Treatment of Xenografts by Transfection with Liposome Vectors

Seven-week-old female BALB/c nu/nu nude mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). The mice were maintained in accordance to institutional guidelines of the Hoshi University Animal Care and Use committee. Mice were inoculated subcutaneously with 1×10^6 AsPC-1 cells that had been resuspended in 100 µl of growth medium. Using Sit-G- and MEL-liposome, four groups were divided according to the treatment schedules: pMK-tk with Sit-G-liposome injection and GCV treatment; pMK-tk with MEL-liposome injection and GCV treatment; PBS injection (50 µl) instead of pMK-tk and GCV treatment; PBS injection (50 µl) instead of pMK-tk and PBS injection instead of GCV treatment. On day 0 after tumor inoculation when tumors had reached a volume of more than 200 mm^3, pMK-tk (20 µg/mouse) with liposome vectors was injected directly into the tumor once every two days 4 times for 7 days (days 0, 2, 4, and 6 after tumor inoculation). Starting 1 day after transfection of pMK-tk with liposome vectors, three to seven tumor/groups were treated intraperitoneally twice a day 4 times for 7 days with GCV (0.4 mg/mouse/day) (days 1, 3, 5, and 7 after tumor inoculation). Next, for long-term observation using Sit-G-liposome, two groups were divided according to the treatment schedules: pMK-tk with Sit-G-liposome injection and GCV treatment; Sit-G-liposome injection and GCV treatment. On day 0 after tumor inoculation when tumors had reached a volume of about 200 mm^3, pMK-tk (20 µg/mouse) with Sit-G-liposome or Sit-G-liposome alone was injected directly into the tumor once every two days 5 times for 9 days (days 0, 2, 4, 6, and 8 after tumor inoculation). Starting 1 day after transfection of pMK-tk with liposome vectors, three to seven tumor/groups were treated intraperitoneally twice a day 5 times for 9 days with GCV (0.4 mg/mouse/day) (days 1, 3, 5, 7, and 9 after tumor inoculation). Tumor volume was calculated using the formula $\pi/6 \times$ larger diameter $\times$ (smaller diameter)^2.

Statistical Analysis

Statistical significance of the data was evaluated by the Student's t-test. A P value of 0.05 or less was considered significant. All experiments were repeated at least two times.

Results

Liposome Formulae Containing Sit-G

It was reported that liposomes composed of Tfx20, DC-Chol, and Sit-G (Tfx20/DC-Chol/Sit-G=1.3/2/1, weight ratio) can form easily dispersed lipoplexes even in the presence of serum (Hwang et al., 2001). To enhance transfection efficiency, we improved formulation with Sit-G. We selected the combination of cationic lipid and helper lipid, DC-Chol/DOPE and DOTAP/Chol, because they were reported to show high transfection efficiency in vivo (Li et al., 1998; Wang et al., 1998; Kawakami et al., 2000). DOTAP is cationic lipid with oleoyl chains and tertiary amine, DC-Chol with cholesterol moiety and quaternary amine. The concentration of Sit-G in the liposome formulae can be increased to 26 mol% maximum (Shimizu et al., 1996). We prepared two formulations of liposome containing 20 to 25 mol% Sit-G in DC-Chol/DOPE (DC-Chol/DOPE/Sit-G (2/2/1, mole) and DOTAP/Chol (DOTAP/Chol/Sit-G (2/1/1, mole). These liposomes were stable and could be stored for several months at room temperature without any change in particle size (data not shown). After addition of Sit-G, the diameter of DC-Chol/DOPE liposomes increased from about 120 nm to 163 nm (Table 1),

Table 1
Diameter, ζ-potential, and transfection activity of Sit-G- and MEL-liposomes and diameter of lipoplexes

