of the herpes simplex virus thymidine kinase (HSV-TK) gene followed by administration of antiviral prodrugs such as acyclovir (ACV) and ganciclovir (GCV). The choice of the agent that can stimulate HSV-TK enzymatic activity is one of the determinants of the usefulness of this strategy. Previously, we found that a diterpenoid, scopadulciol (SDC), produced a significant increase in the active metabolite of ACV. This suggests that SDC may play a role in the HSV-TK/prodrug administration system.

Methods The anticancer effect of SDC was evaluated in HSV-TK-expressing (TK^+) cancer cells and nude mice bearing TK^+ tumors. *In vitro* and *in vivo* enzyme assays were performed using TK^+ cells and tumors. The phosphorylation of ACV monophosphate (ACV-MP) was measured in TK^- cell lysates. The pharmacokinetics of prodrugs was evaluated by calculating area-under-the-concentration-time-curve values.

Results SDC stimulated HSV-TK activity in TK^+ cells and tumors, and increased GCV-TP levels, while no effect of SDC was observed on the phosphorylation of ACV-MP to ACV-TP by cellular kinases. The SDC/prodrug combination altered the pharmacokinetics of the prodrugs. In accord with these findings, SDC enhanced significantly the cell-killing activity of prodrugs. The bystander effect was also significantly augmented by the combined treatment of ACV/GCV and SDC.

Conclusions SDC was shown to be effective in the HSV-TK/prodrug administration system and improved the efficiency of the bystander effect of ACV and GCV. The findings will be considerably valuable with respect to the use of GCV in lower doses and less toxic ACV. This novel strategy of drug combination could provide benefit to HSV-TK/prodrug gene therapy.
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Keywords cancer gene therapy; HSV thymidine kinase; diterpenoid; acyclovir; ganciclovir

Introduction

The herpes simplex virus type 1 thymidine kinase (HSV-TK) is a nucleoside kinase that has a relaxed substrate specificity compared with cellular kinases and can therefore utilize a number of modified nucleosides such as acyclovir

(ACV), ganciclovir (GCV) and 1-β-D-aravinofuranothymidine [1]. In HSV-infected cells, HSV-TK specifically phosphorylates ACV and GCV to their monophosphates, and this activation confers a high degree of selectivity of the drugs [2–4]. Thereafter, the monophosphates are further phosphorylated to the diphosphates (ACV-DP and GCV-DP) and triphosphates (ACV-TP and GCV-TP) by cellular kinases [5–11]. The triphosphates are the fully activated metabolites that are toxic to the virus. Their mechanisms of action appear to be selective inhibition of the viral DNA polymerase and termination of viral DNA chain elongation [8,10,12].

Interestingly, the HSV-TK gene is a 'suicide gene' when used in cancer gene therapy trials of animal models and human [13–17]. That is, if the HSV-TK gene is delivered to actively dividing cancer cells, and ACV or GCV (ACV/GCV) is subsequently administered to the cells, the TK enzyme phosphorylates these prodrugs, yielding toxic metabolites which cause the death of prodrug-treated, HSV-TK-expressing (TK^+) cells. Such a therapy strategy has become a popular method for treating solid tumors such as brain tumors, in which TK-activated GCV metabolites kill only dividing tumor cells while normal brain tissue should not be affected by the treatment. In fact, in many clinical trials of gene therapy, transgene expression has been identified [18–22]. It was initially thought that it is necessary for the HSV-TK gene to enter all the tumor cells for complete cell killing, as reported in the first *in vivo* experiments [23–25]. This was not realistic because current methods of gene delivery using viral or liposomal vectors have poor efficiency that results in limited transduction to a small minority of tumor cells. Thus, the potentiation of the bystander killing effect, which is the effect of causing the death not only of TK^+ cells but also of neighboring non-expressing (TK^-) ones [13,26,27], should have an important meaning in improving the clinical application of HSV-TK/prodrug administration strategies.

After screening antiviral chemicals for more than a decade, we found that scopadulciol (SDC), a tetracyclic diterpenoid isolated from a medicinal plant *Scoparia dulcis* L. [28], produced a significant increase in ACV-TP levels in HSV-infected cells when used in combination with ACV [29], and also in the antiherpes activity of GCV (unpublished data). In the present study, we first explored the mechanism of action of SDC in TK^+ and TK^- cells. SDC was found to stimulate the cytotoxicity of ACV and GCV in TK^+ cells. We further evaluated the antitumor and bystander effects resulting from the selective activation of HSV-TK by SDC in not only *in vitro* but also *in vivo* HSV-TK/prodrug gene therapy. In this study the pharmacokinetics of ACV and GCV monotherapy and SDC/prodrug combination therapy was also examined in mice.

While GCV is a more potent drug than ACV for inhibiting the replication of HSV [7,11] and TK^+ cells [30], the use of GCV with HSV-TK is limited in part by its toxicity. On the other hand, ACV causes much less adverse effects than GCV, the margin of safety being greater than that of GCV. Therefore, if it is possible to lower the dose of GCV or to replace GCV with ACV by enhancing their cytotoxicities, the efficacy of HSV-TK gene therapy could be greatly increased. Our data demonstrate that concomitant use of ACV or GCV with SDC has produced elevated toxicity against TK^+ cancer cells. The combined use of SDC with the prodrugs will be a choice for the improvement of this strategy since the results of early HSV-TK/GCV cancer gene therapy trials in patients with brain tumors and pleural mesothelioma are disappointing [18,31].

Materials and methods

Chemicals

SDC was isolated from *Scoparia dulcis* collected in Taiwan as previously reported [28], or synthesized from scopadulcic acid B [32] as shown in Scheme 1. ACV was purchased from Japan Wellcome Ltd. (Osaka, Japan). GCV was from Syntex (Palo Alto, CA, USA). [8-^{3}H]ACV was obtained from Moravek Biochemicals Inc. (Brea, CA, USA). ACV monophosphate (ACV-MP) was synthesized by phosphorylation of ACV according to the method of Yoshikawa *et al.* [33].

Cells

Human cervical carcinoma (HeLa 229), human bladder carcinoma (EJ-1) and human glioblastoma (PKG-1) cells were obtained from Human Science Research Resources Bank (Osaka, Japan). HEp-2 cells from a tumor of the larynx have been maintained in our laboratory. These cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS).

Plasmid construction

The plasmid pSV2-HSTK-1 containing the HSV-TK gene was used as reported previously [34].

Transfection

Human cancer cells ($1–2 \times 10^5$) were inoculated in 35-mm dishes and cultured for 24 h at 37 °C. The mixture of plasmid DNA and LipofectAmine (Life Technologies, Inc., Gaitherburg, MD, USA) was used for transfection according to the manufacturer's instructions. The transfected cells were cloned three times under selection in 800 μg/ml geneticin to obtain stably HSV-TK-expressing cell lines.

Inhibition of proliferation of cancer cells

TK^+ and parental TK^- cancer cells were inoculated in 24-well plates at a density of 5×10^4 cells per well.

