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Direct Intratumoral Gene Transfer of the Herpes Simplex Virus Thymidine Kinase Gene with DNA-liposome Complexes: Growth Inhibition of Tumors and Lack of Localization in Normal Tissues

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To constitute the site-specific expression of the herpes simplex virus thymidine-kinase (*HSV-TK*) gene in tumor cells, we have assessed the promoter function of the simian virus 40 (SV40) promoter and the 5′ flanking region of c-*erb*B-2 gene using a luciferase-expressing reporter plasmid. After the transfection of the luciferase plasmid directed by the promoter region of c-*erb*B-2 gene, a large amount of luciferase activity was observed in c-*erb*B-2-expressing cells (Colo201, MCF-7, and HEC1-A), while none was detected in cells with no expression of c-*erb*B-2 protein (HRA and KF cells). On the other hand, a high level of luciferase activity was detected in all tumor cell lines tested, when the transfection was performed with SV40 promoter. The repeated transfection of the liposome-conjugated *HSV-TK* gene regulated by the SV40 promoter or by the promoter region of c-*erb*B-2 gene with cultivation in 100 μg/ml of aciclovir for 5 days *in vitro* resulted in growth inhibition for all four cell lines examined or for only c-*erb*B-2-expressing cells in the presence of SV40 promoter or c-*erb*B-2 promoter, respectively. Finally, direct injection of the DNA-liposome complex into established tumors in the presence of 50 mg/kg of aciclovir led to significant tumor volume reduction in all three tumors tested when SV40 promoter was employed. However, this anti-tumor effect was noted only in c-*erb*B-2-positive cells (Colo201 cells) upon intratumoral injection of *HSV-TK* gene regulated by c-*erb*B-2 promoter. In the case of intratumoral gene transfer, foreign DNA was detected in only one of seven mice by polymerase chain reaction (PCR) analysis performed 7 days following injection. When PCR analysis was carried out at 14 or 21 days following injection, no DNA signal was found at all. However, DNA was detected in several normal tissues at all three times tested in the case of intravenous injection. No abnormalities were seen in histologic examinations of normal tissues or in serum biochemical parameters following DNA liposome delivery. These results suggest that the direct gene transfer of *HSV-TK* gene regulated by tumor-specific transcriptional units may be one of the most clinically promising of the selective genetic strategies against cancer.

Key words: Liposome — Herpes simplex virus thymidine kinase — Gene therapy — c-*erb*B-2 promoter

The development of technology to introduce foreign genes into eukaryotic cells has opened up new possibilities for the therapy of cancer. One possible approach to the treatment of localized tumor is to render tumor cells susceptible to normally nontoxic prodrugs by using so-called suicide genes, such as herpes simplex virus thymidine-kinase (HSV-TK).[1–4] To achieve tissue-specific gene transduction, tumor-specific promoters can be utilized to direct expression of a suicide gene introduced by nonspecific transfection methods. For instance, α-fetoprotein or liver-associated albumin promoter region has been employed for hepatocellular carcinoma,[1] tyrosinase transcriptional regulatory sequences for malignant melanoma[5] and the secretory leukoprotease inhibitor (SLPI) for SLPI expressing carcinoma.[6] Recently we have obtained an anti-tumor effect against tumor cells expressing high levels of c-myc protein by transfecting HSV-TK plasmid promoted by the Myc/Max binding sequence, using DNA-liposome complexes.[7]

The c-*erb*B-2 gene encodes a transmembrane glycoprotein which is highly homologous with the epithelial growth factor-receptor and has tyrosine kinase activity.[8] Immunohistological study has revealed that this protein is commonly located in fetal epithelial cells, but is scarcely expressed in normal adult tissues. The c-*erb*B-2 gene has been found to be amplified or overexpressed in many human primary adenocarcinomas, especially those of the stomach, breast, colon and ovary.[9] The differential expression of c-*erb*B-2 gene is regulated at the transcriptional level, probably via the binding of a specific positive transcription factor(s) to the promoter region of the gene, raising the possibility that exogenous *erb*B-2 gene could be actively transcribed in tumors expressing a high level of c-*erb*B-2 protein.[10–13]

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Recently, a polycationic liposome consisting of a positively charged lipid has been developed as a DNA-introducing reagent,[14] and is being used in DNA delivery systems.[15,16] This reagent has numerous advantages over other methods and is simple to use.[17] However, several questions remain regarding toxicity or gonadal localization of *in vivo*-injected DNA-liposome complexes.

In this study, we used the promoter region of human c-*erb*B-2 gene for such an approach, and examined whether the transduction of *HSV-TK* gene into c-*erb*B-2 high-expressing cells under the control of c-*erb*B-2 promoter would generate sensitivity to aciclovir both *in vitro* and *in vivo*. In addition, we examined the toxicity of DNA-liposome complexes injected into tumors *in vivo*.

