Laboratory Investigation

Inhibitory Effects of Toxoplasma Antigen on Proliferation and Invasion of Human Glioma Cells

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Objective : Currently available therapies for human malignant gliomas have limited efficacy. Toxoplasma gondii, an obligate intracellular protozoan parasite, and Quil-A are nonspecific, potent immune stimulants. T. gondii is shown to have antitumor activity in some types of cancers. Therefore, this study is undertaken to evaluate the antitumor effect of Toxoplasma lysate antigen (TLA), alone or in combination with Quil-A, on human glioma U373MG and U87MG cells.

Methods : The in vitro effects of TLA alone or in combination with Quil-A on the proliferation, invasion, and apoptosis of glioma cells were tested using MTT, Matrigel invasion, and DNA fragmentation assays, and the in vivo effects on the growth of gliomas were evaluated in athymic nude mice transplanted with glioma cells.

Results : Treatment with TLA resulted in the suppressed proliferation and invasion of both U373MG and U87MG cells, in a dose-dependent manner. In addition, at high concentration, TLA induced glioma cell apoptosis. When TLA was administered in the mouse glioma model, malignant glioma growth was decreased. The combined treatment of TLA with Quil-A significantly inhibited the proliferation and invasion of cultured cells as well as tumor mass of implanted mice.

Conclusion : TLA inhibits the proliferation and invasion of glioma cells in vitro and in vivo, and these antitumor effects of TLA are significantly enhanced by the addition of Quil-A.

KEY WORDS : *Toxoplasma gondii* · Glioma · Proliferation · Invasion · Apoptosis.

Introduction

Human malignant gliomas are highly aggressive central nervous system tumors. The conventional treatment for patients with malignant gliomas consists of surgery, chemotherapy and radiotherapy. The effect of the currently available therapies is limited, and the prognosis for patients affected by these tumors is usually poor[20,28]. Given the lack of adequate therapy for gliomas, new approaches are necessary. Recent advancements in tumor immunology and tumor molecular biology have provided new information about human tumors. Immunotherapy has been investigated in various tumors as a new treatment modality to reduce injury to normal cells, as well as to increase the benefits of conventional treatment[5,25,26].

Immunotherapy for tumors aims to supplement the weak host immune response to a tumor by providing specific or nonspecific immune stimulants or by the direct administration of cytokines, tumor-specific antibodies, or T cells[1,5,26]. Nonspecific immune stimulants such as *Corynebacterium*, *Mycobacterium*, *Toxoplasma*, Quil-A, or synthetic polynucleotide complex have been used to promote immune responses[1,5,26]. *Toxoplasma gondii* is an obligate intracellular parasite with high affinity for brain cells; it can cause severe lesions in brain tissues. *T. gondii* antigen or *T. gondii* infection can nonspecifically increase the cytotoxicity of host cells and has thus been used widely as an effective immunostimulant in various tumors[6,9,12,23,30,32]. Tumor growth auto-induced in rats by 20-methylcholanthrene was inhibited after the administration of *Toxoplasma* lysate antigen (TLA)[23]. *Toxoplasma* infection and the cell-free extract of this parasite were able to reverse the multidrug resistance of mouse lymphoma and human gastric cancer cells in vitro[32]. These facts suggest the possibility that *Toxoplasma* antigen may be useful as a stimulant of cellular immune responses in humans and as an immunotherapy for cancer. There are, however, few reports of the antitumor activity of *T. gondii* in cerebral tumor cell lines.

The poor prognosis for patients with malignant gliomas is attributable at least in part to this cancer's high proliferation

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rate, resistance to radiotherapy and chemotherapy, and persistent invasiveness into surrounding normal brain parenchyma[20,28]. The poor results with current treatment modalities have generated interest in the use of immunotherapy as an additional mode of treatment. Little is known about the effects of TLA in combination with Quil-A for the treatment of malignant gliomas. To investigate the effectiveness of TLA in treating malignant gliomas, we studied the *in vitro* and *in vivo* antitumor activities of TLA, with and without Quil-A, in two human glioma cell lines, U373MG and U87MG.

