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ORIGINAL ARTICLE

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Dendritic cells pulsed with tumor extract–cationic liposome complex increase the induction of cytotoxic T lymphocytes in mouse brain tumor

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Abstract Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that locate in peripheral organs. It has been thought that a systemic immune response does not play a role in regression of central nervous system (CNS) tumors, because the CNS is an immunologically privileged site. However, recent advances in immunology have led to the possibility of immunotherapy using peripheral DCs against CNS tumors. Here, we investigated whether DCs pulsed with tumor extract could induce an antitumor effect against malignant glioma. Furthermore, we also investigated whether the antitumor effect become higher by pulsation with tumor extract–liposome complex, compared to pulsation with tumor extract alone. As a liposome, we used cationic small unilamellar vesicles composed of *N*-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride (TMAG), dilauroylphosphatidylcholine (DLPC), and dioleoylphosphatidylethanolamine (DOPE) in a molar ratio of 1:2:2. After intracerebral inoculation of mouse malignant glioma GL261 cells into syngeneic C57BL/6 mice, DCs pulsed with extract from the glioma cells by sonication were administered intraperitoneally thrice weekly on days 7, 14 and 21. Tumor growth inhibition was evaluated by measuring the tumor size 1 month after the tumor inoculation. The group treated with DCs pulsed by tumor extract was inhibited in tumor progression compared with the control non-pulsed DCs group, and the group treated with DCs pulsed by tumor extract and liposomes showed substantial tumor volume reductions in all the mice. Among the mice, there were several with no visible masses in their brains. Immunohistochemical study showed that the CD8-positive cytotoxic T cells (CTLs) were strongly recognized among the almost disappearing tumor cells of pulsed DCs groups. The CTLs showed a specific antitumor activity for GL261 mouse glioma cells. These findings indicated that DCs pulsed with tumor extract and liposomes might play an important role in the activation of an immune response in malignant glioma.

Keywords Dendritic cells · Liposomes · Cell extract · Cytotoxic T lymphocyte

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Introduction

Malignant glioma is one of the most formidable neoplasms. Since the tumor aggressively invades to normal brain tissue, conventional treatments – including surgery, radiation therapy and chemotherapy – are largely ineffective. In particular, the delivery of chemotherapeutic agents is thought to be very difficult because of the blood-brain barrier. On the other hand, Sampson et al. [11] and Wakimoto et al. [16] confirmed that systemic immune responses, including cytotoxic T lymphocytes (CTLs), actually function in the brain by some kind of cytokine stimulation in an experimental mela-

noma produced in the brain. We thought that such evidence could probably also apply to malignant glioma.

Immunotherapy consists of antigen-presenting cells, helper T and cytotoxic T lymphocytes, cytokines, and antibodies. All the parts are very important to each other, but in this study we first paid attention to antigen-presenting cells.

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that occur in peripheral organs, where they are exposed to and capture antigens. They then migrate to the lymphoid tissue and present processed antigens in the context of major histocompatibility complex (MHC) class I and II molecules to elicit an antigen-specific T cell response [1, 2, 4]. Other cell types, such as B cells and macrophages, are also competent in capturing and presenting antigens. DCs are more effective and unique in their ability. Therefore, DCs express high levels of MHC class I and II, and the costimulatory molecules required for antigen presentation (CD80, CD86). Because of their unique antigen-presenting feature, DCs represent an especially attractive cell type for the immunotherapy of diseases such as malignant neoplasm [1, 2, 14]. Consequently, peptide- or protein-pulsed, or gene-modified DCs have been used in research models of neoplasm and have been shown to elicit effective anti-tumor responses [3, 5, 12, 13, 17].

