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Rat Glioma Cell Death Induced by Cationic Liposome-Mediated Transfer of the Herpes Simplex Virus Thymidine Kinase Gene Followed by Ganciclovir Treatment

TAZUKA YOSHIDA, MD,[1] MASAAKI MIZUNO, MD,[1,2]* KATSUMI TANIGUCHI, MD,[1] NORIHISA NAKAYASHIKI, MD,[1] TOSHIHIKO WAKABAYASHI, MD,[1] AND JUN YOSHIDA, MD[1]

[1]*Department of Neurosurgery, Nagoya University, Graduate School of Medicine, Nagoya, Japan*

[2]*Department of Molecular Neurosurgery, Nagoya University, Graduate School of Medicine, Nagoya, Japan*

Background and Objectives: We studied antitumor effects and cell death induced by cationic liposome-mediated gene transfer of the herpes simplex virus thymidine kinase (HSV-tk) gene followed by ganciclovir treatment in cultured rat T9 glioma cells and in experimental gliomas produced from this cell line.
Methods: To transfer genes we used small unilamellar cationic liposomes containing *N*-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride. Video-enhanced contrast differential interference contrast microscopy was used for morphologic observations of cultured cells.
Results: When we treated the cells or implanted gliomas with the liposomes and ganciclovir, a strong effect was seen against tumor cells, and survival of tumor-implanted rats was increased. Morphologically, cell death observed after HSV-tk gene/liposome and ganciclovir treatment in the cultured glioma cells included both apoptosis and necrosis.
Conclusions: Introduction of the HSV-tk gene in a DNA-liposome complex followed by ganciclovir treatment induced both apoptosis and necrosis, which together resulted in a potent antitumor effect.
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KEY WORDS: **cells; culture; transplanted brain tumors; video-enhanced contrast differential interference contrast microscopy; apoptosis; necrosis**

INTRODUCTION

Successful gene therapy for malignant tumors requires the development of a safe and effective vector and production of predictable and well-understood antitumor effects. Cationic liposomes are among the most attractive vectors for in vivo experimental gene therapy because they are not infectious and have little immunogenicity or toxicity. Morphologically, cationic liposomes are divided into three main types: small unilamellar vesicles (SUV), large unilamellar vesicles (LUV), and multilamellar vesicles (MLV). SUV bind the genes of interest to their surfaces to produce DNA-lipid complexes. In contrast, LUV and MLV generally entrap the genes within the liposomes, not at the surface. We have found cationic multilamellar liposomes containing *N*-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride (TMAG) to be very useful for gene therapy against experimental brain tumors, especially malignant gliomas [1–5].

In the present study, we investigated whether cationic SUV liposomes containing TMAG are effective against

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*Correspondence to: Masaaki Mizuno, MD, Department of Molecular Neurosurgery, Nagoya University, Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan.

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malignant gliomas. When used to introduce the herpes simplex virus thymidine kinase (HSV-tk) gene before treatment with ganciclovir (GCV). The assessment included morphologic analysis of glioma cell death using video-enhanced contrast differential interference contrast (VEC-DIC) microscopy.

MATERIALS AND METHODS

Cell Line

We used the T9 rat glioma cell line. Cells were maintained in Eagle's minimum essential medium supplemented with 10% fetal calf serum, 0.1 mM nonessential amino acids, 5 mM L-glutamine, and antibiotics (streptomycin, 100 μg/ml; penicillin, 100 U/ml).

Plasmid DNA

We used two plasmids, pVLacZ and pLTRNL. The former, pVLacZ containing the cytomegalovirus promoter-immediate early enhancer and the coding sequence of LacZ, was constructed by Avigen (Alameda, CA). The latter, pLTRNL containing the HSV-tk gene and the neomycin resistance gene between long terminal repeat (LTR) sequences, was a gift from Dr. Nobuhiko Emi at Nagoya University School of Medicine, Nagoya, Japan.