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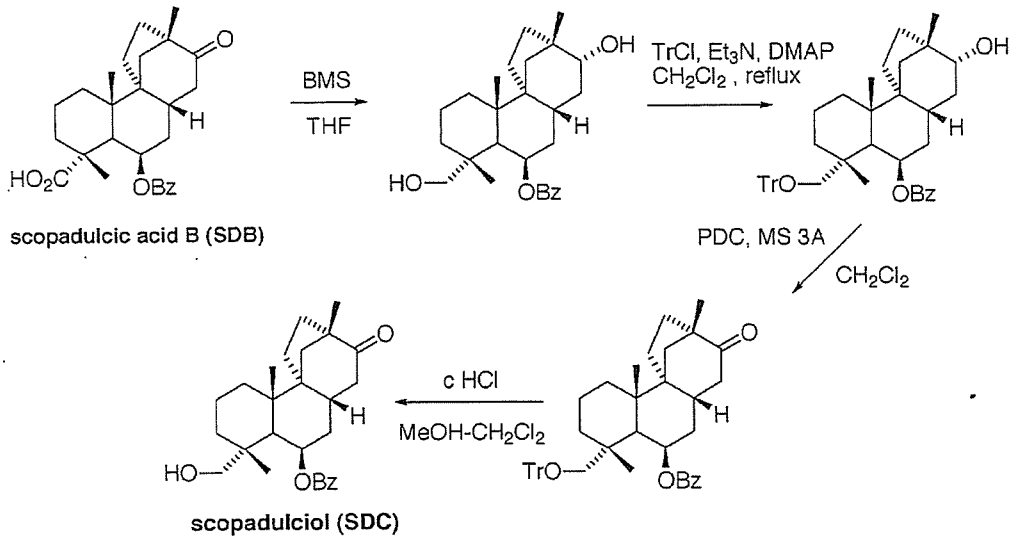

Scheme 1. Semisynthesis of SDC from scopadulcic acid B. BMS, borane/methyl sulfide complex; THF, 5β-pregnane-3α,11β,17α,21-tetrol-20-one; TrCl, trityl chloride; Et_3N, triethylamine; DMAP, dimethylamine pyridine; PDC, pyridimium dichromate; MS3A, molecular sieves 3 Å

After 1 day, the cells were replenished with medium containing varying concentrations of the test compounds. The cells were allowed to grow for 5 days and the viable cell numbers were counted by the trypan blue exclusion method. The 50% inhibition concentration (IC_{50}) was determined graphically.

Assay of kinase activity

For *in vitro* assay of kinase activities, cell pellets were prepared from exponentially growing parental HeLa (HeLa-TK$^-$) or HeLa-TK$^+$ cells, washed twice in ice-cold phosphate-buffered saline (PBS), once in 10 mM Tris (pH 7.5)/5 mM 2-mercaptoethanol/2 mM $MgCl_2$, sonicated for 60 s, and centrifuged at 10 000 rpm for 30 min. Cellular kinase activity in HeLa-TK$^-$ cells was determined using [^{3}H]thymidine as a substrate according to the method of Jamieson *et al.* [35] except that 200 μg of thymidine and 100 μg of bovine serum albumin (BSA) per milliliter were added to the reaction mixture. HSV-TK activity in HeLa-TK$^+$ cells was measured using [^{3}H]ACV as a substrate according to the method of Taylor *et al.* [36] except that 200 μM ACV was added to the reaction mixture. The reaction mixtures were incubated at 37 °C for 90 min in the absence or presence of 0.04 μM SDC, and 20 μl of the samples was spotted onto DE 81 discs (Whatman, Clifton, NJ, USA) to be counted in a scintillation counter. Enzyme activity was calculated and normalized for the amount of protein in each sample.

High-performance liquid chromatography (HPLC) analysis of ACV-MP and ACV-TP

The effect of SDC on the metabolism of ACV-MP was determined in HeLa-TK$^-$ cell lysate. The cell lysate was prepared as described above for cellular kinase activity. An aliqout of the lysate was incubated for 5 h at 37 °C after adding an equal volume of the solution containing 40 mM ACV-MP, 40 mM ATP, 40 mM $MgCl_2$, 0.2 M Tris (pH 8.0) and 200 μg/ml BSA in the absence or presence of 0.01–1 μM SDC. The reaction mixtures were extracted with perchloric acid followed by neutralization with KOH/K_2HPO_4 and centrifugation as reported previously [37]. The supernatants were concentrated to dryness under reduced pressure, redissolved in water, and analyzed with a Shimadzu Prominence HPLC system (Kyoto, Japan). Anion-exchange HPLC was performed on a Whatman Partisil SAX-10 column (4.6 × 250 mm) with a gradient of 10 mM ammonium phosphate (pH 5.5)/5% methanol to 800 mM ammonium phosphate (pH 5.5)/5% methanol. The flow rate was 1.5 ml/min, with 5-min flow of the 10 mM buffer, followed by 30-min flow of the linear gradient to 800 mM buffer. Nucleotides were monitored by UV absorption at 254 nm. The retention times under these conditions were 12.0 min for ACV-MP and 23.7 min for ACV-TP.

Determination of intracellular level of GCV-TP

The quantification of GCV-TP in cell extracts was performed according to the method reported previously [29]. HeLa-TK$^+$ cells were incubated in the absence or presence of GCV (100 μM) and/or SDC (0.02, 0.04 μM) before extraction with 0.5 M $HClO_4$. The supernatants were successively added and treated with KOH, 0.5 M $NaIO_4$ and 4 M methylamine. The separation of dNTP was performed by HPLC using a Partisil SAX-10 column. The retention times for CTP, TTP, ATP, GTP and GCV-TP were 6.8, 8.3, 10.8, 15.1 and 13.9 min, respectively.

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In vitro bystander effects

HeLa-TK$^-$ cells were admixed with HeLa-TK$^+$ cells at various percentages (0, 1, 5, 10, 20 and 100%). The mixtures were inoculated in triplicate at a total of 2×10^4 cells/well into 48-well plates and grown overnight. The cells were then exposed to ACV- or GCV-containing medium at the concentrations of 0, 20, 200 and 1000 μM, or 0, 0.1, 1, 10 and 100 μM, respectively, in the presence of SDC at 0, 0.04 and 0.1 μM. The medium was replenished every 3 days. On the seventh day of exposure, viable cells were counted to determine IC_{50} values.

In vivo experiments in nude mice

Five-week-old female ddY athymic mice were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). The mice were maintained in accordance to institutional guidelines of the Toyama Medical and Pharmaceutical University Animal Care and Use Committee. Mice were injected subcutaneously with 5×10^6 cells that had been resuspended in 200 μl of PBS. The following TK$^+$/TK$^-$ ratios of HeLa cells were injected into the backs of the mice: 100, 10 and 0%. After 10 (0% and 10% TK$^+$ cell-inoculated groups) or 13 days (100% TK$^+$ cell-inoculated groups), when the tumors had reached a volume of more than 100 mm^3, ten tumors/group were treated for 14 days as follows: (1) orally three times a day (every 8 h) with either ACV (4 mg/day), SDC (1 mg/day) or ACV plus SDC; (2) intraperitoneally (i.p.) three times a day with either ACV (2.5 mg/day), SDC (1 mg/day) or ACV plus SDC; (3) i.p. three times a day with ACV (1 mg/day) plus SDC (1 mg/day); (4) i.p. twice a day with either GCV (2.5 mg/day), SDC (1 mg/day) or GCV plus SDC; (5) i.p. twice a day with GCV (1 mg/day) plus SDC (1 mg/day); or (6) i.p. twice a day with GCV (0.25 mg/day) plus SDC (1 mg/day). A control group was treated orally with distilled water or i.p. with PBS. Tumor size was measured once weekly up to 42 days from the start of drug administration. Tumor volume was calculated using the formula $\pi/6 \times$ larger diameter $\times$ (smaller diameter)^2, as previously reported [38].

Bioavailability of ACV and GCV

Mice were treated orally once with ACV (1.33 mg/mouse) or ACV plus SDC (0.33 mg/mouse), or i.p. once with ACV (0.83 mg/mouse), ACV plus SDC (0.33 mg/mouse), GCV (0.83 mg/mouse) or GCV plus SDC. The blood samples were collected at 0.5, 1, 2, 4, 6 and 8 h and immediately centrifuged for 10 min at 1500 rpm to separate the plasma. The plasma samples were subjected to acid protein precipitation by adding an equal volume of 7% perchloric acid. Calibration curves were generated by using samples from spiked blank matrix in the range of 0.1–1.6 μg/ml. The HPLC system consisted of a sorbent delivery system (model LC-10AT, Shimadzu Scientific Instrument, Kyoto, Japan), a degasser, a guard column (4.6 mm i.d. $\times$5 mm) packed with 5 μm ODS resin, a separation column (Shimadzu PREP-ODS(H), 5 μm, 4.6 mm i.d. $\times$250 mm), a UV-vis detector (SPD-6A, Shimadzu), and a data-processing apparatus (Chromatopack CR-6A, Shimadzu). The mobile phase used for the analysis of ACV and GCV was a mixture of methanol and 20 mM phosphate (pH 2.95), in which the mixing ratio was 3 : 97 (v/v), and delivered at a flow rate of 1.0 ml/min. The UV detection was set at 254 nm. Under these conditions, ACV eluted at ~16 min and GCV at ~13 min. The area-under-the-concentration-time-curve (AUC) was determined by the linear trapezoidal rule.

