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RESEARCH ARTICLE

INVESTIGATION OF THE ANTI-TUMOR EFFECTS OF BEVACIZUMAB ON GLIOBLASTOMA CELLS

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ABSTRACT

Aim: Glioblastoma multiforme (GBM) is the most malignant glial tumor. Angiogenesis which provides nutrient and oxygen support to proliferating cells plays an essential role in GBM development, proliferation, and metastasis. The development of antiangiogenetic agents is a promising treatment approach as blood vessels are essential for the vitality of tumor cells. For this purpose, in this study, the effects of bevacizumab on cell viability and apoptosis were analyzed using glioblastoma cells.
Material and method: U-87 MG and T98G cells were treated with various concentrations of bevacizumab for 24 hours, 48 hours, and 72 hours. Cell viability was analyzed after administration of bevacizumab. Cytotoxicity was determined using MTT. Apoptosis rate was determined with cell death detection kit.
Results: Cell viability analysis showed that when 8 mg / ml bevacizumab was administered to glioblastoma cells for 48 hours and 72 hours, cell proliferation was only 50% compared to proliferation of cells without bevacizumab. Additionally, apoptosis rates for U-87 MG and T98G cell lines treated with various concentrations of bevacizumab for 48 hours and 72 hours showed results similar to those of cell viability.
Conclusions: This study showed that while high concentration treatment of U-87 MG cells caused an increase in cell viability in a time-dependent manner, high dose treatment of T98G cells resulted in a decrease in cell viability in a time-dependent manner. This means that some glioblastoma cells can still survive under high doses of bevacizumab. This, in turn, demonstrated that glioblastoma cells developed resistance against bevacizumab.

Key words: Brain tumors, glioblastoma, bevacizumab, apoptosis, drug resistance

INTRODUCTION

The central nervous system is made up of neurons responsible for many tasks. Neurons are surrounded by glial cells which act as supporting cells (1,2). These cells surround the neurons and form the blood-brain barrier. Glial cells are classified as astrocytes, oligodendrocytes, and microglia (3). The World Health Organization (WHO) classifies these tumors from 1 to 4 depending on their characteristics, such as proliferative kinetics, histological characters, and cellular characteristics. While the most common type of brain tumors is astrocytoma, the deadliest is stage

4 primary brain tumor glioblastoma (GBM) (4). Worldwide standard treatment for all patients diagnosed with GBM is surgery followed by simultaneous chemotherapy and radiotherapy (5).

It is thought that the main reason for the failure of chemotherapeutic agents against GBM is the resistance of GBM against apoptosis. Temozolomide (TMZ), which is used as an alkylating agent, can kill GBM cells by stimulating apoptosis and autophagy (6,7). Various signaling pathway inhibitors have been tested in research studies. Furthermore, studies found that when bevacizumab, an anti- vascular endothelial growth factor-A (VEGF-A) monoclonal antibody, is administered with TMZ, it increases survival in GBM patients (8,9,10).

The aim of this present study is to examine the tumor suppressor effects of bevacizumab on glioblastoma cells. For this purpose, glioblastoma cell lines were treated with different concentrations of bevacizumab which was followed by analysis of cell viability and apoptosis rates.

MATERIALS AND METHODS

Cell lines and in vitro culture conditions

Human glioblastoma cell lines U-87 MG and T98G were purchased from ATCC and kept in Dulbecco's Modified Eagle Medium (DMEM) (Gibco; Thermo Fisher Scientific) at 37°C with 10% fetal bovine serum. GBM cells were incubated in a 5% CO_2 incubator.

Reagents

Dulbecco's Modified Eagle's Medium (DMEM), penicillin/streptomycin, 0.5% trypsin/EDTA solution, and fetal bovine serum (FBS) were purchased from Gibco. MTT kit was obtained from Thermo Fisher Scientific. Bevacizumab was obtained from Roche Diagnostics. Cell death detection kit was purchased from Thermo Fisher Scientific.

Cell proliferation measurements by MTT

Before experiments, U-87 MG and T98G cells growing in logarithmic phase were digested with 0.5% Trypsin-EDTA and the cells were pipetted. Cells were carefully counted and $5x10^3$ cells in 100 µL medium per well was seeded into a 96-well plate supplemented with different concentrations (0, 0.5, 1, 2, 4, 8 mg/ml) of bevacizumab. For each concentration, three wells were prepared and a blank control group with culture medium only was also set. Plates were incubated for 24h, 48 h and 72h. After that, cell viability was measured using MTT kit following the manufacturer's instructions. In brief, the medium was removed and replaced by 100 µL of fresh phenol red-free culture medium. 10 µL (10% of the volume of the culture medium) of MTT reagent was gently loaded into the medium in each well. Plates were kept in the incubator at 37℃ for 4 h. 75 µL of medium was removed from each well and then 50 µL of DMSO was added into each well and mixed thoroughly with the pipette. The 96-well plate was then incubated at 37℃ for 10 min. Then the samples were mixed again and the optical density (OD) was measured at 540 nm for each well using a plate reader.