Liposomes are synthetic lipid vesicles able to entrap proteins or genes within their aqueous compartment and/or lipid bilayer. They can be divided electrically into the three formulations of cationic, neutral, and anionic types. The cationic liposomes can be divided morphologically into small unilamellar vesicle (SUV), large unilamellar vesicle (LUV), and multilamellar vesicle (MLV) types. When SUVs are mixed with materials, the SUVs bind them to their surfaces, producing material-lipid complexes. In contrast, the MLV and LUV types generally entrap materials within the liposomes, and not on the surface. In this experiment, we used SUV-type liposomes to bind tumor antigens on their surface. The SUV-type liposomes were used as an efficient immunoadjuvant to enhance the immune response. Nakanishi et al. [9] investigated the relationship between a liposomal surface charge and adjuvant action, and indicated that the positive charge on the surface of liposomes represents an important factor for enhancing their immunoadjuvancy in the induction of antigen-specific immune responses. They also reported that positively charged liposomes containing soluble antigens functioned as a more potent inducer of antigen-specific cytotoxic T lymphocyte responses and delayed-type hypersensitivity responses than negatively charged and neutral liposomes containing the same concentration of antigens [10]. Ishii et al. [6] reported that HIV-1-specific CTL activity was observed to have a stronger effect on immunization with the DNA vaccine and cationic liposomes combination than DNA vaccine alone. These findings prompted us to investigate the effectiveness of DCs pulsed with tumor extract–cationic liposome complex as an adjuvant to induce immune responses for intracranial malignant glioma.

In the present study, we investigated whether a stronger antitumor effect for experimental intracranial glioma was induced by DCs pulsed with tumor extract–cationic liposome complex, compared to DCs pulsed with tumor extract alone. As a liposome, we used our original cationic small unilamellar vesicles composed of *N*-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride (TMAG), dilauroylphosphatidylcholine (DL PC), and dioleoylphosphatidylethanolamine (DOPE) in a molar ratio of 1:2:2.

Materials and methods

Cells

Mouse glioma cell lines, GL261, were used, which are cultured cell lines made from an earlier tumor [16]. The cells were grown in Eagle's minimum essential medium (MEM) containing 10% (v/v) fetal calf serum (FCS), 2 mM nonessential amino acids, 5 mM *L*-glutamine, streptomycin (100 μg/ml) and penicillin (100 U/ml), and were used within one month. The cells express H-2K^b and D^b, but not I-A^b, on their cell surface.

Another mouse glioma cell line, MT539MG, which arose spontaneously in the Vm/DK strain, was obtained from Dr. Y.G. Gillespie (Birmingham, Ala., USA) via Dr. T. Kikuchi (Tokyo, Japan). RMA is a T lymphoma of C57BL/6 origin [7].

Preparation of liposomes

For the preparation of liposomes, TMAG (Sogo Pharmaceuticals, Tokyo, Japan), DLPC (Sigma Chemicals, St. Louis, Mo.), and DOPE (Avanti Polar-lipids, Pelham, Ala.) in a molar ratio of 1:2:2 (total amount, 1 μmol) were dissolved in 0.5 ml chloroform. The solvent was evaporated and the air replaced with nitrogen gas. Then the lipid film was wetted with 1.5 ml phosphate-buffered saline (PBS), and suspended by sonication for 2 min. The liposomes prepared in this study were small unilamellar vesicles (SUV). Liposomes were stable for over 3 months.

DC generation and characterization

After each C57BL/6 mouse was killed, all soft tissues were removed with gauze from the tibias and femurs. The bones were placed in a 60-mm dish with 70% ethanol, washed with RPMI-1640 medium, and transferred to another dish with the same medium. The epiphyses of the bones were cut with sterile scissors and then bone marrow was flushed out with 2 ml RPMI-1640 using a syringe with a 26-gauge needle. The tissue was suspended and the mixture passed through a nylon mesh to remove any pieces of bone or debris. After washing, lymphocytes and Ia$^+$ cells were absorbed by a cocktail of monoclonal antibodies (mAbs). The mAbs were anti-B220 against B cells, anti-Thy1 against T cells and M5/114 against Ia$^+$ cells. Between 7.5×10^5 and 1.0×10^6 cells were placed in 24-well plates in 1 ml of medium with 10^3 U/ml murine recombinant granulocyte-macrophage colony-stimulating factor (mrGM-CSF) added. The culture was fed every 2 days by gently swirling the dishes, aspirating 75% of the medium including granulocytes, and adding fresh medium with GM-CSF. On day 7, floating and loosely adherent cells, which were consistent with DCs, were harvested by gentle pipetting.

The characterization of harvested DCs was performed by flow cytometry. Over 90% of the population of plastic-adherent dendritic cells stained positive for CD11c and CD86 and negative for Gr-1 and B220 (data not shown).