Materials and Methods

Mice

Animal studies were conducted in accordance with the guidelines for animal care of the College of Medicine, Chungnam National University, Animal Care and Use Committee. Congenitally homozygous athymic male nude mice (Balb/c, *nu/nu*; weight, 15~20g) were obtained from the Japan SLC Inc. (Hamamatsu, Shizuoka Prefecture, Japan), kept in groups of eight or fewer, housed in sterile cages, and given autoclaved chow and water *ad libitum*.

Glioma cell culture

The human glioma cell lines, U373MG and U87MG, were obtained from the American Type Culture Collection (American Type Culture Collection, Rockville, MD, USA) and were cultured in RPMI 1640 (GibcoBRL Co., Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS; GibcoBRL) in a humidified atmosphere of 5% CO_2 at 37°C.

Preparation of *Toxoplasma* lysate antigen (TLA)

The RH strain of *T. gondii* was maintained by *in vitro* human fibroblast at 37°C and 5% CO_2. Infected fibroblasts were scraped, forcibly passed first through a 27-gauge needle, and centrifuged at 900×g for 10min using Percoll (Sigma Chemical Co., St. Louis, MO) to pellet parasites. The parasites were disrupted by sonication on ice and centrifuged at 100,000×g for 40min, after which the supernatants were pooled and sterile filtered (Gelman Sciences, Ann Arbor, MI). The protein content was determined by the Bradford method using bovine serum albumin as the standard. Samples were stored in aliquots at -20°C until use.

In vitro cell proliferation assay

Proliferation was evaluated by the microculture tetrazolium (MTT; Sigma) method according to the manufacturer's instructions. The U373MG and U87MG cells were resuspended in complete RPMI 1640 at 1×10^5cells/ml. One hundred $\mu\ell$ of cell suspension was distributed into each well of a 96-well plate. After incubation for 12-18hrs at 37°C and 5% CO_2, TLA (1, 5, and 25μg/ml) or Quil-A (1, 2.5, and 7.5μg/ml; Accurate Chemical and Scientific Corporation, Westbury, NY) was added, and the cells were incubated for 18hrs. Fifty $\mu\ell$ of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT formazan, 2mg/ml; Sigma) was added to each well, and the cells were incubated for another 4 hrs. To dissolve the formazan, 100$\mu\ell$ dimethyl sulfoxide (Sigma) was added, and the absorbance values were read at 540nm using an ELISA plate reader (Molecular Devices, Menlo Park, CA). The proliferation was checked in six wells for each treatment group; proliferation was calculated as the ratio of the absorbance of the treated group divided by the absorbance of the control, multiplied by 100 to give percent proliferation.

In vitro Matrigel migration assay

The invasion of glioma cells in vitro was measured using Matrigel-coated transwell inserts (Costa, Cambrige, MA). Briefly, transwell inserts of 8-μm pore size (diameter, 6.5mm) were coated with a concentration of 100μg/filter of Matrigel. The upper chamber was filled with 1×10^5 glioma cells in 200$\mu\ell$ culture medium supplemented with either TLA (1, 5, and 25μg/ml) or Quil-A (1, 2.5, and 7.5μg/ml). The lower chamber was filled with culture medium containing FBS. Cells were incubated at 37°C and 5% CO_2 for 24hrs. The cells in the lower compartment that had moved through the pores in the polycarbonate membrane were stained with hematoxylin and eosin. The number of stained cells in ten fields was counted under light microscopy (original magnification, ×200). Assays conducted in the absence of TLA or Quil-A (medium-treated) served as negative controls. All experiments were performed in six wells for each sample, and all specimens were counted twice by two independent researchers.

Genomic DNA fragmentation assay

To evaluate apoptosis, DNA was isolated from monolayers of U373MG and U87MG cells supplemented with either TLA or Quil-A by using a G-DEX™ genomic DNA extraction kit (iNtRON Biotechnology, Seoul, Korea), electrophoresed through a 1.5% agarose gel in Tris-borate-EDTA buffer (pH 8.0) at 50 V for 5hrs, and then visualized under UV light after ethidium bromide staining.

In vivo evaluation of antitumor activity

Athymic nude male mice received 2×10^8 U373MG or U87MG cells injected subcutaneously into the right flank.