Data analysis

All values were expressed as mean ± standard deviation (SD). Student's t test was used to compare the values. A value of $p < 0.05$ was considered statistically significant.

Results

Effects of SDC on *in vitro* antitumor activities by ACV and GCV

In HSV-1-infected cells, SDC enhanced the antiviral activities of ACV [29] and GCV (unpublished data). This synergistic action of SDC was suggested to be due to the stimulation of HSV-TK activity, which resulted in increased levels of phosphorylated ACV and GCV. Thus, it might be expected that SDC could increase the susceptibility of TK$^+$ cells to toxic action of these prodrugs by enhanced accumulation of phosphorylated metabolites. We evaluated for inhibitory effects of the combination of SDC and the prodrugs on several human cancer cell lines including HEp-2, EJ-1, HeLa 229 and YKG-1 cells as an *in vitro* model for antitumor assay in the HSV-TK/prodrug administration system. Proliferation of wild-type cells was not markedly inhibited by ACV and GCV with IC_{50} values of 1917–3147 μM and 423–760 μM, respectively (Table 1). After expression of HSV-TK by transfection with the plasmids containing the TK gene, however, these cells became highly susceptible to the prodrugs. That is, the cytotoxic effects of ACV and GCV were intensified by 10- to 26-fold and 9- to 311-fold, respectively, when compared with the IC_{50} values of the wild-type counterparts. GCV produced much higher toxicity on TK$^+$ cancer cells than ACV.

SDC at 0.04 μM alone exerted no toxicity on these cells (data not shown), showing no augmentation of toxic action of both ACV and GCV on wild-type cancer cells (Table 1). On the other hand, the combination of SDC and ACV strengthened the toxicity against TK$^+$ cancer cells, with the IC_{50} values of ACV being 4.0–23 μM.

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Table 1. Cell growth inhibition of ACV and GCV against human cancer cells expressing HSV-TK in the absence or presence of 0.04 μM SDC

Cell line	Cytotoxicity (IC_{50}, μM)[a]			
	ACV	ACV + SDC	GCV	GCV + SDC
HEp - 2	1947 ± 328[b]	2120 ± 191	760 ± 62	677 ± 76
HEp - 2 - HSTK	119 ± 11	8.8 ± 0.70***	6.5 ± 2.1	0.64 ± 0.24**
EJ - 1	1917 ± 150	1963 ± 214	423 ± 63	453 ± 44
EJ - 1 - HSTK	185 ± 38	23 ± 5.1**	49 ± 6.3	1.2 ± 0.16***
HeLa 229	3147 ± 434	3350 ± 656	490 ± 26	488 ± 49
HeLa 229 - HSTK	166 ± 9.1	10.9 ± 3.0***	3.4 ± 0.44	0.15 ± 0.015***
YKG - 1	2193 ± 156	1873 ± 367	497 ± 85	610 ± 61
YKG - 1 - HSTK	83 ± 5.1	4.0 ± 1.9***	1.6 ± 0.42	0.32 ± 0.16***

**$p < 0.01$ vs. prodrug alone; ***$p < 0.001$ vs. prodrug alone.
[a]50% inhibitory concentration (IC_{50}) of ACV or GCV for cell growth inhibition.
[b]Each value is the mean ± SD of triplicate assays.

These levels were 8- to 21-fold lower than those of ACV alone required to inhibit each TK^+ cell line, and were comparable to those observed in GCV-treated cell cultures. Similar results were obtained in the HSV-TK transfectants treated with the combination of SDC and GCV, where the IC_{50} values of GCV (0.15–1.2 μM) were reduced by 5- to 41-fold as compared with those by the treatment with GCV alone. Therefore, the susceptibility of different human cancer cell lines to HSV-TK/ACV- and HSV-TK/GCV-mediated cytotoxic effects was potentiated by the concomitant administration of SDC. Although all cell lines were sensitive to the prodrug, the degree of the sensitivity varied among the cell lines to some extent: their toxic concentrations in EJ-1 were higher than those in HEp-2, HeLa and YKG-1 cells.

Effects of SDC on cellular kinase and HSV-specific TK activities, and GCV-TP formation in cells

Both HSV-TK and cellular kinases are involved in the phosphorylation of ACV and GCV. In order to elucidate the contribution of these kinases in increased susceptibility of HSV-TK transfectants to the prodrugs by SDC, the levels of cellular and HSV-TK enzymatic activities were determined in HeLa-TK^- and HeLa-TK^+ cells, respectively, in the absence or presence of SDC. As shown in Table 2, SDC did not stimulate the enzyme activity in HeLa-TK^- cells. In contrast, phosphorylation of the substrate in HeLa-TK^+ cells was enhanced significantly ($p < 0.05$) by SDC.

Table 2. Effect of SDC on cellular and HSV-derived TK activities

Drug Treatment	Enzyme activity	
	in HeLa-TK^- cells	in HeLa-TK^+ cells
None	526 ± 123[a]	217 ± 53
SDC	541 ± 160	434 ± 54*

Kinase activities were determined in the absence or presence of 0.04 μM SDC, and expressed as the pmol of nucleoside incorporated/min/mg of protein.
[a]Each value is the mean ± SD of triplicate assays.
*$p<0.05$ vs. no drug control.

In order to confirm this effect of SDC, the resultant metabolite of GCV, GCV-TP, was directly measured in HeLa-TK^+ cells by HPLC analysis. The levels of GCV-TP obtained from triplicate determinations were significantly increased to 108 ± 4.5% ($p < 0.05$) or 141 ± 12% ($p < 0.01$) in the cells treated with GCV (100 μM) plus 0.02 μM SDC or GCV plus 0.04 μM SDC, respectively, when the GCV-TP level in the cells treated with GCV alone was taken as 100%.

In another set of experiments, to make clear the effect of SDC on cellular kinases, the conversion of ACV-MP into ACV-TK in HeLa-TK^- cells was determined. Since ACV-MP is suggested not to enter the cells [39], ACV-MP was incubated in the cell lysate followed by HPLC analysis. There was no increase in the amounts of both ACV-MP and ACV-TP when ACV-MP was coadministered with 0.01–1 μM SDC (data not shown).

Effect of SDC on *in vivo* antitumor activities of ACV and GCV

In order to extend the *in vitro* results mentioned above to *in vivo* situations, we used nude mice bearing tumors of HeLa-TK^+ cells. After the cells were injected subcutaneously, drugs were administered successively for 14 days starting from the 13th day of inoculation. In preliminary experiments, different doses of SDC and the prodrugs were studied for the growth of tumors of HeLa-TK^+ cells in nude mice (data not shown). Based on these data, we selected the doses of the drugs that are insufficient for the complete eradication of the tumors. In this study, oral SDC at a dose of 1 mg/mouse showed a tendency of suppressing tumor growth but no statistical significance as compared with no drug control over the observation period of 6 weeks (Figure 1a). Oral ACV at a dose of 4 mg/mouse produced significant reduction in tumor growth as compared with no drug control at days 7 to 42 ($p < 0.001$). When mice were orally treated with the combination of SDC and ACV, significant antitumor effects were observed throughout the periods of study as compared with no drug control ($p < 0.001$). Importantly, SDC enhanced significantly the antitumor effects of

ACV from days 14 ($p < 0.01$) to days 42 ($p < 0.05$) as compared with the animal group treated with ACV monotherapy. In the i.p. administration study, tumor size in mice treated with SDC (1 mg/mouse) increased gradually for the observation period of 6 weeks, while showing significant reduction ($p < 0.05$) during days 14 to 42 as compared with no drug control (Figure 1b). ACV (2.5 mg/mouse) produced significant reduction in tumor growth throughout the period of 6 weeks as compared with the control ($p < 0.001$). The combination of SDC and ACV caused no growth of tumors during the observation periods, showing significant antitumor effects at days 23 and thereafter as compared with ACV monotherapy.

