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Efficacy of intratumoral chemotherapy using arsenic trioxide (As_2O_3) sustained release tablets for the treatment of neurogliocytoma in nude mice

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Abstract. – OBJECTIVE: This study aimed to investigate the inhibiting effect of arsenic trioxide (As_2O_3) on neurogliocytoma in nude mice and the mechanism responsible for this effect.

MATERIALS AND METHODS: Neurogliocytoma implantation models were constructed in nude mice, which were assigned to three groups: the control group, the sustained release tablet-polylactic acid-glycolic acid polymer (50:50) group (PLGA group) and the As_2O_3-polylactic acid-glycolic acid polymer (50:50) (As_2O_3-PLGA group). One tablet of As_2O_3-PLGA was implanted in the tumor of the As_2O_3-PLGA group. Intratumoral implantation was also performed in the other groups using a different type of tablet. The sustained releasing As_2O_3 had an inhibiting effect on the tumors. The TUNEL assay was used to determine the apoptosis rates in the implanted tumors. Immunohistochemical staining and Western blotting was carried out to determine the expression levels of caspase-3 and Bcl-2.

RESULTS: No inhibitory effect was observed on the tumor in the PLGA group, and there was no significant difference between this group and the control group. Subcutaneous tumor growth in nude mice was significantly inhibited in the As_2O_3-PLGA group relative to that in the control group, and the difference was statistically significant ($p < 0.01$). The tumor inhibition rate was 60.8%. The percentage of apoptotic tumor cells in the As_2O_3-PLGA group was 30.8%, which was significantly higher than that in the control group (3.92%) and that in the PLGA group (4.08%). The expression of Bcl-2 in the implanted tumor tissue was significantly reduced, but the expression of caspase-3 increased significantly.

CONCLUSIONS: As_2O_3 has a potent inhibiting effect on the growth of neurogliocytoma in vivo and can induce the apoptosis of tumor cells. The molecular mechanism of this effect may be related to the downregulation of Bcl-2 expression and the upregulation of caspase-3 expression.

Key Words:
Arsenic trioxide (As_2O_3), Polylactic acid-glycolic acid (PLGA), Neurogliocytoma, Nude mice, Apoptosis.

Introduction

Neurogliocytoma is the most common intracranial malignant tumor, and it has a poor prognosis. One of the primary reasons for neurogliocytoma's poor prognosis is its recurrence after tumorectomy and its resistance to treatment. Arsenic trioxide (As_2O_3) can play an active role in the treatment of tumors such as leucocythemia. An *in vitro* study demonstrated that As_2O_3 can inhibit the proliferation of neurogliocytoma cells by inducing apoptosis[1]. Unfortunately, because of its very low transmissivity through the blood-brain barrier (BBB), an effective concentration of As_2O_3 cannot reach the tumor site[2], and thus, its anti-tumor effect *in vivo* cannot be determined. In this study, polylactic acid-glycolic acid (PLGA) polymer[3,4], which is one new nontoxic, nonirritating and highly biocompatible material with biodegradation and sustained release applications, was used to prepare As_2O_3-PLGA sustained release tablets by direct compression, and the tablets were implanted in nude mice models of C6 neurogliocytoma to observe the inhibiting effect of As_2O_3 on the growth of implanted tumors and determine the mechanism of action.

Materials and Methods

Construction of the Implanted Tumor Model In Nude Mice

The surgeries were performed on a superclean bench. Twenty-four BALB/c nude mice were routinely disinfected with 75% ethanol, and then 0.5 ml of a C6 cell suspension (containing 2×10^6 cells in the exponential growth phase) was subcutaneously injected into the outer flank of the forelimb of each nude mouse.

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This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of The First Affiliated Hospital of Harbin Medical University.

Preparation of the PLGA Polymer

As_2O_3 and PLGA were added into an appropriate amount of acetone according to a certain ratio, dissolved by intense mixing, and then placed in a vacuum drying oven. After the acetone and the remaining water had evaporated, the sample was pulverized, blended, and processed into circular films with a diameter of 5 mm and a thickness of 1.5 mm. One tablet weighted 50.0±0.1 mg and contained 1.6 mg As_2O_3. After tissue release for 3 weeks, the preparations were sterilized using ^{60}Co radiation (radiation dosage: 5000 Gy).