Lipids and Ganciclovir

Lipids purchased for preparation of liposomes and their sources were as follows. *N*-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride (TMAG) was obtained from Sogo Pharmaceutical (Tokyo, Japan); dilauroryl phosphatidylcholine (DLPC), from Sigma Chemical (St. Louis, MO); and dioleoyl phosphatidylethanolamine (DOPE), from Avanti Polar Lipids (Pelham, AL). GCV was a kind gift from F. Hoffman-La Roche (Basel, Switzerland).

Preparation of DNA-Liposome Complex

For liposomal transduction of pLTRNL, we used a cationic SUV made up of a mixture of TMAG, DLPC, and DOPE in a molar ratio of 1:2:2, respectively. These lipids were dissolved in 200 μl of chloroform. The solvent was evaporated to produce a lipid film that suspended in 500 μl of phosphate-buffered saline (PBS) and sonicated in a probe-type sonicator to produce small unilamellar vesicles (SUV). Before use, 20 μg of plasmid DNA was mixed with SUV containing 1 μmol of lipid.

Video-Enhanced Contrast Differential Interference Contrast (VEC-DIC) Microscopy

We used video-enhanced contrast differential interference contrast (VEC-DIC) microscopy to observe morphologic changes in rat T9 glioma cells treated with DNA (pLTRNL)-liposome complex. The cells were examined with an inverted Nomarski microscope equipped with a $\times$ 63 DIC objective lens and a $\times$ 2.5 insertion lens (Axiovert 135; Zeiss, Germany). The coverslip, plated with the cultured cells, was fixed with petroleum jelly to a square hole cut in the center of a plastic slide. The optimal image was detected with a 0.5-inch CCD camera (ZVS3C75DEC; Sony, Tokyo, Japan), and image contrast was enhanced with a high-speed digital image processor. The processed image was observed on a slightly overscanned video monitor and simultaneously video recorded on a laser disc or in S-VHS format recorder.

In Vitro Experiments

Transduction efficiency of DNA-liposome complexes. Before assessment of antitumor effect we estimated the transduction efficiency of the DNA-liposome complex. Rat T9 glioma cells (10^5) were plated in a 6-well plate (Falcon 3046, Franklin Lakes, NJ). After 24 hr DNA (pLTRNL)-liposome complex (0.3 μg DNA and 15 nmol lipid/ml) was added to the medium and the incubation was continued for another 48 hr. Transduction efficiency then was evaluated by X-gal staining and by counting the number of blue cells.

Introduction of DNA (pLTRNL)-liposome complex in glioma cells. Rat T9 glioma cells (5×10^4/well) were incubated at 37°C for 24 hr in 24-well plates (Falcon 3047, Franklin Lakes, NJ) and then treated with either PBS, empty liposomes (15 nmol lipid/ml), DNA (pLTRNL; 0.3 μg DNA), GCV (5 μg/ml), or DNA (pLTRNL)-liposome complex (0.3 μg DNA and 15 nmol lipid/ml). The last of these was tested with and without GCV treatment (5 μg/ml). Cell growth was evaluated by 3-(4,5-dimethythiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) method.

Morphologic changes in rat T9 glioma cells treated with DNA (pLTRNL)-liposome complex. To observe morphologic changes in rat T9 glioma cells treated with DNA (pLTRNL)-liposome complex followed by GCV, the cells were plated and cultured in a glass-bottomed well (No. 0, MatTek, Ashland, MA) at a density of 2.5×10^4 cells/well in 2 ml of medium. After 24 hr DNA (pLTRNL)-liposome complex (0.3 μg DNA and 15 nmol lipid/ml) was added to the culture medium. Forty-eight hours later, GCV (5 μg/ml) was added to the well. Morphologic changes then were observed continuously by VEC-DIC microscopy.

In Vivo Experiments

Antitumor effect of DNA (pLTRNL)-liposome complex. We evaluated the antitumor effect of DNA (pLTRNL)-liposome complexes using an experimental rat glioma model. Briefly, rat T9 glioma cells (10^5) were inoculated by stereotactic injection into rat brain 2 mm lateral and 1 mm posterior to the bregma, 4 mm deep to the skull. Male Fisher 344 rats (age, 7 weeks) were used for experiments. Anesthesia consisted of an injection of pentobarbital (50 mg/kg, i.p.).