Liposome	Formula Molar ratio	Diameter (nm)	Polydispersity	ζ-Potential (mV)	Diameter (nm) of lipoplex[a]	Luciferase activity (× 10^7 RLU/mg protein)[b]
Control	DC-Chol DOPE (3/2)	120.2 ± 1.3	0.172	61.5	Aggregation	3.7 ± 1.1
Sit-G	DC-Chol DOPE/Sit-G (2/2/1)	163.7 ± 3.0	0.112	65.5	336.6 ± 20.1	15.1 ± 8.3
MEL	DC-Chol DOPE/MEL (3/2/2)	85.7 ± 9.7	0.293	48.7	306.2 ± 15.6	5.7 ± 1.7

[a]Charge ratio (+/−) of liposome/DNA=3/1 in water, [b]Charge ratio (+/−) of liposome/DNA=3/1 in medium with 10% serum. Data of Sit-G-liposome and MEL-liposome from Fig. 2 (a) and MEL(2) in Fig. 3, respectively. Diameter and luciferase activity are shown as the mean ± *SD* ($n = 3$). ζ-Potential is shown as the mean ($n = 2$).

and that of DOTAP/Chol liposomes from about 85 nm to 175 nm (data not shown). The ζ-potential of these liposomes was about 50 mV to 60 mV.

For optimization of the charge ratio (+/−) of two types of liposome (DC-Chol/DOPE and DOTAP/Chol) containing Sit-G to pDNA, luciferase activity was measured in HepG2 cells transfected with liposomes at various charge ratios (+/−) in medium with or without serum, as shown in Fig. 2. In the presence of serum, the charge ratio (+/−) in the range of about 2/1 to 3/1 showed the highest transfection efficiency, and particularly at the charge ratio (+/−) of 3/1 in liposomes, the transfection efficiency increased in the presence of serum. DC-Chol/DOPE system in the presence of serum seemed to show higher transfection efficiency than DOTAP/Chol one. Tfx20 showed $1.01 \pm 0.04 \times 10^8$ (RLU/mg protein) luciferase activity at the optimal charge ratio (+/−) of 2/1 when incubated in the medium without serum. Sit-G-liposomes (DC-Chol/DOPE/Sit-G [2/2/1], mole, at the charge ratio [+/−] of 3/1 in Fig. 2) showed comparable transfection efficiency ($1.51 \pm 0.83 \times 10^8$ RLU/mg protein) to that by Tfx20 (charge ratio [+/−]=2/1) even in the presence of serum (Table 1). Inhibition of lipofection by serum has often been reported (Faneca et al., 2004). However, Sit-G-liposomes increased the transfection efficiency despite the presence of serum in the medium. In further studies, therefore we used the DC-Chol/DOPE system and a charge ratio (+/−) of 3/1 of liposome to pDNA as Sit-G-liposomes.

Liposome Formulae Containing MEL

A biosurfactant of MEL produced by yeast has mannose and erythritol residues in its molecule; therefore, we expected that this might also be able to form small-sized lipoplexes