In the GCV-treated group, tumor growth was significantly ($p < 0.001$) inhibited at a dose of 2.5 mg of GCV per day throughout the observation period of 6 weeks as compared with no drug control (Figure 2). Combined administration of GCV and SDC gave rise to the most marked decreases in tumor volume over 42 days. There were significant differences between the combination therapy and GCV monotherapy from days 14 ($p < 0.05$) to 42 ($p < 0.001$).

To compare the antitumor efficacy between ACV and GCV, mice bearing tumors of HeLa-TK$^+$ cells were treated with different doses of prodrug alone or the combination of prodrug and SDC (Table 3). When orally administered, a combination of ACV (4 mg/day) and SDC (group 12) showed activity which was higher than GCV (0.25 mg/day) treatment (group 1) and less than GCV (1 mg/day) treatment (group 3) as evaluated by the tumor size at 5 weeks of treatment. This suggested that antitumor efficacy of oral ACV and SDC might be one-quarter of that of i.p. GCV or less. Treatments consisting of i.p. ACV plus SDC (groups 8 and 10), however, produced inhibitory activity that was equivalent to or higher than that by the same doses of GCV alone (groups 3 and 5). In the presence of SDC, GCV treatments (groups 4 and 6) caused approximately 2-fold higher antitumor effect as compared with ACV treatments (groups 8 and 10).

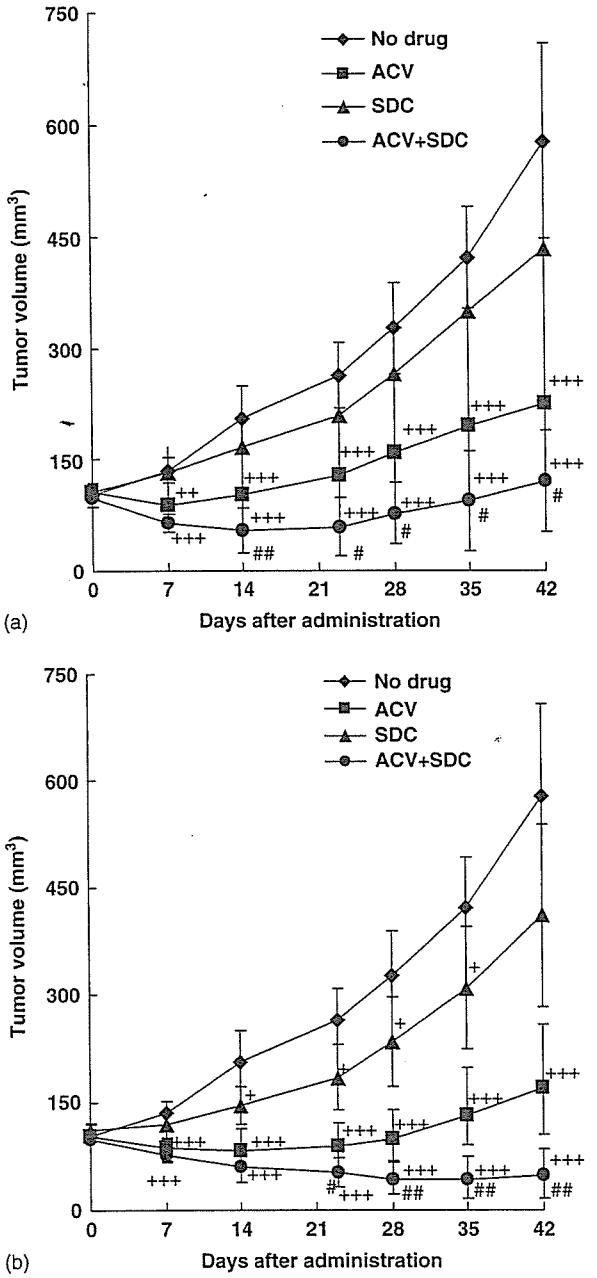

Figure 1. *In vivo* effects of SDC on antitumor actions of ACV in HSV-TK-expressing tumors. SDC (1 mg/day) and ACV (4 mg/day) were orally administered three times a day (every 8 h) (a), or SDC (1 mg/day) and ACV (2.5 mg/day) were intraperitoneally administered three times a day (every 8 h) (b) for 14 days. Bar: mean ± SD from ten determinations. $^{+++}p < 0.001$ vs. no drug control; $^{\#}p < 0.05$ vs. ACV monotherapy; $^{\#\#}p < 0.01$ vs. ACV monotherapy

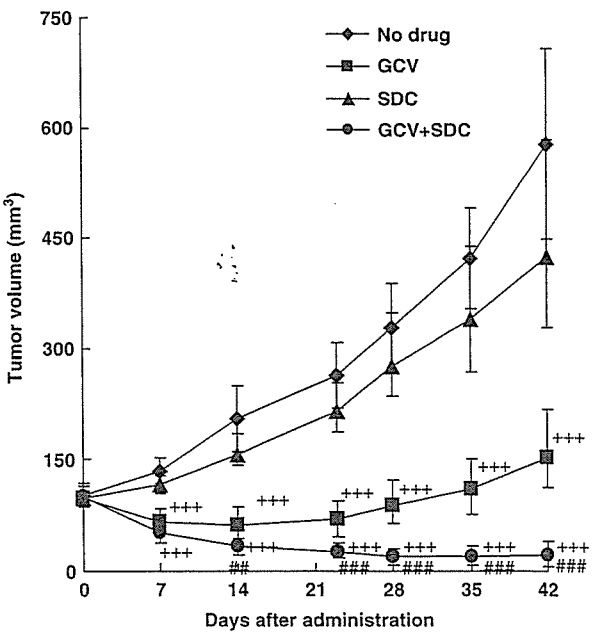

Figure 2. *In vivo* effects of SDC on antitumor actions of GCV in HSV-TK-expressing tumors. SDC (1 mg/day) and GCV (2.5 mg/day) were intraperitoneally administered twice a day (every 12 h) for 14 days. Bar: mean ± SD from ten determinations. $^{+++}p < 0.001$ vs. no drug control; $^{\#\#}p < 0.01$ vs. GCV monotherapy; $^{\#\#\#}p < 0.001$ vs. GCV monotherapy

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Table 3. Comparison of antitumor efficacy between ACV and GCV in the absence or presence of SDC

Group	Treatment	Tumor growth at 5 weeks
1	GCV (0.25 mg/day)	234 ± 44*
2	GCV (0.25 mg/day) + SDC	130 ± 36
3	GCV (1 mg/day)	176 ± 41
4	GCV (1 mg/day) + SDC	28 ± 10
5	GCV (2.5 mg/day)	113 ± 41
6	GCV (2.5 mg/day) + SDC	20 ± 15
7	ACV (1 mg/day)	289 ± 51
8	ACV (1 mg/day) + SDC	64 ± 24
9	ACV (2.5 mg/day)	128 ± 63
10	ACV (2.5 mg/day) + SDC	43 ± 32
11	ACV (4 mg/day)	195 ± 82
12	ACV (4 mg/day) + SDC	96 ± 70

*Percentage of tumor volume when tumor volume at the start of drug treatment was taken as 100%.
Drugs were intraperitoneally administered for 14 days to mice bearing tumors of 100% HeLa-TK$^+$ cells except for groups 11 and 12 where drugs were perorally administered. GCV and ACV were administered twice or three times a day, respectively, alone or in combination with SDC (1 mg/day). Each value is the mean ± SD from ten determinations.

Tumor sizes of mice treated with GCV at doses of 0.25, 1 and 2.5 mg/day were reduced 1.8-, 6.3- and 5.7-fold, respectively, by the addition of SDC (groups 1–6).

