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. 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.

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. Nonspecific immune stimulants such as Corynebacterium, Mycobacterium, Toxoplasma, Quil-A, or synthetic polynucleotide complex have been used to promote immune responses. 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. Tumor growth auto-induced in rats by 20-methylcholanthrene was inhibited after the administration of Toxoplasma lysate antigen (TLA). 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. 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
rate, resistance to radiotherapy and chemotherapy, and persistent invasiveness into surrounding normal brain parenchyma\(^2,28\). 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 treating 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 scrapped, forcibly passed first through a 27-gauge needle, and centrifuged at 900 \(\times\) 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 \(\times\) 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 \(\times\) 10\(^5\) cells/ml. One hundred \(\mu\)l 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 25\(\mu\)g/ml) or Quil-A (1, 2.5, and 7.5\(\mu\)g/ml; Accurate Chemical and Scientific Corporation, Westbury, NY) was added, and the cells were incubated for 18hrs. Fifty \(\mu\)l 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\(\mu\)l 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, Cambridge, MA). Briefly, transwell inserts of 8-\(\mu\)m pore size (diameter, 6.5mm) were coated with a concentration of 100\(\mu\)g/filter of Matrigel. The upper chamber was filled with 1 \(\times\) 10\(^5\) glioma cells in 200\(\mu\)l culture medium supplemented with either TLA (1, 5, and 25\(\mu\)g/ml) or Quil-A (1, 2.5, and 7.5\(\mu\)g/ml) or Quil-A (1, 2.5, and 7.5\(\mu\)g/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, \(\times\)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\(^\text{TM}\) 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 \(\times\) 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 100 μg/mouse of TLA after cell injection (T100-treated group); mice that received 25 μg/mouse of Quil-A after cell injection (Q25-treated group); mice that received 100 μg/mouse of TLA and 1 μg/mouse of Quil-A after cell injection (T100/Q1-treated group); and mice that received 100 μg/mouse of TLA and 25 μg/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:

\[ \text{Tumor volume (mm}^3\) = \text{tumor width (mm)}^2 \times \text{tumor length (mm)} \times 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 25 μg/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).

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; 5 μg/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 25 μg/ml of TLA for 48 hrs 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 25 ug/ml of TLA for 22hrs (Fig. 4A). Apoptosis was noted in cell cultures treated with 25 ug/ml, but not 1 ug/ml, of TLA for 24 hrs. Sometimes, apoptosis was happened in 5 ug/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).

**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 $1,170 \pm 172 \text{mm}^3$ at 3 weeks after injection and $2,520 \pm 120 \text{mm}^3$ 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
The 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 ± 210 mm³ at 3 weeks after injection and 3,450 ± 190 mm³ 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.  

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. Gliomas are
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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 cytokines and account for a significant component to experimental gliomas\(^2,3\). Thus microglial function 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 mice injected with glioma cells\(^4,14,29\). In this experiment, TLA was an effective inhibitor of proliferation and invasion of human gliomas \( \text{in vitro and in vivo} \), 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 \( \alpha\gamma\delta \) integrin 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 Quillaja saponaria Molina. It is known to provide immune potentiating effects of sodium butyrate on proliferation and invasiveness of human 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. and Lunden. 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 non-specific, 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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