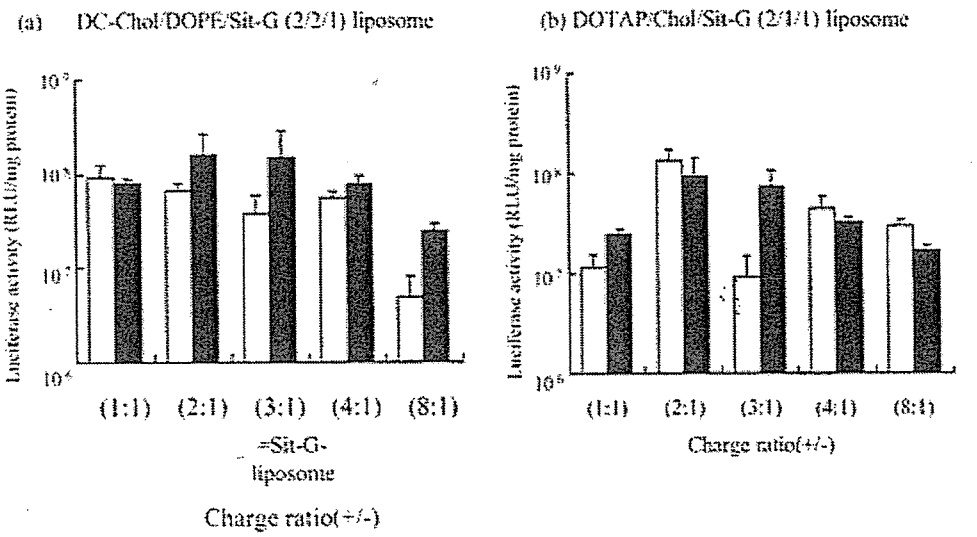

Figure 2. Gene expressions in HepG2 cells transfected with two types of liposomes (DC-Chol/DOPE [a] and DOTAP/Chol [b]) containing Sit-G at various charge ratios (+/−) in medium with or without 10% serum. HepG2 (at a density of 3×10^5 cells/well) was seeded in 12-well plates 24 h before transfection. Liposome/pDNA complexes were diluted with medium with or without serum to a final concentration of 2 μg pDNA in 1 ml medium per well. After transfection in the media without serum for 2 h, 1 ml of the medium was added to the wells and culture was continued for an additional 24 h. For transfection with 10% serum, each cell was incubated for 24 h in medium. Each column represents the mean ± *SD* (n=3–6). (□) without serum; (■) with 10% serum.

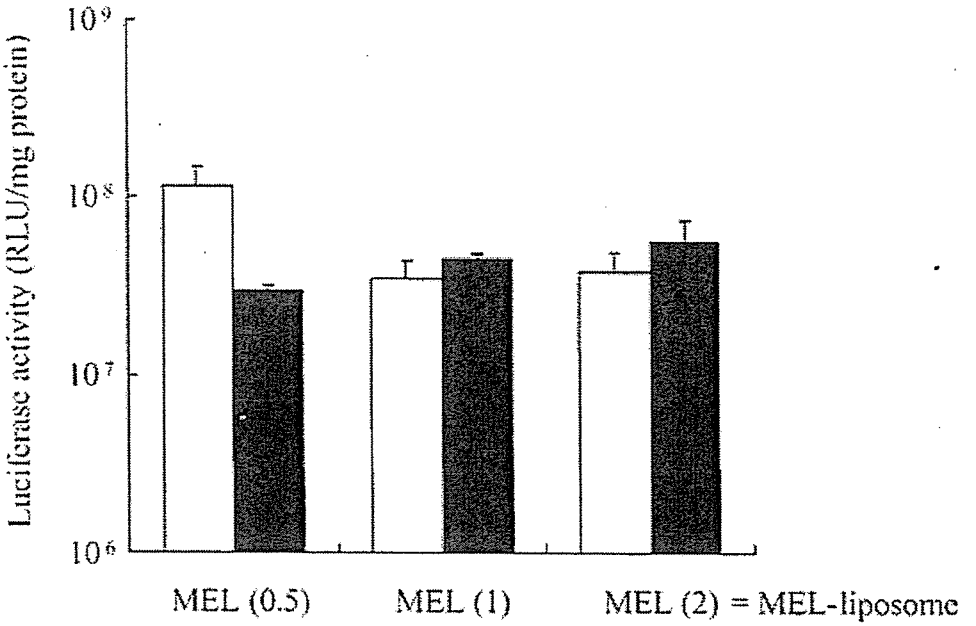

Figure 3. Effect of MEL contained in liposomes (DC-Chol/DOPE/MEL=3/2/0.5–2, molar ratio) on gene expression in HepG2 cells in medium with or without 10% serum. Aliquots of 2 μg of pDNA (pAAV-CMV-luc) were complexed with each liposome at a charge ratio (+/−) of 3 per well. Other experimental conditions were identical to those described in the Fig. 2 legend. Each column represents the mean ± *SD* (n=3–6). MEL (0.5)=DC-Chol/DOPE/MEL=3/2/0.5, molar ratio, MEL (1)=DC-Chol/DOPE/MEL=3/2/1, MEL (2)=DC-Chol/DOPE/MEL=3/2/2. (□) without serum; (■) with 10% serum.

like Sit-G. When the molar ratio of MEL to DC-Chol/DOPE (= 3/2, mole) was changed from 0 to 0.5, 1, and 2 (corresponding to MEL [0], MEL [0.5], MEL [1], and MEL [2] liposomes, respectively), the diameter of liposomes containing MEL was about 80 nm, while liposomes without MEL were about 120 nm in size. These liposome suspensions were stable and could be stored for several months at room temperature without any change in particle size (data not shown).