In vitro potentiation of bystander effects of ACV and GCV by SDC

As transgene expression has generally been limited to a small minority of tumor cells, tumor cell killing via a 'bystander effect' might play a crucial role in effective elimination of tumor tissues. Therefore, we determined the ability of SDC to enhance ACV and GCV toxicities in *in vitro* model systems consisting of mixed cultures of HSV-TK$^+$ and HSV-TK$^-$ (bystander) cells. SDC was not toxic to both TK$^-$ and TK$^+$ cells at the concentrations used (0.04 and 0.1 μM; data not shown). The bystander effects were evaluated in cocultures containing 1, 5, 10 and 20% HeLa-TK$^+$ cells. In the absence of SDC, the IC_{50} values of ACV ranged from 963–427 μM in cocultures with 1–20% TK$^+$ cells (Figure 3a). The simultaneous addition of SDC (0.04 μM) to ACV-treated cocultures significantly reduced the IC_{50} values of ACV by 1.8- ($p < 0.01$), 1.6- ($p < 0.001$), 2.5- ($p < 0.01$) and 2.0-fold ($p < 0.01$) in cocultures containing 1, 5, 10 or 20% TK$^+$ cells, respectively. When SDC was coadministered with ACV at a higher concentration of 0.1 μM, much higher bystander effects were attained depending on a ratio of TK$^+$ cells: 20- ($p < 0.001$), 131- ($p < 0.001$), 569- ($p < 0.001$) and 628-fold ($p < 0.001$) decreases in the IC_{50} values of ACV as compared with those by the treatment with no drug control. As expected, SDC exerted no effect on the cytotoxicity of ACV in parental cells.

GCV showed more potent toxicity than ACV in cocultures with 1–20% HeLa-TK$^+$ cells, its IC_{50} values ranging from 54–1.3 μM (Figure 3b). A combination of GCV and SDC (0.04 μM) decreased the IC_{50} values of GCV depending on the ratio of TK$^+$ cells, i.e., by 1.1-, 1.2-, 1.8- ($p < 0.05$) and 2.2-fold ($p < 0.05$) in cocultures containing 1, 5, 10 or 20% TK$^+$ cells, respectively. A combined use of GCV with a higher concentration of SDC (0.1 μM) produced highly significant ($p < 0.001$) reductions in the IC_{50} values by 193-, 217-, 154- and 50-fold in cocultures containing 1, 5, 10 and 20% TK$^+$ cells, respectively.

In vivo effects of SDC on bystander effects of ACV and GCV

The advantageous action of SDC confirmed in *in vitro* experiments of bystander effects was further evaluated in *in vivo* situations. Mixtures of 10 and 0% HeLa-TK$^+$ cells with parental ones were injected into nude mice. After 10 days of inoculation when the tumors became palpable, the administration of prodrugs alone or drug combination with SDC was started and continued for 14 successive days. In the mice inoculated with parental cells (i.e. 0% TK$^+$ cells), administration of ACV or GCV even in the presence of SDC produced no significant reduction in tumor growth as compared with no drug control (data not shown).

Oral treatment with SDC (1 mg/mouse) alone showed no significant effect as compared with no drug control in the groups inoculated with 10% TK$^+$ cells except for at 42 days ($p < 0.05$) (Figure 4). Oral ACV (4 mg/mouse) alone produced significant decrease in tumor growth in comparison to no drug control at days 7 ($p < 0.05$), 14 ($p < 0.05$), 23 ($p < 0.01$), 28 ($p < 0.01$), 35 ($p < 0.01$) and 42 ($p < 0.001$). Oral ACV in combination with oral SDC resulted in more significant suppression of tumor growth as compared with the control group throughout the period of 6 weeks ($p < 0.001$). In these experimental conditions, SDC potentiated significantly the antitumor effects of ACV at days 23 ($p < 0.05$), 28 ($p < 0.05$), 35 ($p < 0.01$) and 42 ($p < 0.01$) as compared with those attained with ACV alone.

Intraperitoneal administration of GCV (2.5 mg/mouse) alone showed significant differences in tumor growth throughout the period of 6 weeks ($p < 0.01$ to 0.001), as compared with no drug control (Figure 5). By the combined i.p. application of GCV and SDC, significant augmentation of antitumor action of the prodrug was found throughout the observation periods as compared with both no drug control ($p < 0.001$) and GCV monotherapy ($p < 0.05$ to 0.001). Even at 4 weeks after termination of drug administration, no marked growth of tumors was observed in mice treated with the GCV/SDC combination.

Effects of SDC on the bioavailability of ACV and GCV

Single ACV and GCV were administered alone or in combination with SDC at each dose equal to that shown in Figures 1 and 2. The concentrations of ACV

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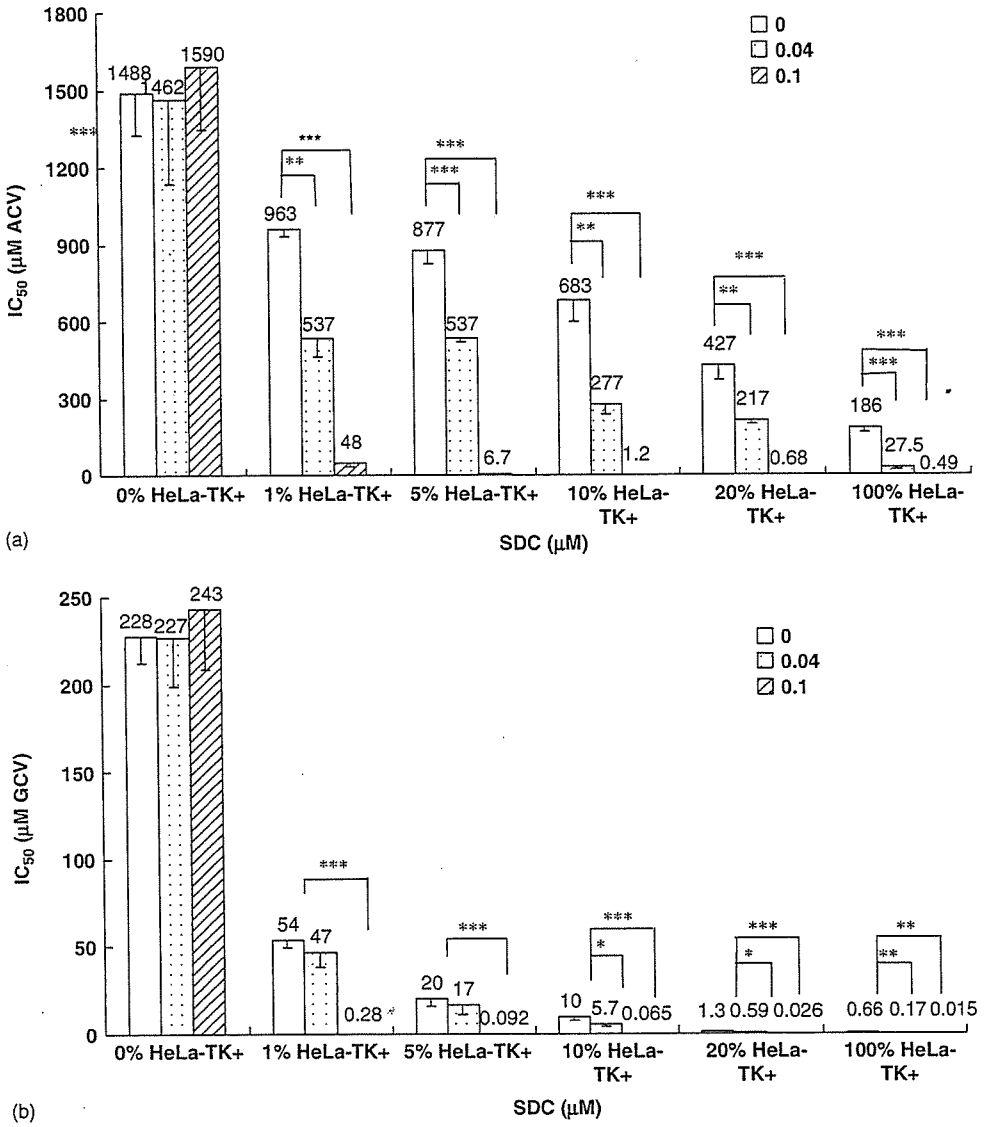

Figure 3. *In vitro* bystander effects of ACV (a) and GCV (b) on the sensitivity of HeLa cell cultures in the absence or presence of SDC. HSV-TK$^+$ and parental cells were cocultured at the ratio of 0 : 100, 1 : 99, 5 : 95, 10 : 90, 20 : 80 or 100 : 0 in the presence of SDC (0, 0.04 or 0.1 μM) and ACV (0–1000 μM) (a) or GCV (0–100 μM) (b). After 7 days of incubation, surviving cells were counted to calculate the IC_{50} values for ACV and GCV. Bar: mean ± SD from three determinations. $^{**}p < 0.01$; $^{***}p < 0.001$

and GCV in the plasma of mice are shown in Table 4. When ACV was administered perorally, the uptake of ACV into the plasma was significantly affected by coadministration of oral SDC after 1 ($p < 0.05$), 2 ($p < 0.01$) and 4 h ($p < 0.05$). Similarly, a marked increase in plasma concentration for ACV was seen at 0.5 and 1 h ($p < 0.05$) after administration when ACV and SDC was coadministered i.p. The resulting AUC for the combination therapy was approximately 2.4-fold higher than that for ACV monotherapy. The uptake of i.p. GCV into the plasma was also significantly affected by i.p. coadministration of SDC at 0.5 ($p < 0.01$), 1 ($p < 0.01$) and 4 h ($p < 0.01$) after administration. The resulting AUC for the GCV/SDC combination was approximately 2.8-fold higher than that for GCV monotherapy.