Animals were divided into six groups: Group 1 (n = 16), receiving a single injection of PBS (20 μl) on Day 4; Group 2 (n = 12), receiving a single injection of empty liposomes (150 nmol lipids/20 μl) on Day 4; Group 3 (n = 12), receiving a single injection of DNA (pLTRNL)-liposome complex on Day 4 with no GCV treatment; Group 4 (n = 12), receiving injections of DNA (pLTRNL)-liposome complex on Days 4 and 8 with no GCV treatment; Group 5 (n = 12), receiving a single injection of DNA (pLTRNL)-liposome complex on Day 4 followed by GCV treatment; Group 6 (n = 14), receiving injections of DNA (pLTRNL)-liposome complex on Days 4 and 8, followed by GCV treatment.

In Group 5, the DNA (pLTRNL; 3.0 μg)-liposome complex (20 μl) was injected stereotactically into tumors inoculated 4 days before. GCV treatment was begun on Day 6 and continued for 5 days (25 mg/kg twice daily, i.p.). In Group 6, DNA (pLTRNL; 3.0 μg)-liposome complex (20 μl) was injected on Days 4 and 8 in the same manner. GCV was injected on Day 6 to 7 and 9 to 10 (25 mg/kg twice daily, i.p.).

Animals were examined daily, and neurological deficits were scored as 0, no deficit; 1, contralateral fore-limb flexion; 2, circling upon tail pinching; or 3, no active movement.

Antitumor effect was evaluated by naked eyes and by measuring the long (a) and short (b) axes in the coronal section of each tumor showing maximal area on day 21 after implantation. The tumor volume (V) was calculated according to the formula, $V(mm^3) = a \times b^2/2$. In addition, the survival times of the treated rats were recorded.

RESULTS

In Vitro Experiments

Transduction efficiency of DNA (pVLacZ)-liposome complex. Forty-eight hours after the addition of DNA (pVLacZ)-liposome complex (0.3 μg DNA and 15 nmol lipid/ml), we confirmed the transduction efficiency (12.4 ± 2.6%) in cultured rat T9 glioma cells by X-gal staining (Fig. 1).

Antitumor effect by DNA (pLTRNL)-liposome complex. As shown in Figure 2, no growth inhibition was observed with PBS or empty liposomes (15 nmol lipids/ml), with DNA (pLTRNL); (0.3 μg DNA) alone, with GCV (5 μg/ml) alone, or with DNA (pLTRNL)-liposome complex (0.3 μg DNA and 15 nmol lipid/ml) in the absence of GCV treatment. In contrast, DNA (pLTRNL)-liposome complex (0.3 μg DNA and 15 nmol lipid/ml) together with GCV treatment (5 μg/ml) reduced growth to less than 80% that seen with other treatments.

Morphologic changes of rat T9 glioma cells treated with DNA (pLTRNL)-liposome complex. Rat T9 glioma cells treated with DNA (pLTRNL)-liposome complex (0.3 μg DNA and 15 nmol lipid/ml) were observed by VEC-DIC microscopy × 2,000 to × 7,500 beginning 8 hr after GCV treatment. As shown in Figure 3, nucleoli became abnormally bright and blebbing of the cell membrane occurred with increasing intensity in increasing numbers of cells over times. At 12 hr some morphologically altered cells had shrunken and developed large membrane outpouchings that we term "ballooning." This sequence is typical for apoptosis. On the other hand, other cells demonstrated different morphologic changes, also shown in Figure 3, which represented necrosis.