MATERIALS AND METHODS

Construction of plasmids Subcloning was carried out using standard recombinant DNA techniques. The expression plasmids used in this study are shown in Fig. 1. The luciferase expression plasmid PGV-B that contains the luciferase coding sequence without any promoter sequence and PGV-P regulated by the simian virus 40 (SV40) promoter and enhancer were purchased from Toyo Inki Mfg. Co., Ltd., Tokyo. PGV-EBP plasmid containing the luciferase sequence under the control of the c-*erb*B-2 promoter was constructed by cloning the 4.6 kb *Eco*R I-*Sma* I fragment of the promoter region of the c-*erb*B-2 gene[11] into the 5′-flanking region of the luciferase coding sequence of PGV-B. The plasmids PGV β-galactosidase (β-Gal) and pEBP β-Gal were constructed by replacing the luciferase gene in PGV-B and PGV-EBP with β-Gal gene in the plasmid pSV β-Gal (Promega, Madison, WI). Plasmids PGV-TK, pSV-TK and pEBP-TK were constructed by replacing the coding region of the luciferase gene (excised by *Hin*d III and *Bam*H I) in PGV-B, PGV-P and PGV-EBP with a 1760 bp BglII-PvuII fragment of HSV106 that contains the HSV-TK coding sequence.

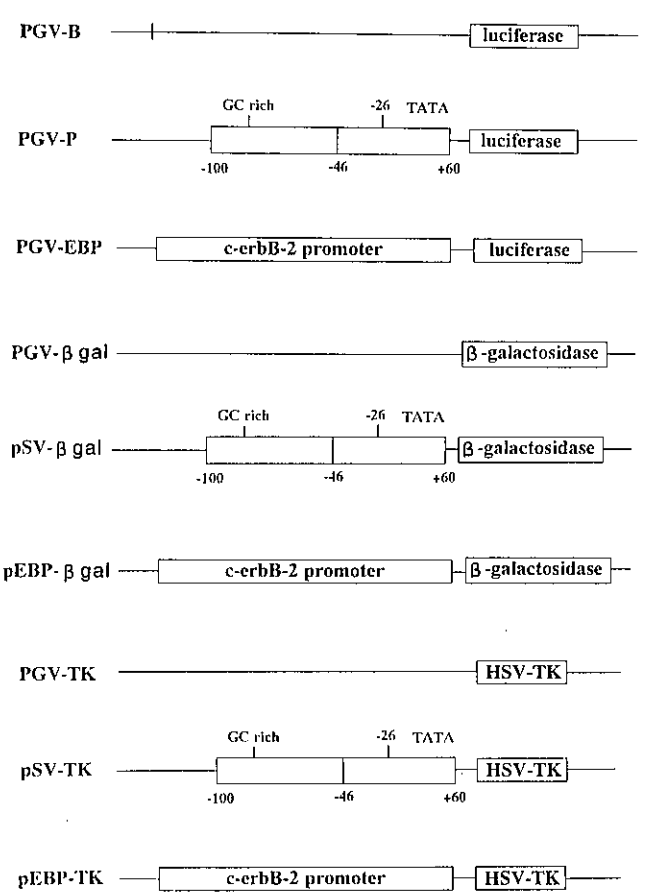

Fig. 1. Structure of expression plasmids. Abbreviations are as follows: TATA, TATA box; GC rich, GC-rich region (CCGCCC)[6]; SV, SV40 promoter; TK, herpes simplex virus thymidine kinase.

Cell culture Human colon adenocarcinoma Colo201 cells[18] were maintained in RPMI 1640 medium with 10% fetal calf serum. Human endometrial adenocarcinoma Hec-1A[19] cells were maintained in MEM medium with 10% fetal calf serum. Human mammary adenocarcinoma MCF-7,[20] and human ovarian adenocarcinoma KF and HRA[21] were maintained in DMEM medium with 10% fetal calf serum.

Flow cytometry Tumor cells (1×10^5) were washed in phenol red-free HBSS containing 1% fetal calf serum and 0.2% sodium azide at 4°C, stained with an appropriate fluorescein isothiocyanate-labeled monoclonal antibody (mAb) (0.1 mg/ml), incubated at 4°C for 45 min, washed twice, and resuspended in 0.5 ml of medium for FACS analysis (Beckton Dickinson and Co., Mountain View, CA). The mAb used was mouse mAb (OM-11-953: IgG) to human c-*erb*B-2 (Cambridge Research Biochemicals, Cambridge, UK).

Transfection DNA was introduced by using LipofectAMINE reagent (GIBCO, Grand Island, NY). LipofectAMINE reagent is a 3 : 1 (wt/wt) liposome formulation of the polycationic lipid, 2,3-diolexyl-*N*-[2-(spermine-carboxamine)ethyl]-*N*,*N*-dimethyl-1,1-propanaminium trifluoroacetate and the neutral lipid, diolexyl phosphatidylethanolamine in membrane-filtered water. Tumor cells (1×10^5) were plated in a 35-mm-diameter tissue culture dish the day before transfection. Three μg of plasmid DNA in 100 μl of opti-MEM (GIBCO) and 10 μg of lipofectAMINE in 100 μl of opti-MEM were mixed gently and incubated for 15–30 min at room temperature. Then 0.8 ml of serum-free medium was added to the DNA-liposome mixture and the DNA-liposome solution (final volume, 1 ml) was added to the culture after the cells had been washed twice with serum-free medium.

The cells were incubated with the DNA-liposome complex for 5–12 h at 37°C in a CO_2 incubator and then the medium was replaced with fresh medium containing fetal calf serum and incubation was continued for 24 to 36 h at 37°C.