Groups and Implantation

When the volume of tumors reached 1200 mm^3, approximately 2 weeks after tumor implantation, the nude mice were randomly assigned to one of three groups, the control group, the retarder-polylactic acid-glycolic acid polymer (50:50) group (PLGA group) and the As_2O_3 sustained release tablet group (As_2O_3-PLGA group). Each group included 8 mice. An incision of approximately 5 mm was made in the skin on the lateral wall of the tumor in each mouse, and after the tumor was separated, one tablet of PLGA or one tablet of As_2O_3-PLGA was implanted in the center of tumor in each mouse in the PLGA and As_2O_3-PLGA groups, respectively. A simulated implantation at the same site was performed in the control group.

Observation and Measurement of Tumors

We routinely observed the behavior, appetite, feces and the urine of nude mice. We also measured the maximum diameter (1) and the minimum diameter (2) of the tumor using a slide caliper and calculated the tumor volume by using the formula $V=1/6\pi ab^2$ [5]. The average tumor volume was then calculated. The tumor inhibition rate was calculated according to the formula: tumor inhibition rate = (volume in the control group – volume in the treatment group)/volume in the control group × 100%.

Histological Examination and Apoptosis Assay

The gross appearance of each tumor was observed at week 3. Tumor tissue samples were harvested, fixed in 10% formalin solution, and embedded in paraffin. After the tissue sections were prepared, they were stained with hematoxylin and eosin, and then a histopathological examination was performed. A TUNEL assay to evaluate the level of apoptosis was performed with an In Situ Cell Death Detection Kit (Roche Co., Ltd). Cells with dark brown nuclei were defined as positive cells. Five visual fields were randomly selected in each section, according to the principle of double blindness, and the number of positive cells among 500 cells viewed at higher magnification was counted by two staff members. These values were averaged.

Immunohistochemical Staining

An S-P immunohistochemistry kit was used. Primary antibodies against Bcl-2 (1:100, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) and caspase-3 (1:100, Santa Cruz Biotechnology, Inc) were incubated with two consecutive pathological sections of the same specimen overnight at 4°C, and then a biotin-labeled secondary antibody and horse radish peroxidase-labeled streptavidin were added. The antibody binding was visualized by adding 3,3'-diaminobenzidine (DAB), and the section was stained with hematoxylin. The specific procedures were carried out in accordance with the instructions for the S-P immunohistochemistry kit. PBS (Phosphate Buffered Saline) was used instead of primary antibody as negative control. Cells with yellow brown cytoplasm or nuclei were considered positive. The percentage of positive cells was calculated after counting 1000 cells at higher magnification (×400) according to the following formula: percentage of positive cells = (number of positive cells/number of total cells) × 100%.

Western Blot Assay

Tumor tissues of three mice were taken and kept in a nitrogen canister. A mortar was cleaned, air-dried at 80°C, and then cooled. The tumor tissue was added to the mortar and crushed together with liquid nitrogen, 2 ml lysis buffer (Beyotime Biotechnology, Beijing, China) code number P0013, 10000 rpm, PMSF: 1.74 ng/ml PMSF contained in 0.01 mM/L) per 1 g tissue. Then the proteins were collected, held at 4°C for 2 h, and centrifuged (10000 rpm) at 4°C

for 4 h. The supernatant was collected in 100 µL aliquots and kept at –20°C[6]. Tumor tissue proteins (50 µg), prepared as described above, were separated by 12% SDS-PAGE (spacer gel: separation gel = 80:120 by volume) and then transferred to a nitrocellulose filter (4°C, 200 mA, 2 h). Evaporated skimmed milk was added dropwise, and the membrane was sealed at room temperature for 1 h. Then, primary antibodies (diluted 1/2000) were added, and the blot was incubated overnight at 4°C. The membrane was then washed and incubated with the secondary antibodies (diluted 1/2000). After treatment for 1 h at room temperature, the nitrocellulose filter was treated with enhanced chemiluminescence (ECL) Plus (Amersham Biosciences, Uppsala, Sweden) for 5 minutes and exposed to X-ray film in the darkroom. The film was then developed and fixed[7].