Vaccination with DCs

Harvested DCs were washed twice and resuspended with MEM. The liposomes, SUV, were used to deliver tumor extract more efficiently into DCs. To produce tumor extract, 10^7 GL261 cells in 500 μl MEM (per mouse) were fragmented using a probe-shape-tipped sonicator in a 15-ml polypropylene tube. Tumor extract was mixed with SUV for 20 min at room temperature. The mixture was then added to DCs and incubated at 37°°C for 25 min. The DCs were washed and resuspended in PBS. One week after tumor inoculation, all mice were injected intraperitoneally thrice weekly (days 7, 14, and 21) with 10^5 DCs in 500 μl PBS per mouse. One week after the third treatment (day 28), all 17 animals were killed and their brains were removed and either frozen in OCT compound for immunocytochemistry or fixed in 10% formalin in 0.1 M phosphate buffer for subsequent hematoxylin and eosin (H&E) staining of coronal sections.

Mice

All mice used in this experimental series were 5- to 7-week-old female C57BL/6 mice (Japan SLC, Hamamatsu, Japan). They were kept under pathogen-free conditions in the animal facility of our institute. Animal experiments were performed according to the principles enunciated in the ''Guide for the Care and Use of Laboratory Animals'' prepared by the Office of the Prime Minister of Japan.

Intracranial tumors

Mice were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg of body weight). After hair shaving and scalp incision, a burr hole was made in the skull 3 mm lateral to the midline and 4 mm posterior to the bregma using a dental drill. The head of each mouse was fixed in a stereotactic apparatus with ear bars. Then 2×10^5 GL261 cells in 2 μl of PBS were injected stereotactically for 4 min at a depth of 2 mm below the dura mater. A sterile Hamilton syringe fitted with a 26-gauge needle was used with a microsyringe pump. The needle was left in the brain for the next 2 min and then slowly withdrawn.

Tumor growth inhibition

Animals were divided into three groups: group A (the control), treated with DCs incubated in MEM containing no FCS ($n=5$); group B, treated with DCs pulsed with tumor extract ($n=7$); group C, treated with DCs pulsed simultaneously with tumor extract mixed with liposomes ($n=5$).

The tumor inhibitory effects were evaluated by direct measuring of tumor size, length (L) and width (W), from H&E staining of coronal sections. The tumor volume (V) was calculated according to the following formula:

$$V(mm^3) = L(mm) \times W(mm)^2/2$$

Statistical analysis was performed using Student's *t*-test. Statistical significance was set at the level of $P<0.05$.

Immunohistochemistry

First, 20-μm frozen sections were cut through the injection site with a cryostat microtome and mounted on silanized glass slides. Sections were fixed for 15 min in acetone at 4°°C and, after washing in PBS, they were blocked with 1% skimmed milk in PBS. The following monoclonal antibodies were used to detect immune system markers: KT174 (rat anti-mouse CD4; Serotec, Oxford, England), and YTS 105.18 (rat anti-mouse CD8; Serotec, Oxford, England). The secondary antibody was biotinylated rabbit anti-rat IgG (H and L chains) absorbed with mouse serum (Vector Laboratories, Calif., USA). The sections were incubated with primary antibodies for 1 h at room temperature, and were then immersed in 0.3% H_2O_2 in absolute methanol for 15 min to block endogenous peroxidase. After being washed three times in PBS, sections were incubated with biotinylated rabbit anti-rat IgG (H and L chains) for 30 min at room temperature. Diaminobenzidine solution was used as a chromagen, and the sections were counterstained with hematoxylin.