In Vivo Experiments

Induction of antitumor effect by DNA (pLTRNL)-liposome complex. The effect of introducing the DNA (pLTRNL)-liposome complex followed by GCV treatment was assessed histologically in rat brain tumors. Tumors could be observed beginning 3 days after implantation of rat T9 glioma cells. At 21 days after implantation, a large mass was confirmed to be present in the right frontoparieto-temporal region in control groups (Fig. 4 and Table I). The tumors were smaller than in control groups when DNA (pLTRNL)-liposome complex was introduced once followed by GCV (Fig. 4 and Table I). The tumor was much smaller than in controls when DNA (pLTRNL)-liposome complex was introduced twice with GCV treatment (Fig. 4 and Table I). Neurological scores are shown in Figure 5. Until 10 days after tumor implantation, any motor deficits were not observed in all animals. As there were no significant differences among Group 1, 2, 3, and 4, we demonstrated the only data of Group 1 as a representative data in Figure 5. The severity of deficits was decreased significantly in rats treated with DNA (pLTRNL)-liposome complex followed by GCV.

Survival curves are shown in Figure 6. When PBS or empty liposomes or a single injection of DNA (pLTRNL)-liposome complex (3.0 μg DNA and 150 nmol lipid) with

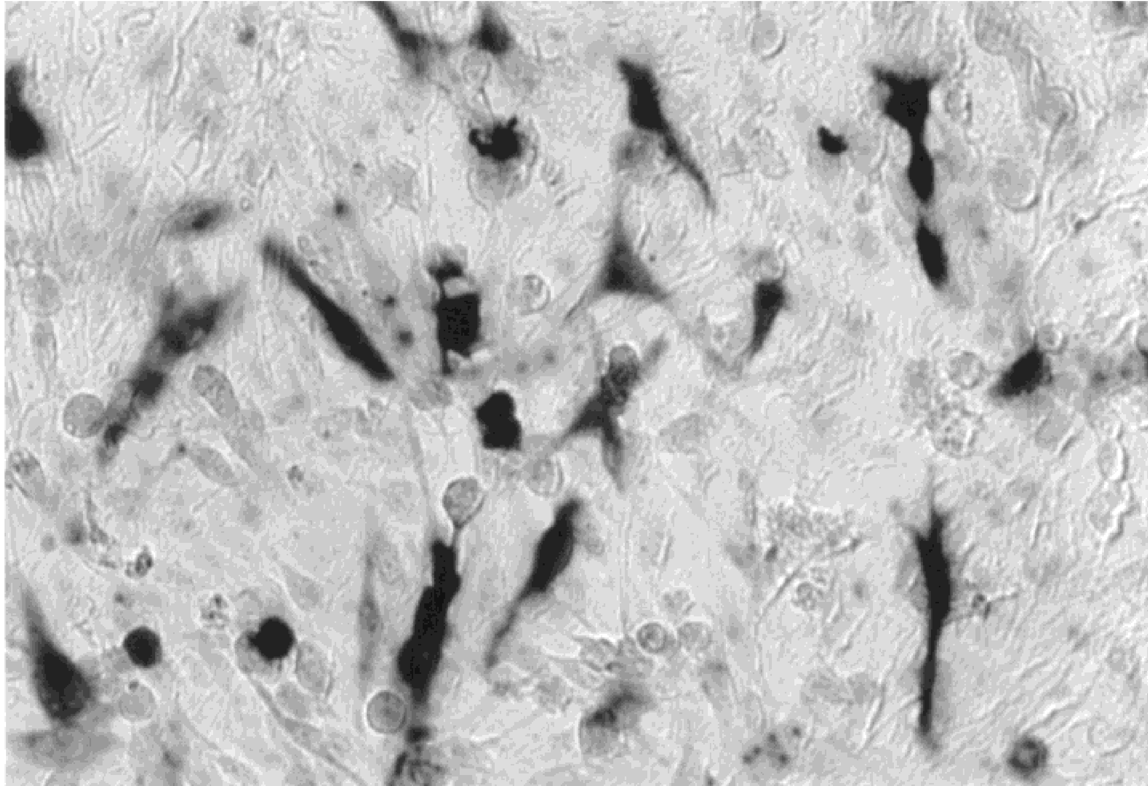

Fig. 1. Transduction efficiency of DNA (pLTRNL)-liposome complex in cultured rat T9 glioma cells. We evaluated the transduction efficiency by X-gal staining and by counting the number of the blue cells. The percentage of blue cells was 12.4 ± 2.6% (n = 5).

no GCV treatment or two injections of DNA (pLTRNL)-liposome complex with no GCV treatment were stereotactically injected into the tumor, all rats died until 29 days. On the other hand, rats treated with DNA (pLTRNL)-liposome complex and GCV administration survived 28.6% (2/7 animals) and 55.6% (5/9 animals) in a single injection and two injections, respectively (Figure 6). Furthermore all these rats had no tumor intracranially. Treated groups had significantly longer survival than any controls.