The effect of MEL in liposomes at various additional ratios of MEL at the charge ratio (+/−) of 3/1 was investigated on gene expression in the medium with or without serum (Fig. 3). When MEL was increased, the transfection efficiency was increased slightly, and MEL (2) showed the highest luciferase activity in the presence of serum. Therefore, in the following studies, we used MEL (2) liposomes as MEL-liposomes.

Control liposomes composed of DC-Chol/DOPE produced aggregation when mixed with pDNA at the charge ratio (+/−) of 3/1, while Sit-G- and MEL-liposomes formed about 300-nm-sized Sit-G- or MEL-lipoplexes, showing high transfection efficiency (Table 1).

Cytotoxicity

We examined the cytotoxicity of liposome vectors (Fig. 4). The concentration of the cationic lipid used in the cell line experiment was about 20 μM in both Sit-G- and MEL-liposomes, resulting in a cell viability of about 70% for Sit-G-lipoplex and 30% for

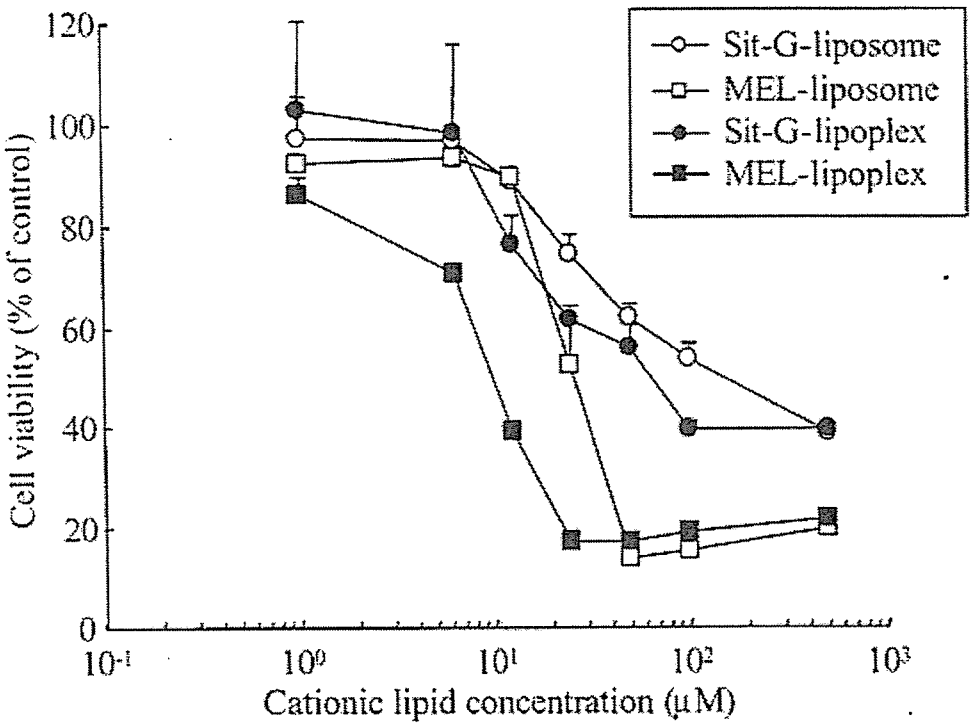

Figure 4. Cytotoxic activity of Sit-G- and MEL-liposomes, and their lipoplexes to HeLa cells. Cells were incubated in medium containing two types of liposomes and lipoplexes for 24 h. Cell viability was determined by WST-8 assay. Data represent the mean ± *SD* (n=4). Sit-G-liposomes: DC-Chol/DOPE/Sit-G=2/2/1 (mole). MEL-liposomes: DC-Chol/DOPE/MEL=3/2/2 (mole). Lipoplex was formed at a charge ratio (+/−) of liposome/DNA=3/1.