Luciferase assay Cells were solubilized in lysis buffer (25 m*M* Tris-HCl, pH 7.4/1% Triton X-100/2m*M* DTT/2 m*M* 1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid/10% glycerol). Cell debris was spun down in a microcentrifuge, and 100 μl of cell extract was taken for luciferase assay. Luciferase activities were measured with a luminophotometer by using the reagents and protocol of a commercial luciferase assay kit (Toyo Inki, Tokyo). Luciferase activity was expressed as a relative light unit, which is the integrated value for 1 s of the light production per 1 mg protein of cell extract. Protein concentration was determined with BCA Protein Assay Reagent (Pierce, Rockfold, IL). The experiments were performed in triplicate.

***In vitro* susceptibility to killing by aciclovir** On day 0, adherent cells were split at a density of 5×10^4–5×10^5 cells per 35 mm plate and incubated at 37°C for 24 h. On day 1, the cells were overlaid with the DNA-liposome complex (3 μg of DNA and 10 μg of LipofectAMINE in 200 μl of opti-MEM) diluted in 1 ml of opti-MEM. After incubation for 5 h at 37°C, the cells were rinsed twice and replaced with growth media containing various concentrations (0, 0.1, 1, 10 and 100 μg/ml) of aciclovir (Wellcome Co., Ltd., Osaka). The same procedure of DNA transfection and exposure to aciclovir as described above was carried out from day 2 to day 5. On day 6, cells were detached from the dishes with 0.25% trypsin. The number of viable cells was determined by means of the trypan blue dye exclusion test and the survival rate was calculated as follows: survival rate (%) = number of aciclovir-treated cells /number of aciclovir-untreated cells $\times100$.

***In vivo* β-Gal plasmid transfection and X-Gal staining** Six days after subcutaneous (s.c.) inoculation of 5×10^6 -1×10^7 cells into nude mice at axillary positions, DNA-liposome complex (3 μg of β-Gal expression plasmid and 10 μg of LipofectAMINE) in 0.1 ml of lactate Ringer's solution was directly injected into established tumors. The tumors were removed 2 days later and resected tumor tissues were frozen in O.C.T. compound after fixation by immersion in a 4% paraformaldehyde solution for 4 h at 4°C. Cryostat sections 5 mm thick were prepared. Slides were incubated in X-Gal overnight and counterstained with hematoxylin.

DNA and reverse transcriptase (RT) PCR analysis Five to seven days after the inoculation of 1×10^7 tumor cells subcutaneously in the axillary position of nude mice, DNA-liposome complex (5 μg of pEBP-TK plasmid and 20 μg of lipofectAMINE) in 0.1 ml of lactate Ringer's solution was administered either by direct injection into established tumors or by intravenous injection via the tail vein using a 21 gauge needle. One, two and three weeks following DNA injection, mice were killed and the tumor, liver, kidney, lung, spleen and ovary were removed. For DNA PCR analysis, genomic DNA from the tumor and tissue samples was isolated. The primers were 5′-CGC-GAA-CAT-CTA-CAC-CAC-AC-3′ (sense) and 5′-GAT-AAA-GAC-GTG-CAT-GGG-AC-3′ (antisense), yielding a 687-bp fragment of *HSV-TK* gene. PCR was performed for 30 cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min), and polymerization (72°C, 2 min). Samples were analyzed by ethidium bromide staining on 1.5% agarose gel. For RT-PCR, total RNA was isolated from the tumors by acid guanidium thiocyanate-phenol-chloroform extraction.[22] After DNase digestion of RNA, random hexamer primers (Amersham Japan, Tokyo) were used to initiate reverse transcription from 5 μg of total RNA with RT. PCR was performed by using the same primers and 25 cycles of denaturation, annealing, and polymerization as described above. PCR products were analyzed by Southern blot hybridization using standard methods with a ^{32}P-labeled HSV-TK cDNA probe.[22]

Intratumoral injection of DNA-liposome complex Tumor cells (1×10^7) were injected s.c. into the axillary position of six-week-old female CD-1 nude mice (*nu/nu*). Three to six days after injection of tumor cells, when the tumor size had reached 50 to 70 mm^3, DNA-liposome complex (3 μg of PGV-TK, pSV-TK or pEBP-TK and 10 μg of LipofectAMINE) in 0.1 ml of lactate Ringer's solution was directly injected into established tumors daily for 14 days, and aciclovir (50 mg/kg) in 100 μl volumes was administered i.p. simultaneously. Control mice received no injection of DNA-liposome complexes into the tumors and were given only aciclovir. Tumor volume was calculated by using a standard formula.

$$\text{Tumor volume} = \text{width}^2 \times \text{length} \times 0.5$$

***In vivo* toxicity assay** Blood was collected from the tail vein immediately prior to inoculation of DNA-liposome complexes and 18–24 days thereafter, and serum samples were stored frozen at −20°C until measurement of tissue-specific enzymes and routine biochemical parameters.