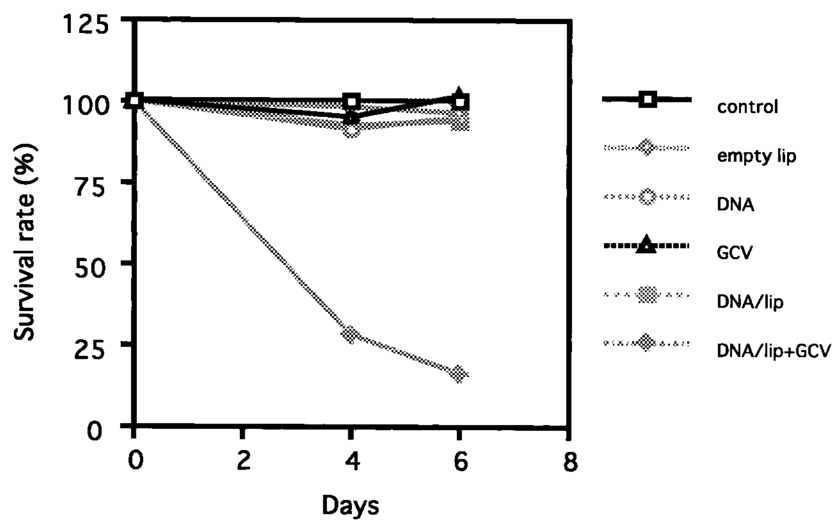

Fig. 2. Growth inhibition in cultured rat T9 glioma cells from introduction of DNA (pLTRNL)-liposome complexes and ganciclovir (GCV) treatment. Columns: 1, phosphate-buffered saline (PBS); 2, empty liposomes (15 nmol lipid/ml); 3, DNA (pLTRNL; 0.3 μg DNA); 4, GCV (5 μg/ml); 5, DNA (pLTRNL)-liposome complex (0.3 μg DNA and 15 nmol lipid/ml) with no GCV treatment; 6, DNA (pLTRNL)-liposome complex (0.3 μg DNA and 15 nmol lipid/ml) with GCV treatment (5 μg/ml). Cell growth was evaluated by MTT method.