MEL-lipoplex. Sit-G- and MEL-liposomes showed decreased cytotoxicity when complexed with pDNA. It might be due to that the positive surface charge of lipoplexes may be decreased by addition of pDNA to liposomes.

In Vitro TK Expression with Liposome Vectors

TK expression by the MK or HSV promoter in HeLa cells was compared by transfection with SitG- and MEL-liposome in medium with 10% serum (Fig. 5). It was confirmed that HeLa cells expressed MK mRNA by RT-PCR analysis (Hattori and Maitani, 2005). Sit-G- and MEL-liposome showed significantly higher pMK-tk activity, about 30 to 40 times than that in nontransduced cells. The Sit-G-liposome showed higher pMK-tk activity than the HSV-tk one.

Suppression of Growth of AsPC-1 Xenografts

It was reported that MK promoter-mediated suicide gene therapy effectively produced cytotoxic effects in AsPC-1 cells (Miyauchi et al., 2001). Therefore, to study the therapeutic potential of liposome vector on the solid tumor model in mice established by AsPC-1 cells, we administered pMK-tk with liposome directly into the tumor and further GCV by i.p. injection (Fig. 6). The treatment schedule consisted of four consecutive intratumoral

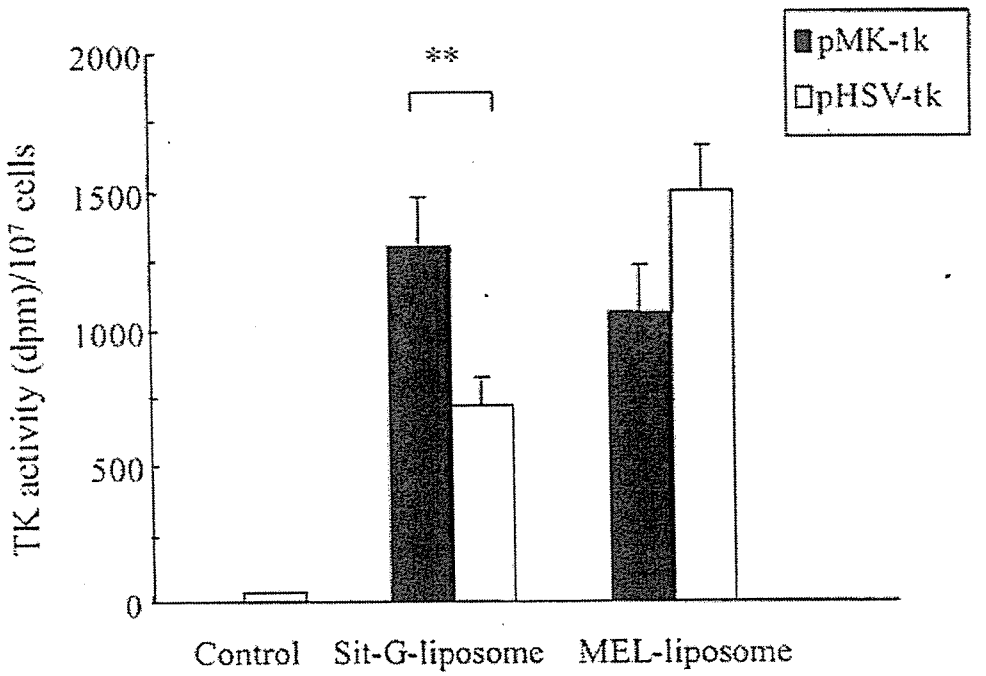

Figure 5. Comparison of TK expression by MK and HSV promoter in HeLa cells transfected with SitG- and MEL-liposome in medium with 10% serum. Cells received no pDNA in control experiments. Aliquots of 2 μg of pDNA (pMK-tk or pHSV-tk) were complexed with each liposome at charge ratio (+/−) of 3 per well. After a 24-h incubation, cells were washed with medium and cell culture was continued for an additional 48 h. Each column represents the mean ± *SD* (n=3). **; $p < .01$.

injections, each of pMK-tk (20 μg/mouse) with two kinds of liposomes or the same volume of PBS as two controls. Of these groups, only the mice treated with transfection by Sit-G-liposome saw delayed tumor growth at 4 to 6 days after starting transfection compared with controls ($p > .05$).