Five groups of five animals each were treated with TLA or Quil-A administered intraperitoneally three times weekly for three consecutive weeks (treatment groups). The treatment was begun the day after cell injection. Control mice were injected with phosphate-buffered saline(PBS). All animals were killed 6 weeks after cell injection. The five groups examined were : mice that received only glioma cells (control group); mice that received 100μg/mouse of TLA after cell injection (T100-treated group); mice that received 25μg/mouse of Quil-A after cell injection (Q25-treated group); mice that received 100μg/mouse of TLA and 1μg/mouse of Quil-A after cell injection (T100/Q1-treated group); and mice that received 100μg/mouse of TLA and 25μg/mouse of Quil-A after cell injection (T100/Q25-treated group).

The extent of tumor growth was measured weekly using sterile metric calipers. Estimations of the tumor volume were made from the linear measurements using the formula[27].

Tumor volume (mm^3) = tumor width $(mm)^2$ × tumor length (mm) ×0.5.

Statistical analysis

The results are expressed as the mean± standard deviation (SD) for each group. Statistical evaluations of the differences in proliferation, invasion, and tumor volumes were determined by the two-tailed Mann-Whitney U test of the nonparametric-independent method and the Student's t-test. Differences between the various groups were considered significant when p values were <0.05.

Results

TLA inhibited proliferation of human glioma cells *in vitro*

To determine whether TLA could suppress the growth of human glioma cells, we first tested the effects of TLA on cell proliferation using the MTT assay. Treatment with TLA for 48 hrs significantly ($p<0.001$) reduced the proliferation rates of both U373MG and U87MG cells in a dose-dependent manner, compared with medium-treated (control) cells (Fig. 1A, B). The numbers of U373MG and U87MG cells were decreased 58.2±1.6% and 47.0±2.3%, respectively, at 25μg/ml TLA. The groups treated with both TLA and Quil-A exhibited significant inhibition of proliferation in comparison to treatment with TLA alone ($0.0001<p<0.001$), and the inhibition tended to increase with increased Quil-A concentration.

High concentration of TLA inhibited the migration of glioma cells *in vitro*

We next determined whether TLA could affect the invasiveness of glioma cells. U373MG or U87MG cells were placed in the upper compartment of a Transwell chamber coated with Matrigel, and the number of cells that infiltrated the lower chamber after 48 hrs was determined (Fig. 2A, B).

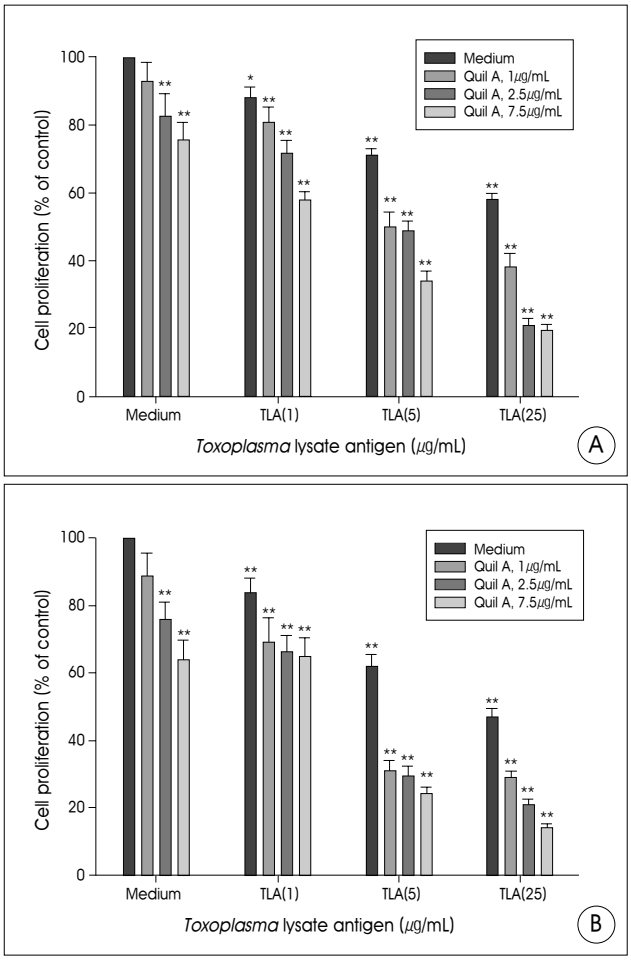

Fig. 1. Growth suppression of human glioma U373MG (A) and U83MG (B) cells by treatment with *Toxoplasma* lysate antigen alone or in combination with Quil-A for 48hrs. The control group, which was set as 100%, was incubated with only medium. Values marked by asterisks differe significantly from medium-treated (control) group (* $p<0.05$, ** $p<0.001$). Each value is the mean ± standard deviation of six different experimental wells.