Discussion

In the present *in vitro* and *in vivo* studies, we evaluated the possible value of SDC in HSV-TK/ACV and HSV-TK/GCV systems by the elimination assay of HSV-TK-expressing cells with drugs.

HSV-infected cells phosphorylated more favorably GCV than ACV as measured by kinase activities [7,8,40], in which GCV has been proven to be a more potent inhibitor of herpes simplex virus (HSV) replication. This fact may be reflected in the *in vitro* results obtained by the cytotoxicity assay with various HSV-TK-expressing cancer cells: GCV was much more potent an inhibitor of HSV-TK gene-transfected cell proliferation than ACV as measured by 50% inhibitory concentrations. In *in vivo* assay systems, GCV was also confirmed to be superior to ACV in

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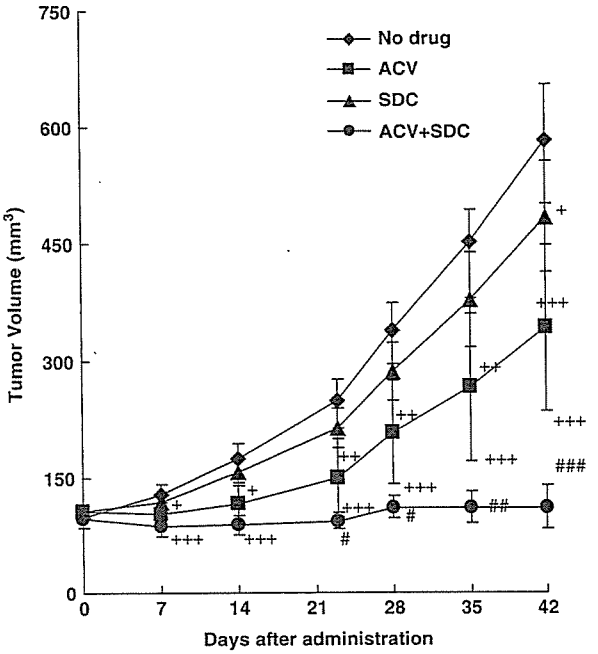

Figure 4. *In vivo* bystander effects of ACV on tumors induced by mixtures of 90% HSV-TK$^-$ and 10% HSV-TK$^+$ HeLa cells in the absence or presence of SDC. ACV and SDC were administered as described in Figure 2a. Bar: mean ± SD from ten determinations. $^+p<0.05$ vs. no drug control; $^{++}p < 0.01$ vs. no drug control; $^{+++}p < 0.001$ vs. no drug control; $^{\#}p < 0.05$ vs. ACV monotherapy; $^{\#\#}p < 0.01$ vs. ACV monotherapy

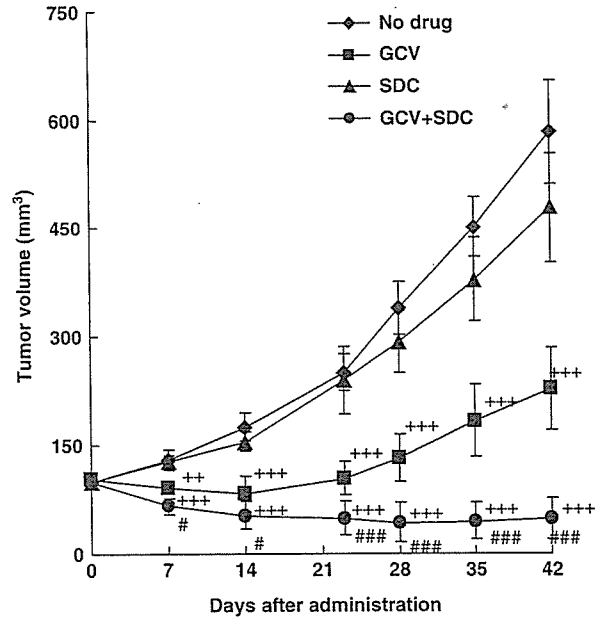

Figure 5. *In vivo* bystander effects of GCV on tumors induced by mixtures of 90% HSV-TK$^-$ and 10% HSV-TK$^+$ HeLa cells in the absence or presence of SDC. GCV and SDC were administered as described in Figure 3. Bar: mean ± SD from ten determinations. $^{++}p < 0.01$ vs. no drug control; $^{+++}p < 0.001$ vs. no drug control; $^{\#}p < 0.05$ vs. GCV monotherapy; $^{\#\#\#}p < 0.001$ vs. GCV monotherapy

antitumor activity. While ACV is a less toxic drug and its margin of safety is greater than GCV, it is normally not effective against HSV-TK-transfected cells because of its high IC_{50}. This suggests that GCV is the prodrug of choice for antitumor therapy using the HSV-TK gene-expressing system, but this approach is limited partly by its toxicity. Therefore, if GCV could be lowered in its dose or replaced by ACV in HSV-TK gene therapy, it would be possible to perform the clinical trials without increasing toxicity. In the present study, we explored with interest the possibility for the novel application of a substance, SDC, in HSV-TK/ACV suicide gene therapy. As a result, when the two prodrugs were i.p. administered at the same doses, ACV, in the presence of SDC, could produce high efficacy that was comparable to monotherapy with GCV (Figures 1b and 2, Table 3). Oral ACV in combination with oral SDC could also produce an anticancer effect that was comparable to GCV monotherapy (Figures 1a and 2, Table 3). These data show that there is the probability to use preferentially an HSV-TK/ACV administration strategy with a combination of SDC in cancer therapy. Furthermore, the potentiation of the antitumor effect of GCV by SDC would contribute to reduced doses of GCV to less toxic levels.

To delineate the mechanism responsible for the potentiation of the toxic action of ACV and GCV by SDC, the kinase activity and the intracellular pool level of GCV-TP in HSV-TK-expressing cells, as well as the conversion of ACV-MP into ACV-TP by cellular kinases, were determined. From these results, it was indicated that HSV-specific TK but not cellular kinases should contribute to the action of SDC that caused the elevation of ACV/GCV cytotoxicities.