CTL generation

C57BL/6 mice that had been inoculated intracranially with GL261 cells were killed on 28 days after tumor inoculation. Aseptically harvested tumors and spleens were teased into each suspension in RPMI-1640 medium supplemented with 10% fetal calf serum and 5×10^{-5} M 2-mercaptoethanol. The cell suspension containing tumor infiltrating lymphocytes (TILs) was concentrated by centrifugation on discontinuous gradients of 45 and 67.5% Percoll solution (Amersham Pharmacia Biotech). This procedure was able to remove $>90\%$ tumor cells based on microscopic examination and at least 1×10^5 TILs were obtained from an animal treated with DCs pulsed with tumor extract/liposome complex. Pooled TILs (1×10^5) or spleen cells (1×10^6) from the treated mice were cultured with 1×10^5 irradiated (100 Gy) GL261 stimulator cells in 24-well plates for 5 days at 37°°C under 5% CO_2 in air (mixed lymphocyte tumor culture [MLTC]). The harvested cells (5×10^4) were maintained as a bulk CTL line by three restimulations (5, 9, and 14 days after MTLC) with 10^5 irradiated (100 Gy) GL261 stimulator cells in the presence of 5×10^6 irradiated (20 Gy) C57BL/6 splenic feeder cells and human interleukin (IL)-2 at a concentration of 30 U/ml in 24-well plates. The phenotype of the CTL line was analyzed by flow cytometry.

In vitro cytotoxic assay

Target cells were labeled by incubating 10^7 cells with 3.7 MBq of $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, Mass.) for 45 min, followed by washing three times. Target cells labeled with ^{51}Cr (1×10^4 cells/100 μl) were incubated with the effector cell suspension (100 μl). After incubation for 3.5 h at 37°°C, the supernatants were removed and their radioactivity was measured. The percentage of specific lysis was calculated by the following formula: [(A-B)/(C-B)]×100, where A is the radioactivity in the supernatant of target cells mixed with effector cells, B is that in the supernatant of target cells incubated alone, and C is that in the supernatant after lysing the target cells with 1% Triton X-100. For antibody (Ab) blocking assay in vitro, 50 μl of Abs (diluted 1:500) were added to the mixture of 1×10^4 labeled target cells (100 μl) and 1×10^5 effector cells (100 μl).

Results

Tumor inhibition

Tumor inhibitory effects were evaluated by calculating tumor volume as described above. The results are shown in Fig.1. In group B, tumor size was decreased compared with the control (group A), although the difference was not significant. Tumors of group C, treated with the liposomal combination, showed a remarkable reduction in volume ($P<0.01$). The mean volumes and standard deviations of groups A, B and C were 78.5 ± 48.8, 34.9 ± 39.5 and 8.9 ± 11.6, respectively. Tumors were found to have disappeared in 3/7 mice of group B and 4/5 of group C when the brain sections were stained with H&E.

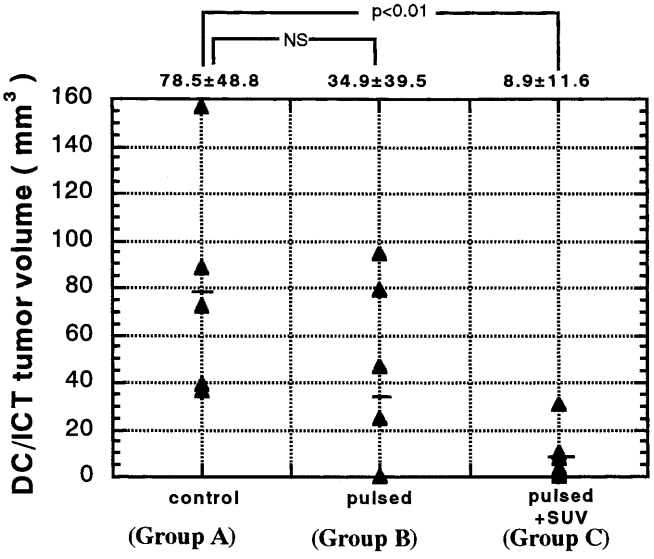

Fig. 1 Inhibition of intracranial glioma treated with dendritic cells pulsed with tumor extract–liposome complex. The mean volumes and standard deviations of *groups A*, *B* and *C* were 78.5 ± 48.8, 34.9 ± 39.5 and 8.9 ± 11.6, respectively. Tumors were found to have disappeared in 3/7 mice of *group B* and 4/5 of *group C* when the brain sections were stained with H&E. *Transverse bar* mean; $^{*}P < 0.01$

Induction of $CD8^+$ cytotoxic T lymphocytes in tumor

In the control (group A), few $CD8^+$ cells were observed in both the intratumoral region and surrounding tissue. On the other hand, numerous $CD8^+$ cells were observed when pulsed DCs were injected intraperitoneally (group B). In group C, far more $CD8^+$ cells were observed than in group B (Fig. 2).