As shown in Fig. 3A and 3B, there was no significant difference in the invasiveness of either U373MG or U87MG cells upon treatment with low concentrations of TLA (T5; 5μg/ml) or Quil-A only, compared with the control group (T5-treated group, $p=0.084$; Q1-treated group, $p=0.106$). However, the number of infiltrating cells was significantly decreased in the presence of 25μg/ml of TLA for 48hrs for both U373MG and U87MG cells ($0.001<p<0.0489$). Moreover, the combined treatment with TLA and Quil-A resulted in significantly greater inhibition than did treatments with TLA or Quil-A alone ($0.0001<p<0.0064$). The invasiveness of both U373MG and

U87MG glioma cells was about 50% inhibited in the groups treated with T25/Q1 and T25/Q7.5, compared with the control group.

Combined treatment with TLA and Quil-A induced apoptosis

We investigated whether TLA alone or in combination with Quil-A can induce programmed cell death in *in vitro* models. Genomic DNA fragmentation was apparent in both U373MG and U87MG cells after treatment with 25μg/ml of TLA for 22hrs (Fig. 4A). Apoptosis was noted in cell cultures treated with 25μg/ml, but not 1 ug/ml, of TLA for 24 hrs. Sometimes, apoptosis was happened in 5μg/ml of TLA. Quil-A-treated U373MG and U87MG cells showed no DNA fragmentation (Fig. 4B). The combined treatment with TLA and Quil-A apparently induced apoptosis in both glioma cell lines (Fig. 4C).

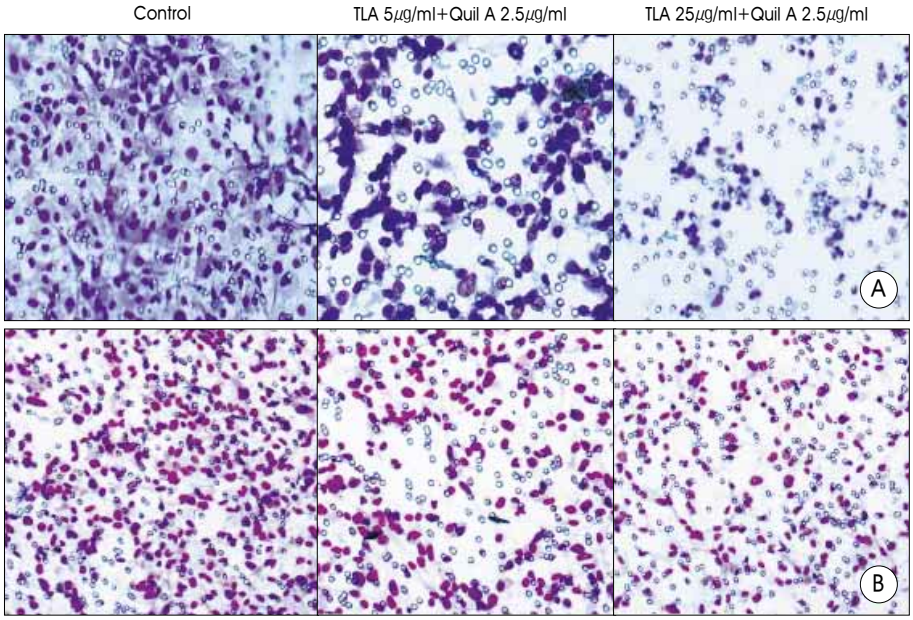

Fig. 2. Photomicrographs showing the infiltration of U373MG (A) and U87MG (B) cells in a Transwell assay. Cells were plated in the upper Transwell chamber and allowed to infiltrate through a Matrigel-coated membrane in the presence of *Toxoplasma* lysate antigen alone or in combination with Quil-A. Infiltrating cells were stained with Diff-Quik. Original magnification, ×400.