Statistical Analysis

All data were expressed as the mean ± standard deviation (SD). First, the homogeneity test for variance was performed for all data, and then SAS.9.1.3 statistical software was used for the analysis of variance for factorial designs and the significance test. $p < 0.05$ indicated a statistical difference among different factors, and $p < 0.01$ denotes a significant difference.

Results

Effects of As2O3-PLGA on the Growth of Implanted Neurogliocytomas in Nude Mice

The rate of tumor growth was significantly lower in the As_2O_3-PLGA group, and the tumor volume in this group was markedly smaller than those in the control group ($p < 0.01$) and the PLGA group ($p < 0.01$). There were no statistically significant differences between the PLGA group and the control group at various time points ($p > 0.05$) (Figure 1A). The average tumor inhibition rates at week 1, week 2, and week 3 after treatment in the As_2O_3-PLGA group were 47.1%, 77.3%, and 86.1%, respectively, and the tumor inhibition rate in the PLGA group was less than 10%. The difference in the inhibition rate between these two groups was statistically significant ($p < 0.01$). This result demonstrates that As_2O_3 inhibited the growth of the neurogliocytomas and that the inhibiting effect was related to As_2O_3 and not to PLGA (Table I).

Pathological Examination and TUNEL Assay of Implanted Tumor Tissue

Tumor tissues were sampled for pathological examination after 3 weeks of treatment in each group. There were no significant differences between the PLGA group and the control group, which both exhibited abundant tumor cells, significant heteromorphism in the cell nuclei and many capillary vessels (Figure 1A). Significantly fewer tumor cells were observed in the As_2O_3-PLGA group, and karyopyknosis, chromatin agglutination and marginalization, crescent-shaped nuclei, and even occasional nuclear fragmentation were observed in the tumor cells, indicating that these cells were undergoing apoptosis (Figure 1B). The TUNEL assay results for peripheral tumor tissue sections showed that the positive stain rate in the control group was 3.92 ± 0.41% and that the positive stain rate in the PLGA group was 4.10 ± 0.17%; there was no significant difference between these two groups ($p > 0.05$) (Figure 1C). The positive stain rate in the As_2O_3-PLGA group was 30.0 ± 0.89%. The values for this group were significantly different from those of the other two groups ($p < 0.01$) (Figure 1D), indicating that As_2O_3-PLGA sustained release tablet can induce apoptosis in neurogliocytomas.

Immunohistochemical Assay

There were no significant differences with respect to the percentages of Bcl-2- and/or caspase-3-positive cells between the PLGA group and the control group at week 3 after treatment ($p > 0.05$) (Figure 2A and C). The percentage of Bcl-2-positive cells in the As_2O_3-PLGA group was significantly lower than that in the control group ($p < 0.0001$) (Figure 2B), and the percentage of caspase-3-positive cells in the As_2O_3-PLGA group was significantly higher ($p < 0.0001$) (Figure 2D) than that in the control group (Table II).

Western Blot Assay

A Western blot analysis of two proteins in the neurogliocytoma tissue of nude mice showed that the expression level of Bcl-2 was significantly downregulated and the expression level of caspase-3 protein was significantly upregulated in the As_2O_3-PLGA group. The concentrations of the two proteins were not significantly different between the PLGA group and the control group (Figure 3).