TILs and spleen cells obtained from the mice treated with DCs pulsed with tumor extract alone or with tumor extract–liposome complex were stimulated twice by co-culturing with irradiated GL261 cells in the presence of syngeneic feeder cells, and the blastic cell lines generated were characterized (Table 1). We could not induce blastic cell lines from tumor and spleen of untreated mice by the same procedures. Most of the blastic cells expressed CD3 (data not shown), and the CD4/CD8 ratios of these CD3-positive cells were presented in Table 1. In cultures of TIL and spleen of these experiments, $CD8^+$ cells were predominantly induced (82–97%). Their cytotoxic activities against GL261 cells were indicated in Fig.3. Although MT539MG and RMA cells expressed a higher level of H-$2K^bD^b$ than GL261 (data not shown), they were not lysed by the CTL lines from tumor and spleen of mice treated with DCs.

The cytotoxic effector cells were determined by Ab blocking tests. Cytotoxicity against GL261 was blocked by Abs against CD3, TCR$\alpha\beta$, CD8, H-$2K^bD^b$ and H-2^b, but not those against CD4, TCR$\gamma\delta$, H-2^d, H-2^k, I-A^b and asialoGM1.

Discussion

In this study, we investigated the effectiveness of brain tumor immunotherapy initialized by pulsed DCs with

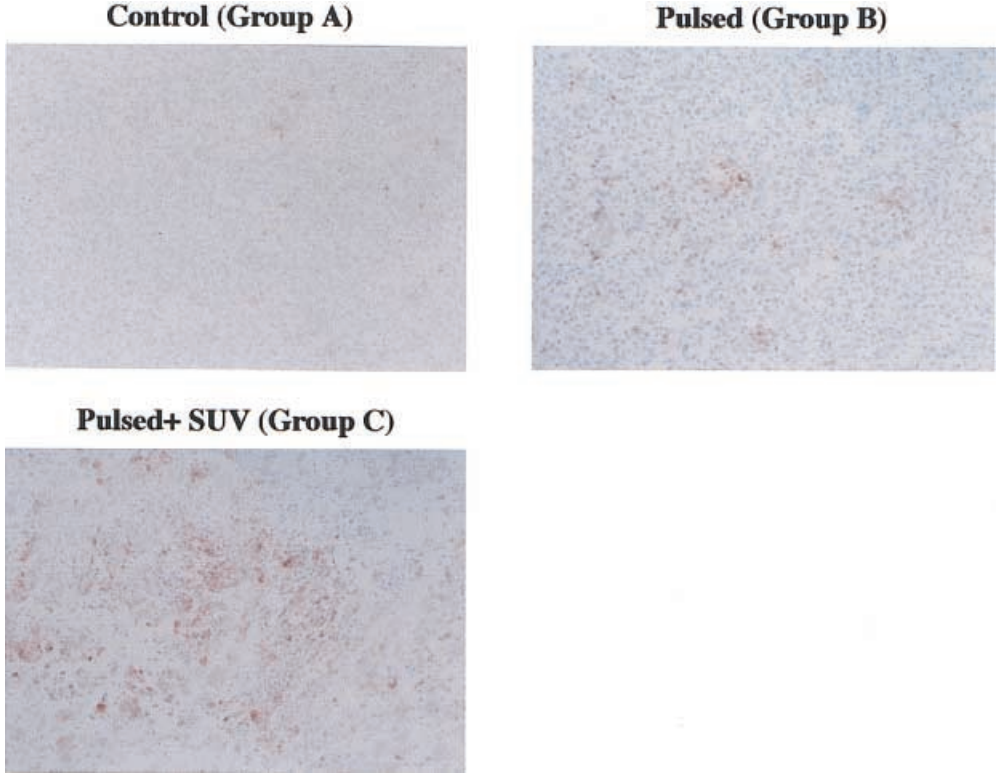

Fig. 2 Photographs showing immunohistochemical staining of intracranial gliomas (×100). Immunohistochemical study shows that the CD8-positive cytotoxic T cells (CTLs) were more strongly recognized in an experimental glioma treated with DCs pulsed by tumor extract–liposome complex (*Group C*), when compared to tumor extract alone (*Group B*) or no treatment (*Group A*)