TLA reduced glioma growth *in vivo* in athymic nude mice

The effect of TLA on glioma growth in vivo was tested in athymic nude mice. TLA decreased glioma growth in subcutaneous models, and the effect was dose-dependent. Athymic nude mice receiving only U87MG cells (U87MG control) began dying by 5 weeks of cell injection, and 60% of those mice were dead at 6 weeks. However, athymic nude mice receiving U373MG cells (U373MG control) did not die during the experimental period, nor did mice that received U373MG or U87MG cells and treatment with TLA, Quil-A, or a combination of both (T100-, Q25-, T100/Q1-, and T100/Q25-treated group). Detectable tumor masses did not develop until 1 week after the injection of U373MG or U87MG cells. By 2 weeks after cell injection, all mice had palpable tumor masses. The skin overlying the tumor began to ulcerate at 4 weeks after cell injection (Fig. 5A, B, C).

In control mice receiving U373MG cells, the tumor volumes were 1,170 $\pm$ 172mm^3 at 3 weeks after injection and 2,520 $\pm$ 120mm^3 at 6 weeks (Fig. 6A). Compared with controls, the tumor volume was decreased significantly in T100-treated mice (p=0.014) but not in Q25-treated mice (p=0.1664). The

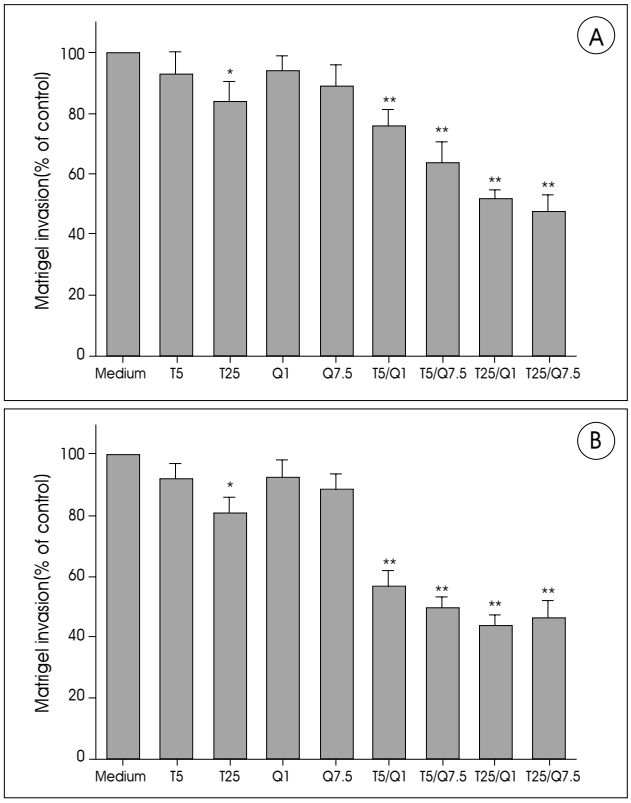

Fig. 3. The suppression of invasiveness of human glioma U373MG (A) and U87MG (B) cells on a permissive substrate by *Toxoplasma* lysate antigen alone or in combination with Quil-A. Values marked by asterisks differe significantly from medium-treated (control) group (* p<0.05, ** p<0.001). Each value is the mean $\pm$ standard deviation of six different experimental wells.