RESULTS

Expression of c-*erb*B-2 gene in tumor cell lines To evaluate the surface expression of c-*erb*B-2 protein on the cell lines, Colo201, Hec1-A, MCF-7, HRA and KF, flow cytometric analysis employing mouse mAb was performed. As shown in Fig. 2, anti-c-*erb*B-2 mAb reacted strongly with Colo201, Hec1-A and MCF-7 cells. Virtually all the cells were positive for c-*erb*B-2 protein as

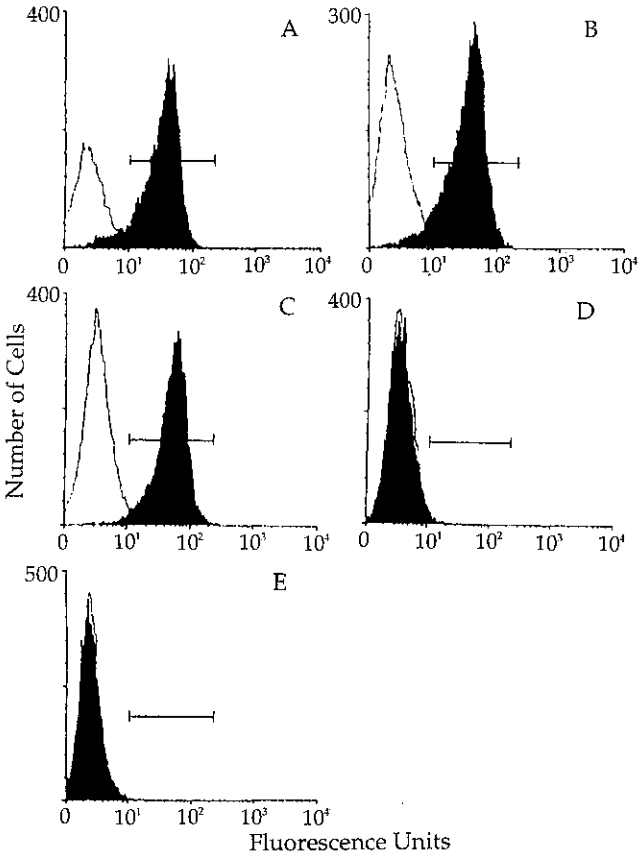

Fig. 2. Cell surface expression of the c-*erb*B-2 protein. Single cell suspensions derived from different monolayer cultures by treatment with trypsin and EDTA were stained with either FITC-labeled irrelevant mouse monoclonal antibody or FITC-labeled mouse monoclonal antibody to human c-*erb*B-2 protein (OM-11-953) and analyzed by flow microfluorometry in a FACS II system. Cell lines: A, Colo201, B, MCF-7; C, Hec1A; D, KF; E, HRA.

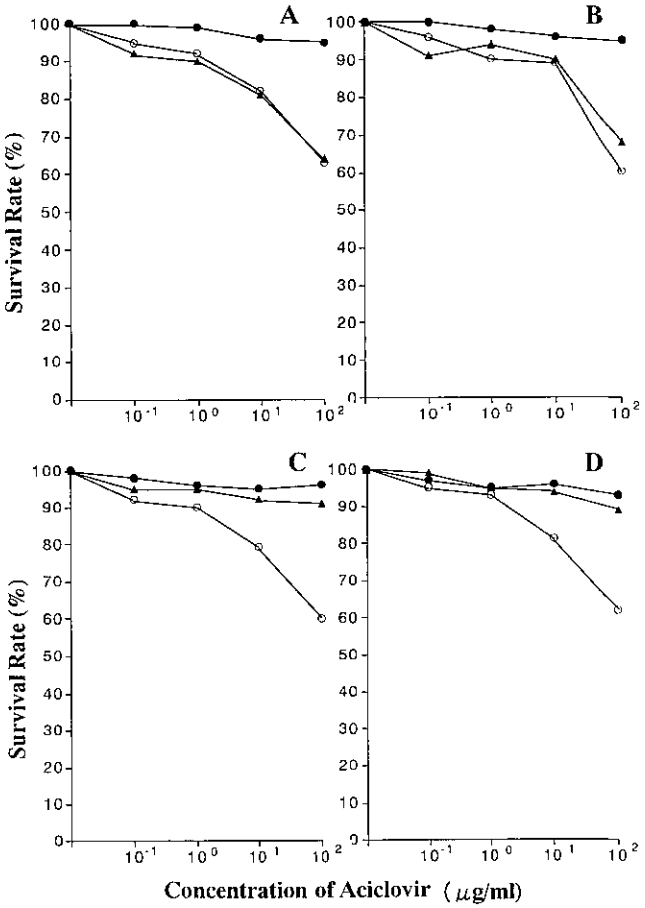

Fig. 3. Effect of HSV-TK transfection and aciclovir administration *in vitro*. Cells were transfected with PGV-TK, pSV-TK or pEBP-TK plasmid and liposomes daily for 5 days and exposed to 0, 0.1, 1, 10 and 100 μg/ml of aciclovir for 5 days. Cell lines; A, Colo201; B, MCF-7; C, KF; D, HRA; ●, PGV-TK; ○, pSV-TK; ▲, pEBP-TK.

evidenced by a single fluorescent cell peak. In contrast, neither HRA nor KF cells showed binding by mAb indicating the absence of expression of c-*erb*B-2 protein on these cell lines.

Transcriptional activity of c-*erb*B-2 promoter region To examine transcriptional activity of the SV40 promoter and c-*erb*B-2 promoter, we transfected three reporter plasmids into various tumor cell lines. The transcriptional activity of c-*erb*B-2 promoter as shown by luciferase assay was restricted to tumor cell lines expressing c-*erb*B-2 protein, although SV40 promoter expressed high levels of luciferase activity in all five cell lines (Table I). The level of transcriptional activity of c-*erb*B-2 promoter in these c-*erb*B-2 positive cell lines was 50 to 75% of that of SV40 promoter, suggesting that the function of c-

Table I. Transcriptional Activity of SV40 Promoter and the Promoter Region of c-*erb*B-2 Gene

Cell line	Luciferase activity (RLU)		
	PGV-B	PGV-P	PGV-EBP
Colo201	4.2±1.2	1882±320	1419±203
MCF-7	7.3±2.3	2783±421	1981±196
HEC-1A	4.2±1.0	2071±515	1047±103
HRA	6.6±4.1	1269±347	8.8±1.0
KF	6.0±3.6	2148±402	8.3±2.3

Luciferase activity was expressed as a relative light unit (RLU), which is the integrated value for 1 sec of the light production per 1 mg protein of cell extract. Values are expressed as mean±SD.