Because of the poor efficiency in current methods of gene delivery by viral or liposomal vectors into target tissues, it was hoped that the bystander effect might compensate for such a low efficacy. As shown in the present study, SDC has the ability to enhance ACV and GCV toxicities in mixed cultures of HSV-TK$^+$ and HSV-TK$^-$ cells as evaluated both *in vitro* and *in vivo* model systems. Therefore, the potentiation of the bystander killing effect of the prodrugs caused by SDC can be expected to improve the clinical application of HSV-TK/prodrug administration strategies. Thus far, transfer of phosphorylated nucleotides between cells has been shown to be mainly through gap junctional intracellular communication (GJIC) [41]. This phenomenon has been considered to explain the bystander effect involved in HSV-TK/prodrug gene therapy [42–45]. In fact, in the cells with poor GJIC expression, the bystander effect by GCV was enhanced after transduction of gap junction protein genes such as connexins 26 and 43 [45–47]. However, the HeLa cells used in our study are poorly communicating cells, in which the bystander effect is quite limited [45,47]. In spite of poor GJIC, the prodrugs showed dose-dependent bystander killing in HeLa cell populations in the present study. There is another theory proposed to explain the bystander killing: phagocytosis by HSV-TK$^-$ bystanders of apoptotic vesicles containing toxic GCV phosphates from HSV-TK$^+$ cells [26]. At present,

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Table 4. Effects of SDC on the bioavailability of acyclovir (ACV) and ganciclovir (GCV) as expressed by plasma AUC

Drug therapy	Route of administration	Time (h) after drug administration					$AUC_{0\rightarrow 6}$ (h·μg/ml)	$AUC_{0\rightarrow \infty}$ (h·μg/ml)
		0.5	1	2	4	6		
ACV	oral	1.20[a] ± 0.03	0.55 ± 0.02	0.16 ± 0.03	0.11 ± 0.04	0.02 ± 0.03	1.767	1.796
ACV + SDC	oral	1.14 ± 0.10	0.81* ± 0.12	0.28** ± 0.02	0.18* ± 0.02	0.03 ± 0.01	2.225	2.280
ACV	intraperitoneal	4.33[a] ± 2.4	0.22 ± 0.06	0.17 ± 0.04	ND	ND	3.647	3.704
ACV + SDC	intraperitoneal	7.35 ± 2.33	1.11* ± 0.46	0.21 ± 0.05	ND	ND	8.843	8.922
GCV	intraperitoneal	7.55[b] ± 1.79	2.55 ± 0.87	0.56 ± 0.06	0.80 ± 0.01	ND	9.199	9.248
GCV + SDC	intraperitoneal	20.26** ± 2.54	9.45** ± 1.65	0.60 ± 0.01	0.16** ± 0.02	ND	25.451	25.525

ND, not detected.
[a]plasma concentration of ACV (μg/ml)(mean ± SD)($n = 3$).
[b]plasma concentration of GCV (μg.ml)(mean ± SD)($n = 3$).
*$p < 0.05$ vs. monotherapy.
**$p < 0.01$ vs. monotherapy.

while SDC augmented the bystander effects of ACV and GCV, it is not clear whether the compound might be involved in the expression of GJICs and/or phagocytosis of neighboring cells.

Drug combination is one strategy to overcome the toxicity of prodrugs and insufficient gene delivery into targets. Among such trials, tumor cell killing was enhanced by increasing GJIC function with the treatment with specific chemicals such as retinoids [48–50] or by increasing tumor cell apoptosis using inducers of apoptosis with GCV [51]. Involvement of different antitumor agents such as topotecan [52], a gap junction inducer [53], thymidylate synthase inhibitors including 5-fluorouracil [54], and a polycation which induces adenovirus-mediated gene transfer [55], have also improved the efficacy of HSV-TK suicide gene therapy. Hydroxyurea, a ribonucleotide reductase inhibitor, was shown to increase the GCV-TP levels in HSV-TK-expressing cells by reducing cellular dGTP pools in *in vitro* models [56], and to delay tumor growth *in vivo* [57]. These trials have never directly taken part in the expression of HSV-TK on which the suicide gene therapy system relies. Recently, we have reported that ponicidin, a natural diterpenoid, stimulates the activity of the HSV-TK enzyme [34]. However, this compound is less effective than SDC for exerting biological functions and, in addition, there are problems in providing a sufficient supply of this compound by methods of isolation from the plant, biotechnology or chemical synthesis. In contrast, SDC can produce higher antitumor potency in HSV-TK gene therapy, and importantly, can be constantly obtained by the biotechnological process using leaf organ culture systems and partial synthesis from scopadulcic acid B, which is biosynthesized much more abundantly in organ-cultured tissues from *Scoparia dulcis* [58], in a high yield, as shown in Scheme 1 in the present study.

Because the safety of a drug is the most important concern in the application to humans, we evaluated *in vivo* subacute toxicity of SDC by i.p. injection of a 2-fold higher dose (2 mg/mouse) to mice for 10 days. All animals survived the treatment schedule without any reduction in body weight, and there were no abnormal signs for at least 1 month of the observation period (data not shown).

The significant increase in AUC observed in mice treated with the ACV/GCV and SDC combination could result in increased disposition of ACV and GCV in the tissues of tumor-bearing mice. While it is not yet clear to what degree SDC could affect the antitumor effects of the prodrugs by increasing their bioavailability, these effects might play an important role in increased antitumor activities of the prodrugs when given in these combinations.

In summary, SDC is a candidate for combinatory therapy of HSV-TK-mediated cancer cell killing. We think that there is much room for further improvement of antitumor effects by drug combination therapy using SDC with the prodrugs when an appropriate medication schedule is investigated.

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Liposome Vector Containing Biosurfactant-Complexed DNA as Herpes Simplex Virus Thymidine Kinase Gene Delivery System

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For injectable-sized liposome complexed with DNA (lipoplexes) with high transfection efficiency of genes, we initially prepared small-sized liposomes by addition of biosurfactant. For selectivity of gene expression, the thymidine kinase (MK-tk) gene controlled by midkine was used for herpes simplex virus thymidine kinase (HSV-tk) gene therapy. Liposomes composed of 3([N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol), L-dioleoylphosphatidylethanolamine (DOPE), and a biosurfactant, such as β-sitosterol β-D-glucoside (Sit-G) for Sit-G-liposomes and mannosylerythrytol lipid A (MEL) for MEL-liposomes, produced about 300-nm-sized lipoplexes. Sit-G- and MEL-liposomes showed higher transfection efficiency of the luciferase marker gene and thymidine kinase activity in the presence of serum in the cells. The treatment with transfection of MK-tk gene by Sit-G-liposome and injection of ganciclovir significantly reduced tumor growth in a solid tumor model, compared with that by Sit-G-liposome alone. This finding suggested that Sit-G-liposome is a potential vector for HSV-tk gene therapy.

Keywords cationic liposome, sterylglucoside, biosurfactant, gene delivery, herpes simplex virus thymidine kinase, midkine

Introduction

In gene therapy, gene transfer approaches employ mostly viral vectors because of their high gene transfer efficiency, but concerns have been raised regarding their safety. Cationic

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liposomes constitute the most promising alternative to the use of viral vectors for gene therapy and have been studied in several clinical trials (Alton and Geddes, 1995). Often, commercially available cationic liposomes are mixed with plasmid DNA (pDNA), resulting in large aggregated liposome-pDNA complexes (lipoplexes) (Sternberg et al., 1994; Lai and van Zanten, 2002). These systems work satisfactorily in vitro because large complexes are often more efficient in transfecting cells in vitro (Felgner et al., 1994). However, such lipoplexes cannot be injected in blood vessels and yield very low-level transfection efficiency in vivo (Egilmez et al., 1996). Regarding the circulation lifetime of liposomes in terms of their size, it has often been considered that smaller-sized particles, of the order of about 300 nm, are preferable to larger particles. Liposome vectors should fulfill several minimal requirements (i.e., injectable particle size after forming lipoplexes, high transfection efficiency and selectivity).

In many cases, the small-sized lipoplexes are formed using small-sized liposomes prepared by sonication and extrusion through filters of decreasing size and/or using of pDNA-condensing agents such as polyamines (Gao and Huang, 1996). However, these preparation methods have many processes. To protect larger-sized lipoplexes, we prepared initially small-sized liposomes by an ethanol injection method without using extruder or polymers and just by addition of β-sitosterol β-D-glucoside (Sit-G) or mannosylerythrytol lipid A (MEL) to liposomes, because Sit-G is a good dispersant (Hwang et al., 2001) and MEL produces small-sized lipoplexes with high transfection efficiency in vitro (Inoh et al., 2001). An ethanol injection method is an easy method leading spontaneously to smaller particles (70 to 100 nm) without sonication (Maitani et al., 2001).