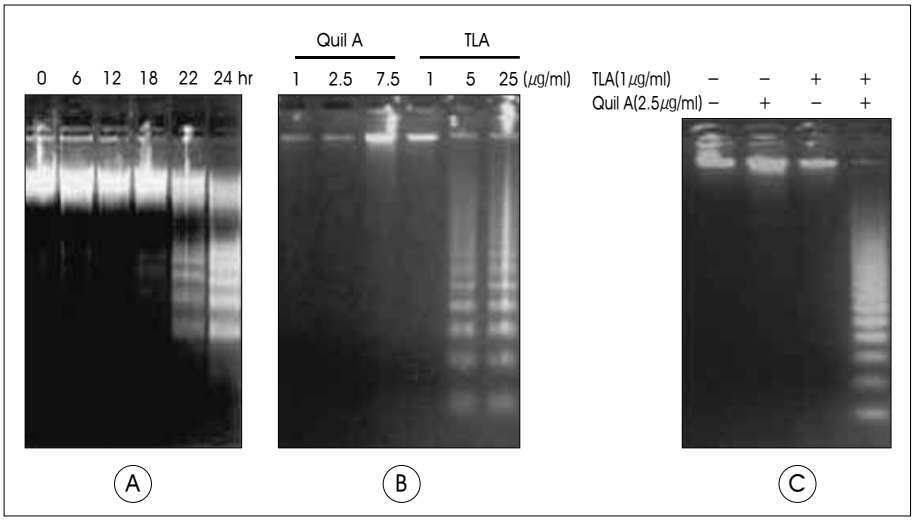

Fig. 4. Apoptosis-inducing effects of *Toxoplasma* lysate antigen alone or in combination with Quil-A in glioma cells. The fragmentation of genomic DNA was analyzed by agarose gel electrophoresis and ethidium bromide staining. A : time dependence of apoptosis induction by *Toxoplasma* lysate antigen in U373MG cells. B : concentration dependence of apoptosis induction by *Toxoplasma* lysate antigen or Quil-A in U373MG cells. C : apoptosis-inducing effects of the combined treatment with *Toxoplasma* lysate antigen and Quil-A in U373MG cells. The results represent three independent experiments.

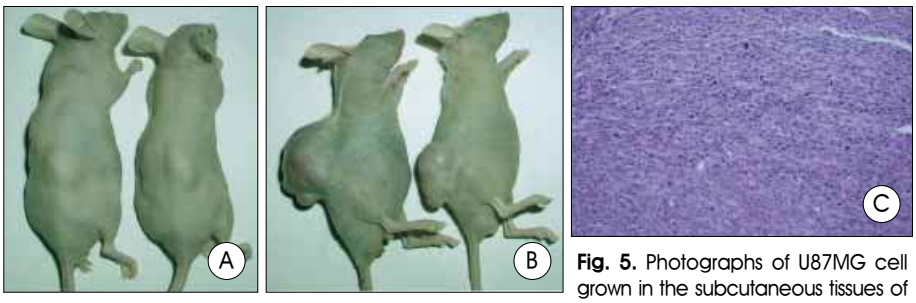

Fig. 5. Photographs of U87MG cell grown in the subcutaneous tissues of athymic nude mice. A and B, tumor mass of athymic nude mice subcutaneously injected with U87MG cells at day 14 (A) and 28 (B). C : histopathologic finding of tumor mass from athymic nude mice injected with human glioma U87MG cell line. Original magnification, ×400.

growth of the tumor masses in the T100/Q1-treated and T100/Q25-treated groups was also significantly inhibited compared with tumor growth in the control group ($0.013 < p < 0.017$), and there was a significant difference in growth between the T100/Q1- and T100/Q25-treated groups ($p=0.016$).

The tumors of athymic nude mice injected with U87MG cells were larger than the tumors of mice injected with U373MG cells, but the growth patterns of all groups receiving U87MG cells were similar to those of the groups receiving U373MG cells. The tumor volumes of the U87MG control group were $1,635 \pm 210 mm^3$ at 3 weeks after injection and $3,450 \pm 190 mm^3$ at 6 weeks (Fig. 6B), whereas the tumor volume with T100 treatment was significantly decreased($p=0.018$), but not with Q25 treatment ($p=0.589$). The tumor masses of the T100/Q1-treated and T100/Q25-treated groups also showed significantly inhibited growth compared with that of the control group ($p=0.025$ and $p=0.014$, respectively).

Discussion

Gliomas are the most common primary brain tumor in adults, constituting 40% of all central nervous system tumors, and the limitations of surgery, radiation therapy, and chemotherapy are well known. Therefore, immunotherapy has been suggested as an additional mode of treatment. In the present study, we examined the *in vitro* and *in vivo* effects of *Toxoplasma* antigen alone and in combination with Quil-A on malignant glioma growth. TLA inhibited glioma cell proliferation and migration *in vitro*. In athymic nude mouse malignant glioma models, TLA also significantly reduced glioma growth *in vivo*. Moreover, the addition of Quil-A to the TLA treatment significantly inhibited *in vitro* proliferation and invasion as well as tumor mass size in glioma cell-implanted mice in comparison to treatment with TLA alone. Apoptotic effects were evident upon treatment with TLA in combination with Quil-A. Our data indicate that the administration of TLA alone or in combination with Quil-A significantly inhibits glioma cell proliferation and migration *in vitro* as well as suppresses human malignant glioma growth *in vivo*.