*erb*B-2 promoter was weaker than that of SV40 even in cells expressing high levels of c-*erb*B-2 protein. No transcriptional activity of c-*erb*B-2 promoter was observed in tumor cell lines that do not express c-*erb*B-2 protein.

***In vitro* susceptibility to killing by aciclovir** *In vitro* sensitivity to killing by aciclovir was assessed to determine whether or not the introduced pEBP-TK plasmid could render tumor cells sensitive to aciclovir. As foreign genes transfected with liposome complexes are generally retained in the cytoplasm and their expression is transient, we conducted repeated gene transfer of the HSV-TK plasmid for 5 days. As shown in Fig. 3A, the survival rate in 10 and 100 μg/ml aciclovir was 81% and 64 % for pEBP-TK transfected Colo201 cells, and 82% and 63% for pSV-TK transfected Colo201 cells, respectively. In pEBP-TK and pSV-TK transfection experiments into MCF-7 cells, the respective rates for 10 and 100 μg/ml of aciclovir were 90% and 68%, and 89% and 66% (Fig. 3B). However, in the case of HRA and KF cells that express extremely low levels of c-*erb*B-2 protein, a reduced survival rate was observed only with pSV-TK transfected cells (at 100 μg/ml of aciclovir, 60% for HRA cells and 62% for KF cells), but not with pEBP-TK transfected cells (at 100 μg/ml of aciclovir, 94% for HRA and 90% for KF cells) (Fig. 3, C and D). When the *PGV-TK* gene was transfected into the cells, high survival rates at various concentrations of aciclovir were observed (the survival rates at 100 μg/ml of aciclovir were 96%, 98%, 98% and 97% for Colo201, MCF-7, HRA and KF cells, respectively).

***In vivo* β-Gal activity** To evaluate the *in situ* transfection efficiency, liposomes conjugated with a plasmid encoding β-Gal were injected once into established tumors, Colo201, HRA and KF, in nude mice. Two days later, tumor tissues were resected and X-Gal staining was performed on 5 μm thick cryostat sections. In the case of PGV-β-Gal plasmid transfection, the percentage of blue-stained cells was less than 0.1% in all three cell lines. X-Gal-positive cells after *pSV-β-Gal* gene transfection amounted to 0.9%, 0.8% and 1.0% of Colo201, HRA and KF cells, respectively. In the case of pEBP-β-Gal transfection, substantial numbers of cells were stained only among c-*erb*B-2-positive Colo201 cells (1.1%), but not HRA or KF cells ($P<0.1\%$) (Table II).

Table II. *In Vivo* Transfection Efficiency

Cell line	Transfection efficiency		
	PGV-β gal	pSV-β gal	EBP-β gal
Colo201	<0.1	0.9	1.1
HRA	<0.1	0.8	<0.1
KF	<0.1	1.0	<0.1

Injection of plasmid DNA was performed into tumors in nude mice 6 days after tumor cell inoculation. Tumors were removed and X-gal staining was carried out 48 h after DNA transfection, as described in "Materials and Methods." At least 1,000 cells were counted and the percentage of blue-stained cells was calculated.

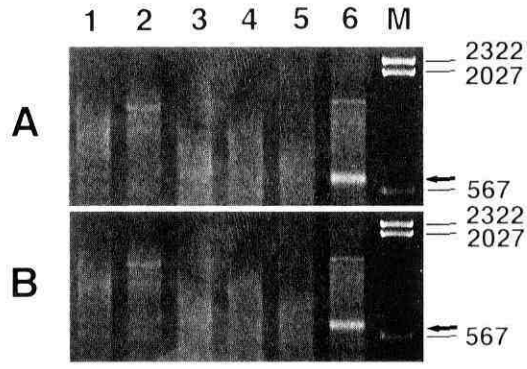

Fig. 4. Detection of HSV-TK DNA in mouse tissues following direct intratumoral gene transfer. HSV-TK plasmid DNA (major band with expected size of 687 bp fragment) was not detected in lung (lane 1), liver (lane 2), kidney (lane 3), spleen (lane 4) or ovary (lane 5). The presence of HSV-TK DNA in transduced tumor tissue (lane 6) was detected 7 days following injection. Gene transduction was performed into 2 cell lines; Colo201 cells (A) with high expression of c-*erb*B-2 protein and HRA cells (B) with no expression of c-*erb*B-2 protein.