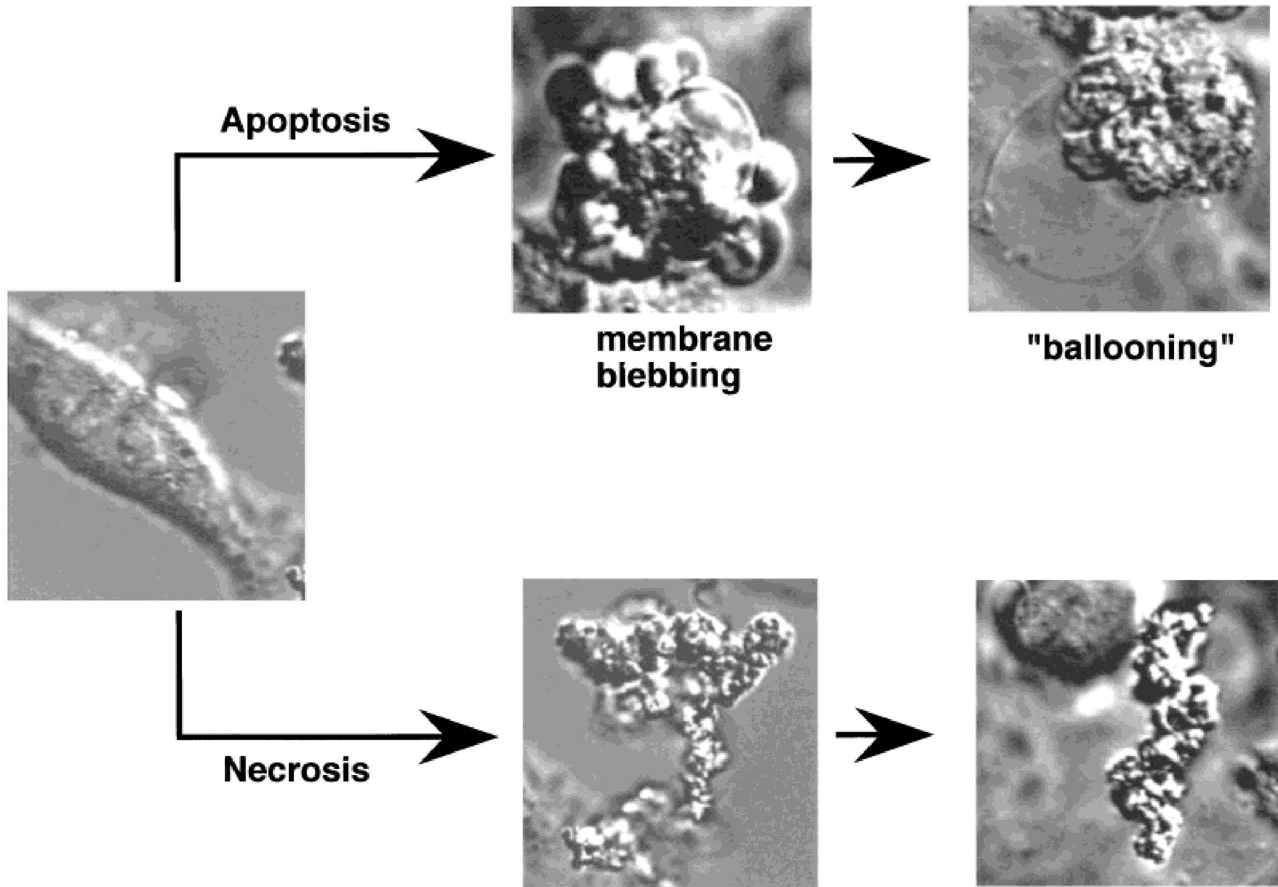

Fig. 3. Morphologic changes in cultured rat T9 glioma cells after introduction of DNA (pLTRNL)-liposome complex and ganciclovir treatment. Video-enhanced contrast differential interference contrast microscopy was used. Both apoptosis and necrosis resulted (original magnification, × 2,000).

DISCUSSION

We have tested the efficacy of multilamellar liposomal gene transfer and reported antitumor effects from introducing the β-interferon (IFN) gene in both cultured glioma cells and an experimental glioma model [1–5]. In the present study, we investigated the effectiveness of an unilamellar liposome, SUV containing the cationic reagent TMAG, on cultured rat glioma cells and in an experimental glioma model. In the rat experimental