To observe the change in tumor growth for long-term use of Sit-G-liposome, we administered pMK-tk (20 μg/mouse) with Sit-G-liposome or Sit-G-liposome alone directly into the tumor and further GCV by i.p. injection (Fig. 7). The treatment schedule consisted of five consecutive intratumoral injections, each of pMK-tk or the same volume of Sit-G-liposome suspension as a control. The mice treated with transfection of pMK-tk by Sit-G-liposome showed significantly reduced tumor growth compared with those treated with Sit-G-liposome alone at 30 days after starting transfection ($p < .05$). This finding suggested that the antitumor response was caused not by toxicity of Sit-G-liposomes but by transfection of Sit-G-lipoplex against the tumor.

Discussion

Among synthetic vectors, lipoplexes have been the most frequently employed and the most intensively studied. Lipoplexes are large, heterogeneous, and unstable, especially at high pDNA concentrations. They form as a result of electrostatic binding between cationic liposomes and negatively charged pDNA, and they are inherently difficult to manipulate. To protect larger-sized lipoplexes, we prepared initially small-sized liposomes by addition

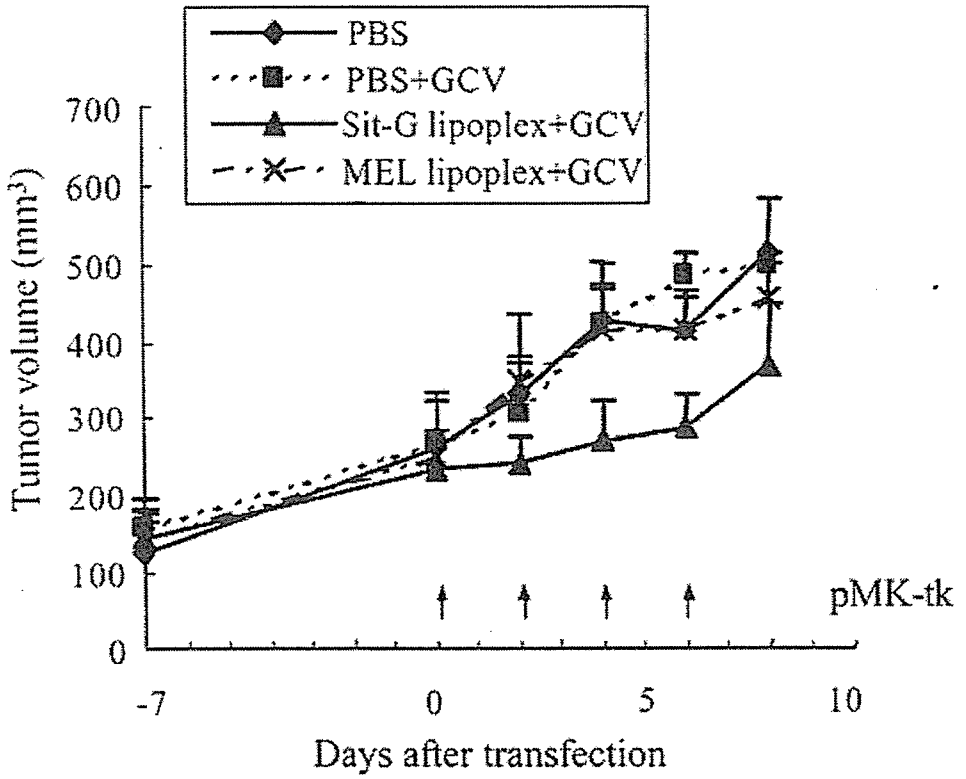

Figure 6. Effect of liposome vectors with pMK-tk administered directly into the tumor on tumor growth in HSV-tk gene therapy. Seven-week-old female BALB/c nude mice were s.c. inoculated with 1×10^6 AsPC-1 cells. On day 0 after inoculation of tumor cells, pMK-tk (20 μg/mouse) with liposome vectors were administered directly into the tumor measuring with 0.6 to 0.7 cm in diameter in animals once every two days 4 times for 7 days. Animals inoculated with liposome vectors were further treated with GCV starting 1 day after gene transfection. GCV was administered by i.p. injection twice a day 4 times for 7 days at a dose of 0.4 mg/mouse/day. Two control groups were treated by injection of PBS (50 μl) instead of pMK-tk and injection either of GCV (0.4 mg/mouse/day) or PBS. Tumor volume was measured using calipers and calculated. Each point represents the mean ± *SE* (n=3–7).

of Sit-G or MEL to DC-Chol/DOPE liposomes and could prepare about 300-nm-sized lipoplexes, whereas lipoplexes of DC-Chol/DOPE liposomes as a control resulted in aggregation in medium with serum. Sit-G and MEL may be good dispersants in preparing lipoplexes.

The early condition of incubation of lipoplexes, with the cells in the presence of serum or not, is important for change of sizes of lipoplexes because large complexes are often more efficient in transfecting cells in vitro (Felgner et al., 1994). The transfection efficiency may be reflected from uptake of lipoplexes to the cells until about 3 h after incubation because transfection was observed 6 h after incubation (data not shown). The transfection efficiency of the luciferase marker gene by Sit-G- and MEL-liposome was enhanced in the presence of serum, comparable to that by Tfx20, a commercial gene transfection reagent in the absence of serum. One of the reasons is that Sit-G may form easily dispersed lipoplexes in the presence of serum (Hwang et al., 2001), suggesting that the glycoside residue in Sit-G molecule prevents the interaction of lipoplexes with serum.