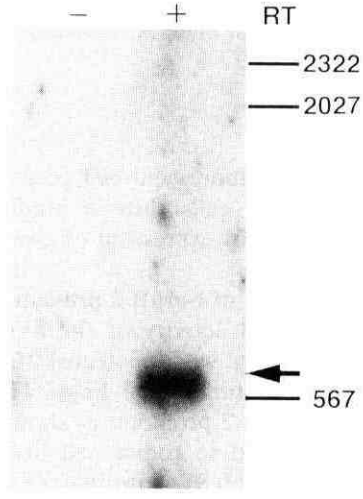

Fig. 5. Expression of HSV-TK DNA in tumors of Colo201 cell lines. Southern blot analysis showing a 687 bp band of RT-PCR products in tumor tissue of Colo201 cells with reverse transcriptase (+) or without reverse transcriptase (−).

Distribution of DNA-liposome complexes after intravenous or intratumoral administration To determine whether DNA-liposome transfection resulted in uptake of plasmid DNA into normal tissues including gonadal tissue, we performed PCR analysis of tumor and normal tissues. As shown in Fig. 4, in mice subjected to direct gene transfer into tumor tissues, a major band with the expected size of 687 bp was observed only in tumor tissues of either Colo201 or HRA, but not in liver, kidney, spleen, lung or ovary from the same mice 7 days following injection. To confirm the expression of HSV-TK gene following direct intratumoral injection of DNA-liposome complex, we performed RT PCR. Using the RT-PCR technique, we detected 687 bp exogenous HSV-TK mRNA in Colo201 tumor (Fig. 5), The findings from direct gene transfer or intravenous injection using DNA-liposome complexes are summarized in Table III. In the intravenous injection model, plasmid DNA was occasionally detected in liver, lung and spleen tissue of treated mice, while PCR signals were not detected in tumor, kidney or ovary at any time tested. In contrast, in the case of intratumoral injection, plasmid DNA was detected only in tumor tissue, but not in normal tissues such as liver, kidney, lung, spleen and ovary at any time tested. Exceptionally, plasmid DNA was detected once in the liver of one of seven treated mice 7 days following DNA transfection, although m-RNA of plasmid DNA was not detected by means of RT-PCR (data not shown).

Table III. Localization of Plasmid DNA by PCR Analysis after Direct Gene Transfer *in Vivo*

Organ	Site of introduction					
	Intravenous			Intratumoral		
	Days after injection			Days after injection		
	7 ($n=6$)	14 ($n=7$)	21 ($n=5$)	7 ($n=7$)	14 ($n=8$)	21 ($n=3$)
Tumor	0/6	0/7	ND	7/7	4/8	3/3
Lung	3/6	3/7	1/5	0/7	0/8	0/3
Liver	2/6	1/7	1/5	1/7	0/8	0/3
Kidney	0/6	0/7	0/5	0/7	0/8	0/3
Spleen	3/6	2/7	1/5	0/7	0/8	0/3
Ovary	0/6	0/7	0/5	0/7	0/8	0/3

Mice were injected with 0.1 ml of the mixture of DNA and liposome, and DNA was extracted from the indicated tissues for analysis by PCR. Mice received injection into the tail vein or into Colo201 tumors previously inoculated into the axillary position. Tumor diameter at the time of injection was 50–80 mm. ND: not detected.

Tumor regression following direct intratumoral injection of pEBP-TK and pSV-TK Since MCF7 and Hec 1A cells could not establish tumors in nude mouse, Colo201, HRA and KF cells were employed in this *in vivo* experiment. When the tumor size reached 50–70 mm^3 in volume (3 to 6 days after the inoculation of tumor cells at