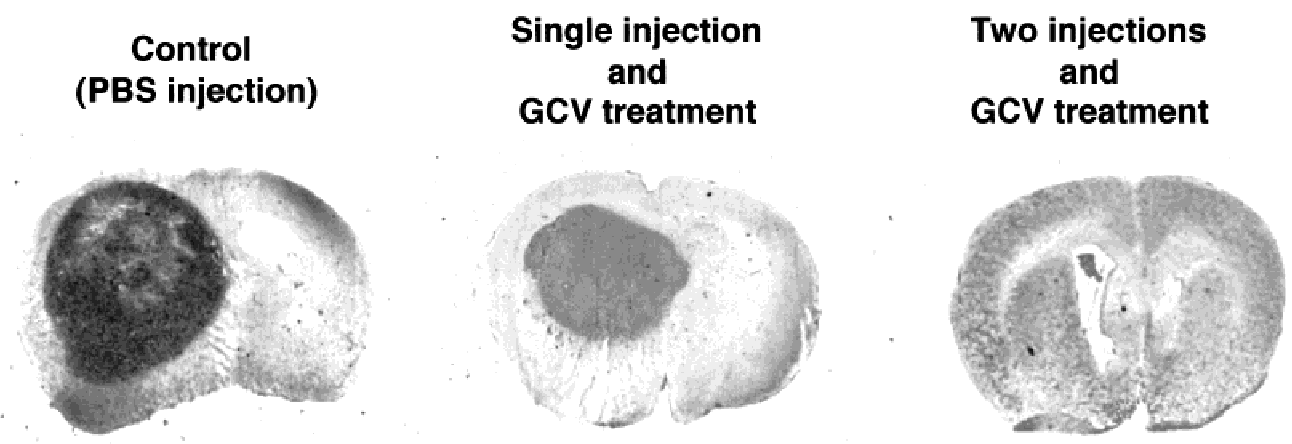

Fig. 4. Histologic findings 21 days after implantation of an experimental glioma subsequently treated with DNA (pLTRNL)-liposome complex and ganciclovir. One treatment with the complex is composed with two treatments, as well as with no treatment (control) in representative rat brains (hematoxylin and eosin).

TABLE I. Antitumor Effect of DNA(pLTRNL)-Liposome Complex, Followed by Ganciclovir Treatment on Intracerebrally Transplanted T9 Rat Glioma

Group	No. of animals	No. of no tumor-bearing animals	Tumor size (mm^3)
1	6	0	193.2 ± 13.3
2	5	0	185.8 ± 11.8
3	5	0	179.4 ± 15.0
4	5	0	191.9 ± 9.7
5	5	1	142.0 ± 24.1
6	5	2	27.2 ± 11.5

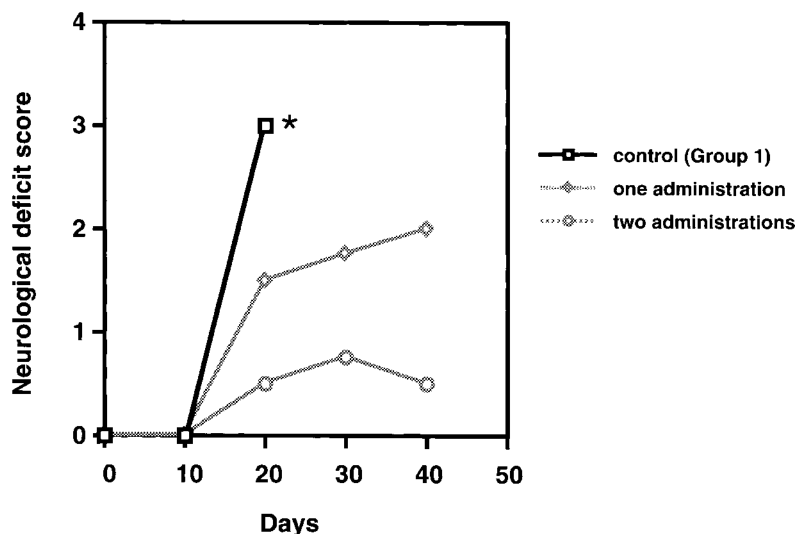

Fig. 5. Development of neurological deficits in treated and control rats with experimental gliomas (n = 5 per group). The treatment consisted of either a single administration of DNA (pLTRNL)-liposome complex (0.3 µg DNA and 15 nmol lipid/ml of medium) together with ganciclovir treatment (5 µg/ml), or two such administrations of the complex with ganciclovir. The symbol indicates that poor neurological condition precluded further evaluation.