Tumors frequently interfere with the development and function of immune responses such as delayed-type hypersensitivity, target cell cytotoxicity, and lymphocyte proliferation[1]. On the basis of advanced cellular and molecular immunology, the potential antitumor activity of cancer immunotherapy has been investigated in various forms. The ability of different infections to suppress neoplastic growth has been well documented. This phenomenon has been traditionally attributed to a concomitant infection-induced, cell-mediated, antitumor immunity. Infection with *T. gondii* results in strong cell-mediated immune responses, especially T cell mediated immune responses, and produces abundant cytokines[21]. Gliomas are

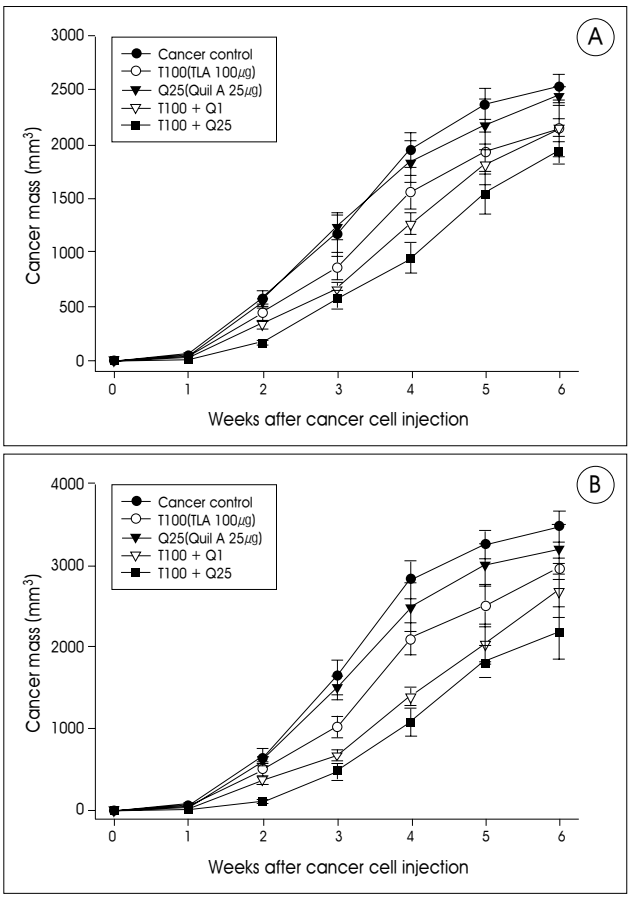

Fig. 6. Tumor size of athymic nude mice subcutaneously injected with U373MG (A) or U87MG (B) cells and treated with *Toxoplasma* lysate antigen alone or in combination with Quil-A. The data are represented as mean ± standard deviation of two separate experiments.

contained various cell types, including microglia, a type of differentiated tissue macrophage, and lymphocytes and so on. Microglia are capable of secreting a variety of immunomodulatory cyokines and account for a significant component to experimental gliomas[2,3]. Thus microglial fuction in gliomas may be important in the development of immunotherapy strategies. In the present study, TLA treatment of U373MG or U87MG cells significantly inhibited the proliferation and invasion of these glioma cells, and this inhibition was significantly enhanced by the addition of Quil-A. Similar results were found in athymic nude mouse injected with glioma cells. These results indicate that TLA alone or in combination with Quil-A may activate cell-mediated immune responses, subsequently inducing anti-proliferative and anti-invasive activity, as well as apoptosis[10,15,24].

There have been many reports on antitumor agents of human gliomas, and these agents have been divided into a few categories according to their main action mechanisms. The first group consists of some angiogenesis inhibitors, including human PEX, p16/INK4a, and AMG-1470, that can inhibit the proliferation of glioma cells[16,27,31]. Human PEX, derived from human glioma cells in culture, was a potent inhibitor of angiogenesis, proliferation and migration of tumor and endothelial cells, as well as a synergistic agent with low dose chemotherapy[27]. AGM-1470, a fungus-derived inhibitor of angiogenesis, significantly inhibited glioma growth and prolonged the survival of implanted mice[31]. In the second group, the administration of some immune modulating agents resulted in a significant inhibition of gliomas either *in vitro* or *in vivo*. Some members of this group are interferon, tumor necrosis factor, transforming growth factor β antagonist, IL-2, lymphocyte-activating killer (LAK) cells, BCG, and monoclonal antibodies to tumor[5,8,11,14,19,29]. LAK cells added to glioblastoma T98G and U373MG cells adhered to the cells and killed[8,14,29]. TLA and Quil-A are also included this category, so there have been many reports on the immune-modulating effects of TLA and Quil-A[6,9,12,15,23,24,30,32]. However, there are few studies using TLA or Quil-A in glioma models. In this experiment, TLA was an effective inhibitor of proliferation and invasion of human gliomas *in vitro* and *in vivo*, and its antitumor inhibitory effects were significantly enhanced by Quil-A. The third group of antitumor agents, which includes chemical and signaling-related agents such as $\alpha\gamma\beta$ 3 intergrin inhibitor (IS201), a differentiation-inducing agent (sodium butyrate), the combination of clotrimazole and cisplatin, and a nonsteroidal antiestrogen compound (Tamoxifen), inhibited the growth and invasion of gliomas[4,5,13,17,33].