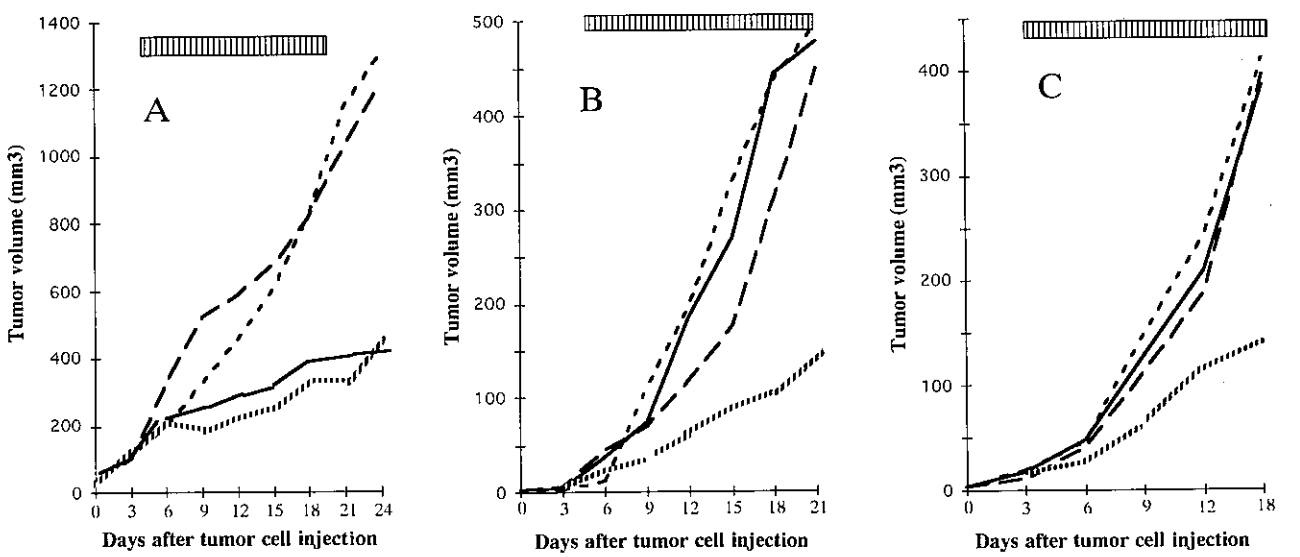

Fig. 6. Antitumor effect of intratumoral gene transfer of HSV-TK gene *in vivo*. Three to six days after tumor cell inoculation, DNA-liposome complex (plasmid DNA 3 μg plus lipofectAMINE 10 μg) was directly injected into established tumors daily for 14 days and aciclovir (50 mg/kg) was administered i.p. at the same time. Treatment groups ($n=6$/each) were injected with pSV-TK plus aciclovir (IIII) or pEBP-TK plus aciclovir (—). Control groups ($n=6$/each) were injected with aciclovir only (--) or PGV-TK plus aciclovir (····). Cell lines: A, Colo201; B, HRA; C, KF.

Table IV. Evaluation of Serum Biochemical Parameters after *in Vivo* Gene Transfer in Nude Mice

	pEBP-TK + Liposome + Aciclovir ($n=4$)	Liposome + Aciclovir ($n=4$)	Aciclovir only ($n=4$)	Saline control ($n=6$)	Preinjection ($n=12$)
GOT (IU/liter)	155.2	152.6	125.9	123.4	132.4
GPT (IU/liter)	35.8	34.3	40.0	41.6	39.4
LDH (IU/liter)	1935.4	1964.5	2140.1	2200.6	1938.1
ALP (IU/liter)	145.0	150.0	185.0	180.0	161.4
T-Bil (mg/dl)	0.3	0.3	0.3	0.3	0.3
BUN (mg/dl)	22.5	22.5	21.5	21.0	22.0
Creatinine (mg/dl)	0.1	0.1	0.2	0.1	0.1
Weight of liver (g)	1.50	1.47	1.40	1.42	
Weight of spleen (g)	0.21	0.15	0.16	0.17	
Weight of kidney (g)	0.38	0.43	0.49	0.48	
Whole body weight (g)	22.0	24.0	24.0	22.5	

Blood samples were obtained from CD-1 female nude mice prior to intratumoral injection and at termination after 18–24 days.

axillary positions in nude mice), established tumors were treated with repeated injections of DNA-liposome conjugate with the simultaneous administration of aciclovir for 14 days. In this experiment, we used two control groups, of which one received PGV-TK plasmid injection into tumors with aciclovir and the other received only aciclovir ($n=6$/groups), as a preliminary study revealed no difference among PGV-TK injection plus saline administration, PGV-EBP plus aciclovir administration, pEBP-TK plus saline administration and only saline administration, in terms of growth rate (data not shown). As shown in Fig. 6A, the growth of Colo201 tumors injected with pSV-TK or pEBP-TK plus aciclovir started to be suppressed after the fifth or sixth injection. On day 24, the reduction of tumor size in the pSV-TK or pEBP-TK treated groups was statistically significant when compared with the control groups ($P<0.01$). The rates of tumor reduction in the pSV-TK and pPEB-TK groups were almost equal and no statistically significant difference was observed. The median values of tumor volume in Colo201 on day 24 were 430 mm^3 (pSV-TK plus aciclovir), 384 mm^3 (pEBP-TK plus aciclovir), 1193 mm^3 (PGV-TK plus aciclovir) and 1255 mm^3 (aciclovir only). In contrast, in the cases of KF and HRA cells with no expression of c-*erb*B-2 protein, suppression of tumor growth was noted only in the pSV-TK treated group, but not in the pEBP-TK- or PBV-TK-treated group (Fig. 6, B and C). The tumor volume in the pSV-TK-treated group was clearly reduced (153 mm^3 on day 21 for HRA and 149 mm^3 on day 18 for KF) as compared with the control groups (500 mm^3 on day 21 for HRA and 430 mm^3 on day 18 for KF), and the difference between the two groups is statistically significant ($P<0.05$). No structural destruction was observed by histopathological analysis in tumor tissues. In accordance with the results of *in vitro* experiments, the injection of HSV-TK plasmid regulated by the promoter region of the c-*erb*B-2 gene in the presence of aciclovir had an anti-tumor effect against cells expressing high levels of c-*erb*B-2 protein, while the SV40 promoter actively drove the HSV-TK gene in all tumor cells examined and consequently reduced the tumor burden.

Organ toxicity after *in vivo* gene transfer To determine whether there is any tissue toxicity associated with the use of liposomes for the gene transfer, organs and blood samples from experimental animals were analyzed and compared with those from normal controls. No histopathological abnormalities or changes of weight of major organs, such as liver, kidney and spleen, were found in the experimental animals (Table IV). In addition to this analysis of organ toxicity, we performed routine analysis of biochemical parameters of blood samples. Blood samples collected from animals given DNA-liposome complex and 50 mg/kg of aciclovir daily for 14 days were compared with those of control animals. No statistically significant difference in liver and renal functions was found among the four groups: DNA, liposome and aciclovir group, liposome and aciclovir group, aciclovir group and saline control group (Table IV).

DISCUSSION

The capability to introduce a suicide gene into tumor cells *in vivo* is critical for the establishment of an effective gene therapy for cancer. To achieve tumor-specific expression, we must deliver the suicide gene directly to tumor cells, and in consequence, the introduced gene has to be actively transcribed only in the target cells by an

appropriate promoter or enhancer system. In this study, we have investigated the feasibility of tumor-specific expression of the *HSV-TK* gene directed by the promoter region of the c-*erb*B-2 gene and the ability of such expression to enhance the sensitivity of tumor cells to aciclovir.