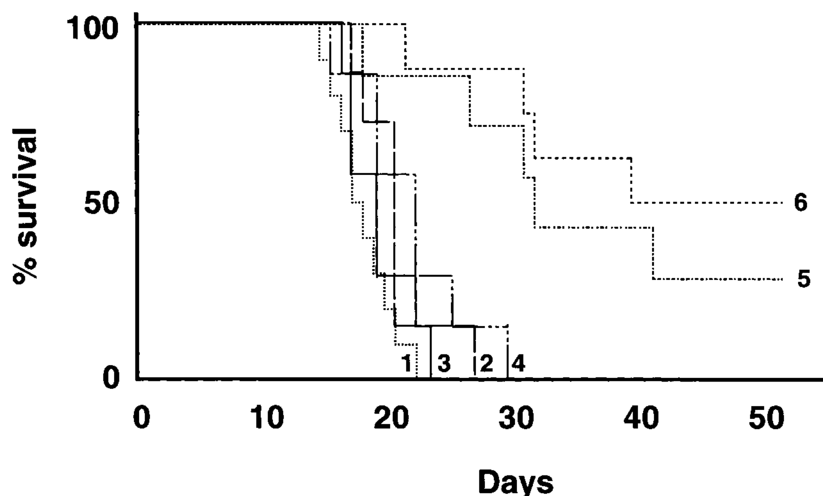

Fig. 6. Kaplan-Meier survival curves of rats treated once or twice with DNA (pLTRNL)-liposome complex and ganciclovir (GCV) compared with untreated controls. Group 1 (n = 10), receiving a single injection of PBS (20 µl) on Day 4; Group 2 (n = 7), receiving a single injection of empty liposomes (150 nmol lipids/20 µl) on Day 4; Group 3 (n = 7), receiving a single injection of DNA (pLTRNL)-liposome complex (3.0 µg DNA and 150 nmol lipid) on Day 4 with no GCV treatment; Group 4 (n = 7), receiving injections of DNA (pLTRNL)-liposome complex on Days 4 and 8 with no GCV treatment; Group 5 (n = 7), receiving a single injection of DNA (pLTRNL)-liposome complex on Day 4 followed by GCV treatment (25 mg/kg i.p.); Group 6 (n = 9), receiving injections of DNA (pLTRNL)-liposome complex on Days 4 and 8, followed by GCV treatment.

glioma model, we obtained an antitumor effect from the introduced HSV-tk gene followed by GCV treatment that was at least as potent as the effect of the previous reported human IFN-β gene (“xenogene”) introduced with MLV-liposomes [6]. The safety of cationic SUV liposomes requires further careful study, however, because one report has suggested that SUV liposomes have more potential for toxicity than MLV liposomes in in vitro experiments [7].

The greatest problems in human gene therapy using viral vectors are low transduction efficiency and poor distribution of the transduced gene within the target tissue [8]. The same drawbacks are likely with DNA/liposome complexes. Zhu et al. have described continuous micro-infusion of the DNA/liposome complex into the brain as an alternative means of administration to introduce genes more effectively with less toxicity [9]. Improvements in injection methods may help to overcome the problems of toxicity of the complexes and unpredictable distribution of transduced genes.

Video-enhanced contrast differential interference contrast (VEC-DIC) microscopy is a video microscopy technology that has advanced rapidly since 1980. The resolution now attainable by this approach, on the order of 1 μm, permits continuous observation of organelles in a viable cell over time. In the present study, morphologic changes in cultured rat T9 glioma cells treated with the HSV-tk gene and GCV were observed continuously. We confirmed that HSV-tk gene introduction followed by GCV treatment induced both apoptosis and necrosis simultaneously in cultured rat T9 glioma cells. By this microscopic technique, apoptosis is characterized by an increase of brightness at the circumference of nuclei and in nucleoli, membrane blebbing, cell shrinkage, formation of apoptotic bodies and “ballooning” as shown in Figure 3. We suspect that “ballooning” is a final step in the process of apoptosis; these observations will be described in detail elsewhere. As opposed to apoptosis, Melcher et al. have emphasized necrosis, demonstrating that induction of necrosis is highly important in activation of tumor immunogenicity via heat-shock protein [10]. Induction of necrosis in glioma cells, in addition to apoptosis by the HSV-tk gene and subsequent GCV treatment, is believed to add to the efficacy of this therapy by activation of the host immune system that attacks nonnecrotic tumor cells as well as necrotic ones (the “bystander effect”). We plan to examine activation of the immune system by gene therapy for gliomas in detail. If use of DNA-liposome complexes incorporating HSV-tk gene followed by GCV treatment is able to strongly activate the immune system, such a strategy may prove highly effective against malignant gliomas.

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