Malignant gliomas are known to invade surrounding normal brain tissue, which prevents complete surgical resection and results in a high frequency of local recurrence. Glioma cells have been shown to infiltrate on permissive substrates, such as collagen Type IV, laminin, and fibronectin, in the brain[5]. Here, we demonstrated that treatment with a high concentration of TLA significantly suppresses the invasion of glioma cells through pores of a fibronectin-coated membrane. Invasiveness involves cell motility, adhesion to and detachment from substrates, and cytoskeletal organization. Thus, TLA alone or in combination with Quil-A is likely to affect one or more of these functions in tumor cells, although further studies are needed to delineate the involvement of TLA in these aspects of invasiveness. Also, successful migration and invasion of glioma cells requires their resistance to apoptosis after the cell has detached from the primary tumor tissue. In the this study, the combined treatment with TLA and Quil-A apparently induced apoptosis, suggesting that TLA and Quil-A could inhibit the invasion of glioma cells.

In the present study, we used TLA and Quil-A as immune stimulants. No obvious signs of toxicity, such as weight loss,

inactivity, opportunistic infections, or reduced appetite, were observed in the groups of animals treated with either TLA or Quil-A. When the animals were killed, their major organs were examined for the occurrence of any gross pathological changes, and no signs of distress or disease were documented. Quil-A is a type of saponin derived from the bark of *Quillaia saponaria* Molina. It is known to provide immune potentiating responses and, hence, can be useful as adjuvants. Although its function has not been well characterized, it is thought to induce antigen presentation, cytokine production, and T lymphocyte cytotoxicity[7,15]. In our study, treatment with a high concentration of Quil-A significantly inhibited the proliferation and invasion of glioma cells. Moreover, these phenomena were significantly increased by the combined treatment with TLA and Quil-A. These findings are similar to those of reports by Khan et al.[18] and Lunden[22]. Namely, the protective immune responses elicited by *Toxoplasma* antigens in the presence of Quil-A were more potent than those elicited by the parasite antigen alone. Also Quil-A was increased the apoptosis when it was treated with TLA. We found that injection with *Toxoplasma* antigen effectively blocked neoplastic growth of human glioma U373MG and U87MG cells. It may be possible that new immunostimulating molecules could be identified from *Toxoplasma* organisms.

Conclusion

Gliomas are the most common primary brain tumor in adults, constituting 40% of all central nervous system tumors. Currently available therapies for human malignant gliomas have limited efficacy. Therefore, immunotherapy has been suggested as an additional mode of treatment. *Toxoplasma gondii*, an obligate intracellular protozoan parasite, and Quil-A are nonspecific, potent immune stimulants. *T. gondii* was shown to have antitumor activity in some types of cancers. In the present study, we examined the *in vitro* and *in vivo* effects of *Toxoplasma* lysate antigen (TLA) alone and in combination with Quil-A on malignant glioma growth. Treatment with TLA resulted in the suppressed proliferation and invasion of both U373MG and U87MG cells, in a dose-dependent manner. In addition, at high concentration, TLA induced glioma cell apoptosis. When TLA was administered in the mouse glioma model, malignant glioma growth was decreased. The combined treatment of TLA with Quil-A significantly inhibited the proliferation and invasion of cultured cells as well as tumor mass of implanted mice. Our data indicate that the administration of TLA alone or in combination with Quil-A significantly inhibits glioma cell proliferation and migration *in vitro* as well as suppresses human malignant glioma growth *in vivo*.

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