First, we confirmed that the promoter region of the c-*erb*B-2 gene has transcriptional activity in tumor cells expressing high levels of c-*erb*B-2 protein. Secondly, we demonstrated that c-*erb*B-2-promoted *HSV-TK* gene transduction rendered only cells with high levels of c-*erb*B-2 protein chemosensitive to aciclovir in *in vitro* experiments. In contrast, in the case of pSV-TK plasmid transfection, aciclovir administration *in vivo* and *in vitro* showed significant toxicity in all cells tested, including HRA and KF cells with no expression of c-*erb*B-2 protein, indicating that the SV40 promoter is transcriptionally active in a wide variety of tumor cells. Nevertheless, tumor-specific expression directed by c-*erb*B-2 promoter or Myc/Max binding sequence is considered to be more convenient for the clinical trial of gene therapy, since it is difficult to eliminate the possibility that the surrounding normal cells could be harmed by the administration of aciclovir when the *HSV-TK* gene promoted by the SV40 promoter is introduced.

Although it is possible to express introduced genes in a tissue-specific fashion by using cell-specific promoters or enhancers, the establishment of technology to introduce foreign genes into target tissues has been difficult. As new approaches to site-specific gene expression *in vivo*, non-viral DNA delivery and expression systems have been developed, such as cationic liposomes,[14–16] lipopolyamine[23] and lipopolylysine.[24] Cationic lipids can be formulated which contain hydrolyzable bonds, so that biodegradation can occur after they have fused with the cell membranes and delivered recombinant genes to cells. These gene transfer systems are effective in terms of transfection in episomal forms and are universal as regards cell type. They have other advantages over the retroviral-mediated gene transfer system, such as capability to transfer a gene into nondividing cells and no risk of helper virus production. In addition, the risk of insertional mutagenesis associated with gene transfer using a DNA-liposome complex is extremely low compared with that which would be expected in retrovirus gene tranfer protocols.[25, 26] The DNA/liposome ratio and the absolute concentration of the DNA/liposome complex are critical for achieving high rates of transfection efficiency. We found the optimal conditions were 3 μg of DNA and 10 μg of lipofectAMINE in 1 ml of opti-MEM to achieve maximum transfection efficiency in HeLa cells, which can be transfected easily by conventional calcium phosphate precipitation methods. For our *in vivo* study, we employed 3 μg of DNA and 10 μg of lipid in 0.1 ml of lactate Ringer's solution, which showed maximum transfection efficiency in our previous *in vivo* study.[7] Despite low transfection efficiency, the tumor burden on nude mice was reduced by the repeated direct intratumoral injection of DNA-liposome complexes, indicating that expression of the *HSV-TK* gene in only a very small number of tumor cells was sufficient to induce an antitumor effect. This "bystander effect" is thought to generate cytotoxicity to nearby non transduced cells, which is extremely important, because it is not necessary to deliver the suicide gene to every tumor cell *in vivo*.[27–29] The mechanism of the bystander effect is still unknown, although several possible explanations have been put forward, such as the formation of apoptotic vesicles by neighboring HSV-TK-non expressing cells, the transfer of toxic molecules from HSV-TK-expressing cells to non-expressing cells through gap-junctions, and/or the enhancement of immune response to the release of cytokines by apoptotic cell death.[30–32] In our experiment, the aciclovir-exposed *in vivo*-transduced tumors revealed chromatin condensation and vesicle formation, which are characteristic features of apoptosis (data not shown). This bystander effect might have contributed to tumor regression in our experiments *in vivo* and *in vitro*.

For clinical applications, the potential long-term complications of intratumoral gene transfer with DNA-liposome complexes have to be addressed. In this study, the toxicity of the cationic lipid mixture and the uptake of foreign DNA in normal tissues including gonadal tissue were evaluated. We found no evidence of acute adverse reaction in mice. There was no mortality or chronic toxicity detectable by biochemical or histopathological analysis in mice given intravenous or intratumoral injection of DNA-liposome complexes. Finally, we performed PCR analysis to examine the possibility of transfection into normal organs following either intratumoral or intravenous gene transfer. PCR analysis revealed that DNA was localized frequently in lung, liver and spleen after intravenous injection of DNA-liposome complex. It is not clear whether DNA is localized in these organs because they are the first tissues encountered by the DNA in the circulation or because the DNA-liposome complex has a specific affinity for these tissues. In contrast, we found by PCR analysis that DNA is localized only in tumor tissues after intratumoral injection with DNA-liposome complexes, except for a PCR signal observed in the liver of one of seven mice in the PCR experiment performed at one week after injection. Since PCR technology can detect DNA at an extremely low copy number, these data suggested that gene transfer in normal tissues is unlikely to occur in the case of the intratumoral gene transfer method.

The major concern in the utilization of DNA for therapeutic purpose is to eliminate the possibility of intro-

ducing genes into normal tissues, including gonadal tissue. Taken together with related reports that uptake of DNA in normal organs was minimal in animal studies,[33–35] our finding suggests that DNA conjugated with cationic lipid is a promising approach to the delivery of suicide genes to a specific tumor site, and clinical trials of cancer therapy using this system may be justified.

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