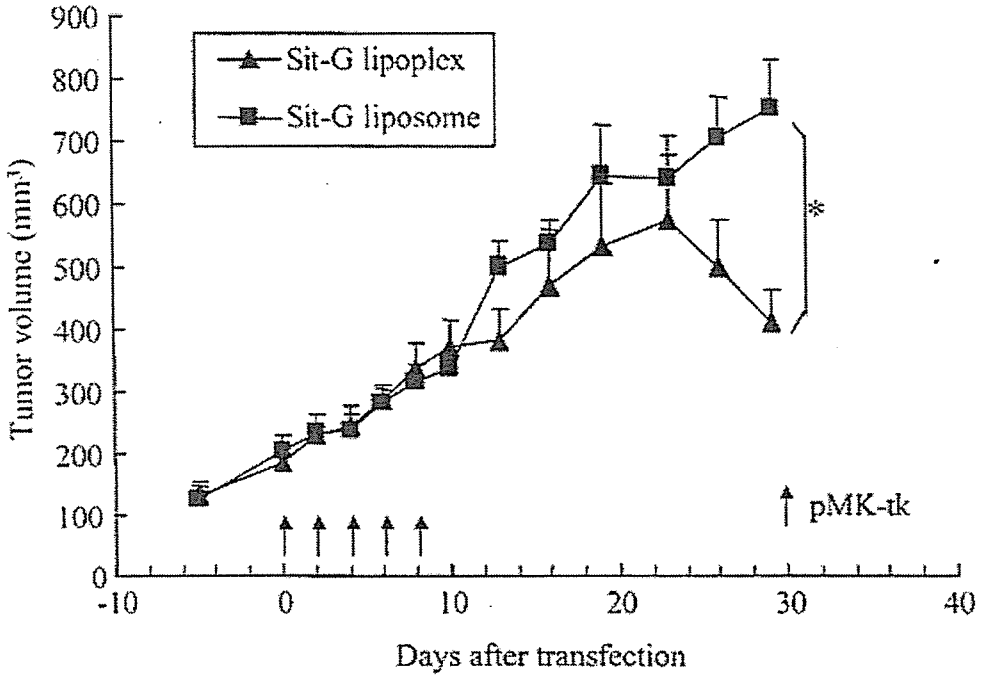

Figure 7. Effect of Sit-G-liposome vector with pMK-tk administered directly into the tumor on tumor growth in HSV-tk gene therapy. Seven-week-old female BALB/c nude mice were s.c. inoculated with 1×10^6 AsPC-1 cells. On day 0 after inoculation of tumor cells, pMK-tk (20 μg/mouse) with liposome vector or liposome alone was administered directly into the tumor measuring with 0.5 to 0.6 cm in diameter in animals once every two days 5 times for 9 days. Animals inoculated with liposome vector were further treated with GCV starting 1 day after gene transfection. GCV was administered by i.p. injection twice a day 5 times for 9 days at a dose of 0.4 mg/mouse/day. Each point represents the mean ± *SE* (n=3–7). *; $p < .05$.

This indicates the usefulness of Sit-G- and MEL-liposomes as transfection reagents. However, the exact mechanism involved in the enhancement of gene expression by serum is not known.

The size of lipoplexes, about 300 nm, seemed rather large, but the lipoplexes can be used for intratumoral and intravenous injection. The size of the lipoplex is an important factor in transfection. Large lipoplexes were reported to contribute to the enhanced gene expression in vitro (Felgner et al., 1994; Zhang et al., 1997; Ross and Hui, 1999; Turek et al., 2000). Here, the size of our lipoplexes was almost 300 nm. Therefore, size effect of transfection may be avoided. Cellular mechanisms of Sit-G- and MEL-liposomes might be different.

In consideration of selectivity and safety, we selected the MK promoter for the application of suicide gene therapy. MK, a growth/differentiation factor, is expressed predominantly in various types of human tumors, whereas its expression in adult normal tissues is highly restricted (Tsutsui et al., 1993). TK expression by pMK-tk mediated by Sit-G- and MEL-liposomes was confirmed in HeLa cells to be comparable to that by pHSV-tk with the HSV promoter. This finding