Table 1 CTL activity of TIL and spleen cells against GL261 glioma cells

Experiment		CTL activity against GL261* (% lysis, E/T ratio = 20)	Phenotype (%) CD4	CD8
Experiment 1	TIL	81	3	97
	Spleen	80	4	96
Experiment 2	TIL	62	12	88
	Spleen	41	18	82

*CTL activity was measured on day 21 after initiation of MLTC (day 5)

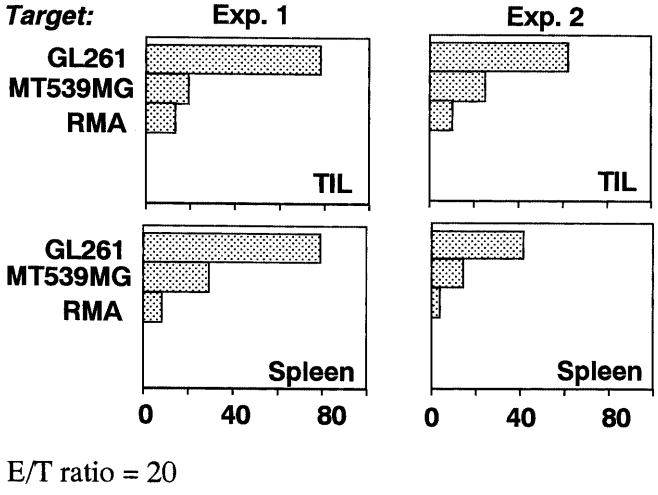

Fig. 3 Lytic activities of TIL and spleen cells for GL261 cells. TIL and spleen cells were obtained from mice treated with DCs and were stimulated with irradiated GL261 in the presence of syngeneic splenic feeder cells. Higher cytotoxic activities against GL261 cells were confirmed in TIL and spleen cells. Although MT539MG and RMA cells expressed a higher level of H-2K^{b}D^b than GL261, they were not lysed by the CTL lines from tumor and spleen of mice treated with DCs

the assistance of cationic liposomes. After intracerebral inoculation of mouse malignant glioma GL261 cells into syngeneic C57BL/6 mice, DCs pulsed with extract from the glioma cells by sonication were administered intraperitoneally thrice weekly on days 7, 14 and 21. Tumor growth inhibition in the treatment group was evaluated by measuring the 2 axes of each tumor in the H&E stained specimen one month after tumor inoculation. The group treated with pulsed DCs was inhibited in tumor progression compared with the non-pulsed DC control group, and the group treated with pulsed DCs by an efficient delivery system of liposomes obtained successful tumor volume reductions in all mice ($P < 0.01$). In addition, no visible tumor masses were found in several brains of the treated mice. Immunohistochemical study showed that the CD8-positive T cells were strongly recognized in the almost disappearing tumor cells of pulsed DCs groups. In addition, TILs and spleen cells obtained from the mice specifically induced cytotoxic activity against GL261 cells in vitro, although MT539MG and RMA cells expressed higher levels of H-2K^{b}D^b than GL261 (data not shown). Of course, we confirmed that these experiments are reproducible. These findings indicate that GL261-induced T cell lines recognize the antigen(s) expressed on GL261, but not on MT539MG or RMA. Furthermore, the cytotoxic activity was cancelled by anti-CD3, TCR$\alpha\beta$, CD8, H-2K^{b}D^b and H-2^b antibodies, but not by anti-CD4, TCR$\gamma\delta$, H-2^d, H-2^k, I-A^b and asialoGM1 antibodies. This shows that CD8-positive cells with a typical CTL phenotype predominantly contributed to the cytotoxicity. Liau et al. [8] reported the same phenomenon, i.e., that dendritic antigen-presenting cells pulsed with acid-eluted peptides derived from autologous tumors represent a promising approach to the immunotherapy of established intracranial gliomas.

These results suggest that immunotherapy using DCs pulsed with tumor extract–cationic liposome complex, may become a useful therapy for malignant gliomas.

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