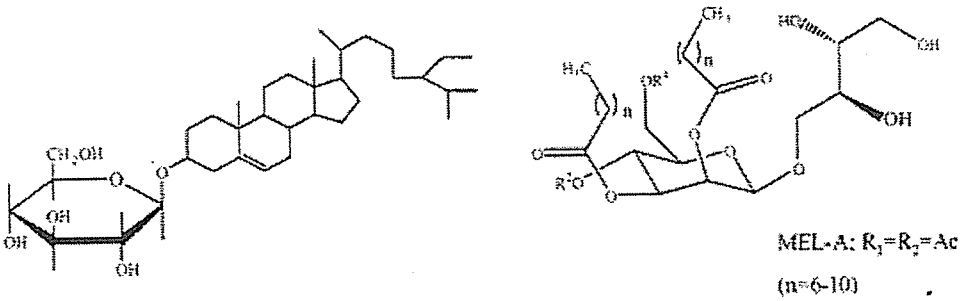
Tissue-targeted gene expression is an important issue for improvement of safety in gene therapy such as herpes simplex virus thymidine kinase (HSV-tk) gene therapy. Preferential expression of a suicide gene, HSV-tk, in tumor cells contributes to the safety and the efficacy of suicide gene therapy because nonspecific cytotoxicity to healthy cells can be minimized (Miller and Whelan, 1997). Expression of the midkine (MK) gene in human adult tissue is low and is restricted. However, its increased expression was observed in a variety of human tumors (Tsutsui et al., 1993; Miyauchi et al., 2001). A MK promoter may increase the selective thymidine kinase (TK) expression to the tumor.

In our study, liposome vector for lipoplexes was studied to optimize the formulae of injectable-sized lipoplexes with high transfection efficiency and to examine liposome-mediated TK gene expression controlled by the MK promoter in cell culture and in vivo growth inhibition of tumors in HSV-tk gene therapy.

Materials and Methods

Materials

3β[*N*-(*N'*,*N'*-Dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) and dioleoyl-3-trimetylammonium propane (DOTAP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Avanti Polar Lipids. (AL, USA), respectively. Sit-G was obtained from Essential Sterolin Product (Midland, South Africa). MEL (Fig. 1) was supplied by Dr. Kitamoto (National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan). Tfx20, commercial gene transfection reagent, was purchased from Promega (Madison, WI, USA). The Pica gene luciferase assay kit was purchased from Toyo Ink Mfg. Co. Ltd. (Tokyo, Japan). Bicinchonic acid (BCA) protein assay reagent was purchased from Pierce (Rockford, IL). The pDNA encoding the luciferase marker gene (pAAV-CMV-Luc) was supplied by Dr. Tanaka in Mt. Sinai School of Medicine



β-Sitosterol β-D-glucoside (Sit-G) Mannosylerythritol lipid A (MEL)

Figure 1. Chemical structures of β-sitosterol β-D-glucoside (Sit-G) and mannosylerythritol lipid A (MEL).

(NY, USA). pMK-tk plasmid coding for TK gene under the control of human midkine (MK) promoter, stretching from bp −2285 of 5′-upstream flanking region to bp 34 of exon 1 of the human MK gene, was inserted into pLXSN vector in the opposite direction to the long terminal repeat (Miyauchi et al., 2001). pHSV-tk plasmid coding for TK gene under the control of HSV promoter was constructed by insertion of 3.6-kb Bam HI fragment including HSV-1 TK gene into pSV2-neo plasmid (Hayashi et al., 2000). [^{3}H]Aciclovir (ACV) was obtained from Moravek Biochemicals, Inc. (Brea, CA). Ganciclovir (GCV) was kindly supplied by Glaxo Smith Kline Co. (Helis, UK). All reagents were of analytical grade. RPMI-1640 medium and fetal bovine serum (FBS, serum) were purchased from Invitrogen Corp. (Carlsbad, CA, USA).

Preparation of Liposomes

Liposome formulae were prepared with lipids (e.g., Sit-G-liposomes=DC-Chol/DOPE/Sit-G=2/2/1, molar ratio=10:5.4:13.8, weight [mg]) in 4 ml water by a modified ethanol injection method (Maitani et al., 2001). Aliquots of 1 μl of liposome suspensions were equivalent to about 4.7 nmol as positive charge of Sit-G-liposomes. Tfx20 particles were prepared according to the manufacturers' protocol.

Measurement of Size and ζ-Potential

The mean particle size and ζ-potential of liposomes and lipoplexes in water were determined using a light-scattering instrument (ELS-800, Otsuka Electronics Co., Ltd., Osaka, Japan) by a dynamic laser light-scattering method and an electrophoresis light-scattering method, respectively, at 25 ± 1°C.

Cell Culture

A human hepatoblastoma cell line, HepG2, was obtained from Riken Cell Bank (Ibaraki, Japan). A human cervix carcinoma cell line, HeLa 229, and a human pancreatic carcinoma cell line, AsPC-1, were supplied by Human Science Research Bank (Osaka, Japan) and Chiba Cancer Center Research Institute (Japan), respectively. Cells were maintained in RPMI1640 supplemented with 10% (v/v) heat-inactivated (56°C, 30 min) FBS (growth

medium) in a humidified incubator (5% CO_2) at 37°C. All cells were scraped off with trypsin-EDTA (0.05% trypsin, 53 mM EDTA-4Na) and seeded in growth medium 1 day before the experiment. For luciferase gene transfection, HepG2 cells were seeded at a density of 3×10^5 cells/well in 12-well tissue culture plates. For TK gene transfection, HepG2 and HeLa 229 cells were seeded at a density of 6×10^5 cells/dish in 35-mm culture dishes.

Transfection of Cells

Lipoplexes at charge ratios (+/−) of 1/1, 2/1, 3/1, 4/1, or 8/1 of cationic lipid to pDNA were formed by addition of liposomes to pDNA (1.26, 2.53, 3.79, 5.05, 10.1 µl of Sit-G-liposome suspension to 2 µg pDNA per well) with gentle shaking and leaving at room temperature for 10 to 15 min. Lipoplexes were diluted with medium containing no or 10% serum to a final concentration of 2 µg pDNA per well. After transfection in the medium without serum for 2 h, 1 ml of the growth medium was added to the wells and culture was continued for an additional 24 h. For transfection with 10% serum, each cell with lipoplexes was incubated for 24 h in the growth medium.

Expression Assays

Luciferase expression was measured according to the luciferase assay system. Incubation was terminated by washing the plates three times with cold PBS. Cell lysis solution (Pica gene) was added to the cell monolayers and subjected to one cycle of freezing (−70°C) and thawing at 37°C, followed by centrifugation at 13,000 rpm for 10 min. The supernatants were stored at −70°C until the assays. Aliquots of 10 µl of the supernatants of cell lysates were mixed with 100 µl of luciferase assay system (Pica gene) and relative light units (RLU) were measured with a chemoluminometer (Wallac ARVO SX 1420 multilabel counter, Perkin Elmer Life Science, Co. Ltd., Kanagawa, Japan). Protein concentration of the supernatants was determined with BCA reagent, using BSA as a standard, and RLU/mg protein was calculated.

An assay for TK expression was performed as follows: after a 24-h incubation, cells were washed with medium and cell culture was continued for an additional 48 h. Buffer (10 mM Tris-HCl pH7.5, 2 mM $MgCl_2$, 5 mM β-mercaptoethanol) was added to the washed cells and subjected to three cycles of freezing (−70°C) and thawing at 37°C, sonicated for 60 seconds, followed by centrifugation at 20,000xg for 30 min. The supernatants were stored at −80°C until the assays. Aliquots of 20 µl of the supernatants of cell homogenates were assayed by the TK enzyme assay using [^{3}H]ACV as a substrate (Hayashi et al., 2000).

Cell Viability Assay

HeLa 229 cells were seeded at a density of 1×10^4 cells per well in 96-well plates and maintained for 24 h before transfection in Minimal Essential Medium (MEM, Life Technologies, Inc., Grand Island, NY, USA) supplemented with 5% FBS. The culture medium was replaced with MEM (100 µL) supplemented with 5% FBS including various concentrations of liposomes or lipoplexes. The cells were incubated for 24 h. The number of surviving cells was determined by a WST-8 assay (Dojindo Laboratories, Kumamoto, Japan). Cell viability was expressed as the ratio of the A_{450} of cells treated with the liposomes or lipoplexes to that of the control samples.