Evaluation of apoptotic cell death

The first event seen in apoptosis is DNA fragmentation that follows the release of nucleosomes into the cytoplasm. In this study, apoptosis was evaluated using the Cell Death Detection ELISA Plus Kit (Roche Applied Science, Germany) according to the manufacturer's instructions. The principle of ELISA method is based on measuring mono and oligonucleosomes found in the cytoplasmic fractions of apoptotic cell lysates by means of monoclonal antibodies that recognize DNA and histones. At the end of the experiment, 100 µL of the lysis solution was added to the cells and kept for 30 minutes. Cell lysates belonging to all groups were centrifuged for 10 minutes at 200 x g. 20 µL of the obtained cytoplasmic fraction was transferred to the ELISA plate. 80 µL of the mixture containing peroxidase-linked anti-DNA and biotin-labeled anti-histone was added on it and kept at room temperature for 2 hours. At the end of this period, the wells were washed with the washing solution and ABTS (2.29 Azino-di- [3 ethylbenzothiazoline sulfonate] diammonium salt) solution was added. The reading was performed at 405 nm wavelength (490 nm as reference). Apoptotic cell death was given by proportioning the absorbance found to the absorbance of the control.

Statictical analyses

Data analyses were carried out using SPSS 25.00 statistics program. All experiments were conducted in triplicate and the results are expressed as the mean±SD. Student's t-test was used to compare the results between two groups. Two-way ANOVA followed by Bonferroni post-tests was applied to

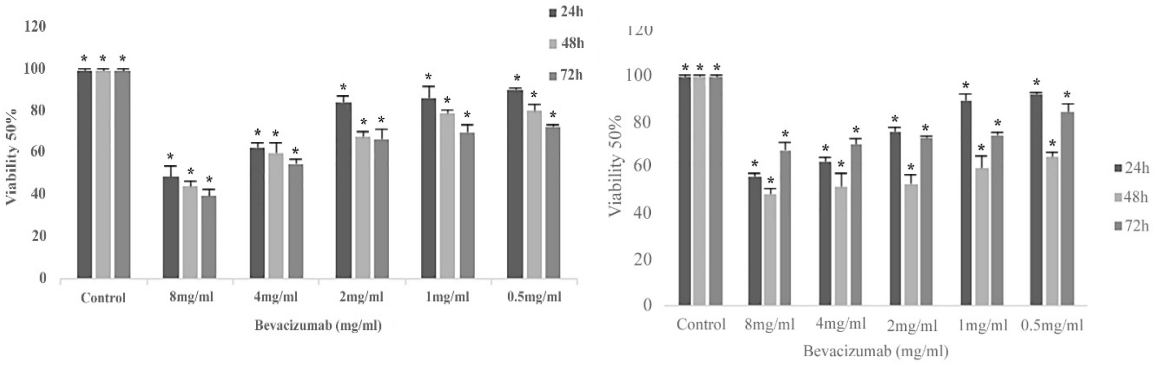

Figure 1. The anti-proliferation effects of bevacizumab on glioblastoma cells. (a) T98G cells were treated with different concentrations of bevacizumab for 24 h, 48 h and 72h, (b) U-87 MG cells were treated with different concentrations of bevacizumab for 24 h, 48 h and 72h and then the cell viability was measured using MTT kit. Error bars represent mean ± SD. P-values were determined by one-way ANOVA followed by Tukey's post hoc test. *P<0.05 vs. control group.

determine significant differences between different treatments in cancer cells ($p < 0.05$ was considered as significant).

RESULTS

Bevacizumab suppressed the proliferation of glioblastoma cells

Clinically, the monoclonal antibody bevacizumab is used as an angiogenesis inhibitor. However, it is unclear whether it could directly suppress the proliferation of tumor cells. In order to examine the effects of bevacizumab on glioblastoma cells, U-87 MG and T98G cells were treated with different concentrations of bevacizumab for 24, 48 and 72 hours and then cell proliferation and viability were examined using MTT kit. For T98G cells; it was observed that as bevacizumab concentration increased, cell viability decreased. Proliferation suppression increased even more when the duration of drug treatment was extended to 48 hours. When 8mg / ml of bevacizumab was administered for 48 hours, glioblastoma cells showed only about 50% proliferation compared to that of cells without bevacizumab (Figure 1a). For U-87 MG cells, it was observed that cell viability decreased with increasing bevacizumab concentration. Proliferation suppression was further increased when the drug treatment duration was extended to 72 hours. When 8 mg / ml of bevacizumab was administered for 72 hours, glioblastoma cells showed only about 50% proliferation compared to that of cells without bevacizumab (Figure 1b). These results show that bevacizumab was able to directly suppress the proliferation of U-87 MG glioblastoma cells in a dose and time-dependent manner.

Bevacizumab promoted the apoptosis of glioblastoma cells.