suggested that the MK promoter driving MK-positive cells could achieve tissue-targeted gene expression. Although luciferase expression of Sit-G-liposome was higher than that of MEL-liposome, TK expression by these liposome vectors was not significantly different, not correlating with the luciferase transfection

efficiency. These findings strongly suggested that Sit-G- and MEL-liposomes could be applicable to in vivo HSV-tk gene delivery.

Compared with Sit-G- and MEL-lipoplex, Sit-G-lipoplex was less cytotoxic. Sit-G-liposome reduced significantly tumor size by day 30 after the start of transfection of pMK-tk gene therapy, compared with Sit-G-liposome alone. These findings suggested that cytokines induced by a nonspecific manner by intratumoral injection of lipoplex might not contribute the reduction of the tumor size. The reason that the effect of Sit-G-liposomes on tumor growth suppression was different from that of MEL-liposome vector is not clear, but it might be related to the fact that inhibition of enhanced glucose transport in GCV-treated cells increased apoptosis (Haberkorn et al., 2001) with glucose residues in Sit-G in nanoparticles and liposomes (Nakamura et al., 2003). Our finding suggested that HSV-tk gene therapy could be performed by repeated intratumoral administration of Sit-G-liposome vector system. Further study of the optimization of the formulae of Sit-G-liposome and therapeutic design is needed to be used in vivo for this therapy.

We have shown that injectable lipoplexes (about 300 to 350 nm) could be obtained by addition of Sit-G or MEL to cationic liposomes. Smaller-sized Sit-G- and MEL-lipoplexes might be obtained by the dispersity of the sugar residue of Sit-G and MEL in the presence of serum. These liposome vectors showed high transfection of the luciferase gene and TK activity in the cells. Sit-G-liposome is a promising vector in gene-based therapy.

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