Besides its anti-proliferation effects, we also investigated whether bevacizumab could promote apoptosis in glioblastoma cells. T98G cells were treated with various concentrations of bevacizumab for 72 hours. This was followed by measuring apoptosis using ELISA method. Without bevacizumab, T98G cells showed a very low percentage of apoptosis. On the other hand, even a low dose (2mg / ml) of bevacizumab was able to significantly induce high levels of apoptosis (Figure 2). However, the rate of apoptosis decreased in high concentrations. This means that some glioblastoma cells can still survive even when high doses of bevacizumab are administered, and that T98G cells may have gained resistance to bevacizumab (Figure 2a).

As for U-87 MG cells, they showed a very low percentage of apoptosis without bevacizumab. However, even a low dose (2mg / ml) of bevacizumab could induce high levels of apoptosis to a significant level (Figure 2b).

DISCUSSION

The current treatments used for glioblastoma have many drawbacks such as low efficacy, various side effects, and resistance to chemotherapeutic drugs. All these problems cause high mortality and high glioblastoma recurrence. Despite the advances in developing new chemotherapeutic agents, resistance of cancer cells to chemotherapy is still a very important issue for both clinicians and drug developers; and this could seriously reduce the effectiveness of anticancer drugs in clinical practice.

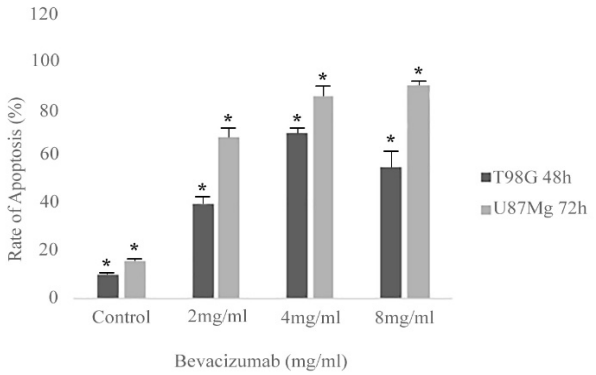

Figure 2. Bevacizumab induced apoptosis in T98G cells in a concentration-dependent manner at 72h and Bevacizumab induced DNA fragmentation in U-87 MG cells in a concentration-dependent manner at 48h *p < 0.05.

One study examined the effectiveness of the combination of irinotecan and bevacizumab in the treatment of GBM. In this phase II prospective study, 85 different and recurrent brain tumor patients were included (GBM, glioma WHO grade III, glioma WHO grade I and others). İt was found that bevacizumab and irinotecan combination was well tolerated by the patients. Additionally, it was observed that most of the patients in the glioblastoma and glioma WHO grade III groups achieved disease stabilization (11).

İn another study 921 patients with glioblastoma brain tumor diagnosed from 26 countries were divided into 2 groups; the first group received adjuvant therapy + bevacizumab and the second group received adjuvant therapy + placebo. In this Phase III study, it was found that the bevacizumab group (n = 458) had a significantly increased survival and a slowed down brain tumor formation compared to the placebo (n = 463) group (12)

Huan et al. using U-87 MG cells, it was observed that bevacizumab caused direct anti-proliferation and pro-apoptosis effects on glioblastoma cells by providing downregulation of anti-apoptotic proteins and regulating pro-apoptotic proteins (13). In our present study, the effect of bevacizumab, an anti-angiogenic reagent, on tolerance induction in T98G and U-87 MG glioblastoma cells was investigated. It was found that bevacizumab can directly suppress the proliferation of glioblastoma cells in a dose and time-dependent manner. Meanwhile, bevacizumab was able to promote the apoptosis of tumor cells. However, apoptosis of glioblastoma cells decreased when treated with high doses of bevacizumab, indicating that some glioblastoma cells could tolerate bevacizumab. To the best of our knowledge, the first study to report that autophagy plays a role in the tolerance of glioblastomas to bevacizumab was conducted by Huan et al. (14). Therefore, they showed that inhibition of autophagy is acceptable as a new way to overcome the tolerance of glioblastomas to anti-angiogenic agents.

Bevacizumab has been shown to reduce VEGF and pro-angiogenetic molecules in glioblastoma cell lines and may be a promising agent for these therapeutic targets.

Figure 1(a-b). The anti-proliferation effects of bevacizumab on glioblastoma cells. (a) T98G cells were treated with different concentrations of bevacizumab for 24 h, 48 h and 72h, (b) U-87 MG cells were treated with different concentrations of bevacizumab for 24 h, 48 h and 72h and then the cell viability was measured using MTT kit. Error bars represent mean ± SD. P-values were determined by one-way ANOVA followed by Tukey's post hoc test. *P<0.05 vs. control group.

Figure 2. Bevacizumab induced apoptosis in T98G cells in a concentration-dependent manner at 72h and Bevacizumab induced DNA fragmentation in U-87 MG cells in a concentration-dependent manner at 48h *p < 0.05.

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