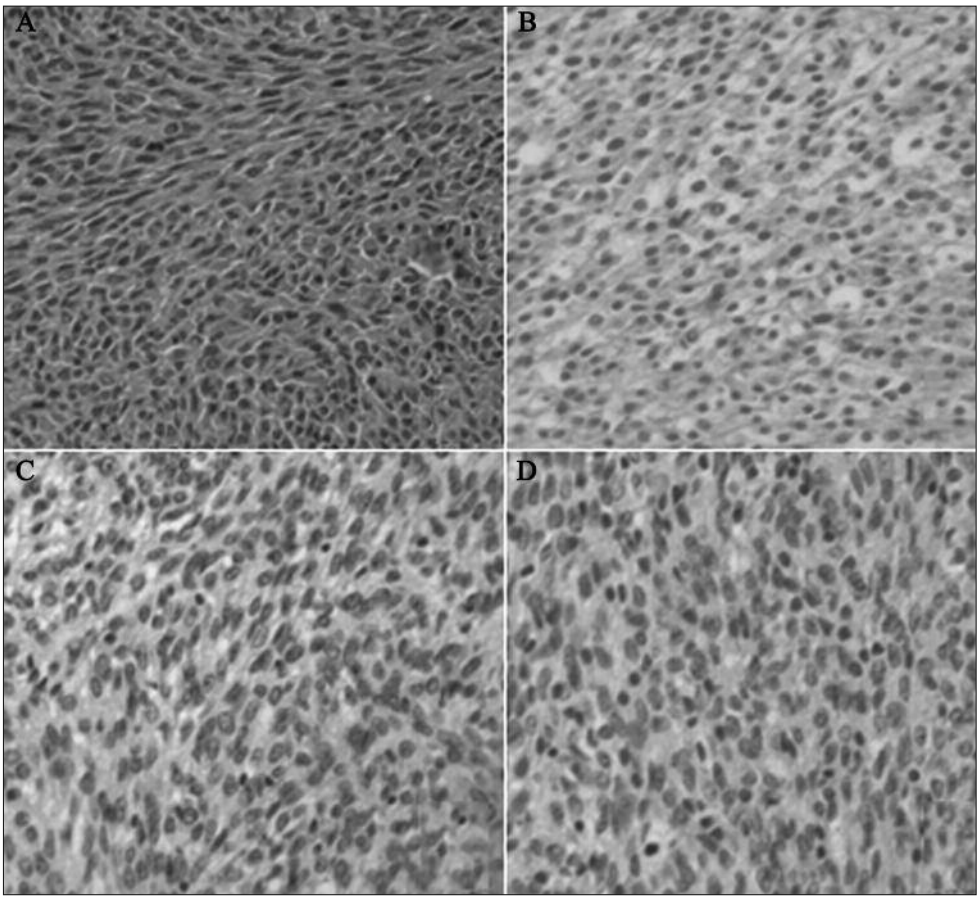

Figure 1. Histology and TUNEL staining of implanted tumor in the control group (HE×400) and As_2O_3-PLGA group. ***A,*** Abundant tumor cells displaying heteromorphism of the nuclei and more capillary vessels. ***B,*** Significantly fewer tumor cells, karyopyknosis and chromatin agglutination and marginalization are present in the tumor cells, which thus display signs of apoptosis. ***C,*** Cells in which the nuclei were stained dark brown was defined as positive cells; these scattered cells accounted for approximately 3.92% of all cells. ***D,*** There were fewer cells, which were scattered. The cells in which the nuclei were stained dark brown were defined as positive cells, and these cells accounted for approximately 30.80% of all cells.

Discussion

Neurogliocytoma is the most common malignant tumor of the central nervous system, and it cannot be completely removed by surgery because of its characteristic infiltrative growth[8,9]. Although chemotherapy is an effective way to eliminate the residual tumor cells, its clinical efficacy is greatly restricted because of its significant systemic toxic side effects and the difficulty of the drugs in passing through the BBB[10,11]. Local chemotherapy with a sustained release agent,

Table I. Volume measurements for implanted tumors in each group.

Group	Average volume of the implanted tumors (mm^3) (mean ± SD)		
	Week 1 after tumor implantation	Week 2 after tumor implantation	Week 3 after tumor implantation
Control	2416.2 ± 56.6	5210.4 ± 78.5	6200.7 ± 86.7
PLGA	2387.8 ± 30.2	5190.6 ± 89.2	6280.8 ± 66.3*
As_2O_3-PLGA	1278.4 ± 20.8	1180.5 ± 30.4	867.5 ± 10.8**

Note: Compared with the control group, $*p = 0.8399 > 0.05$; Compared with the control group, $**p < 0.0001$.

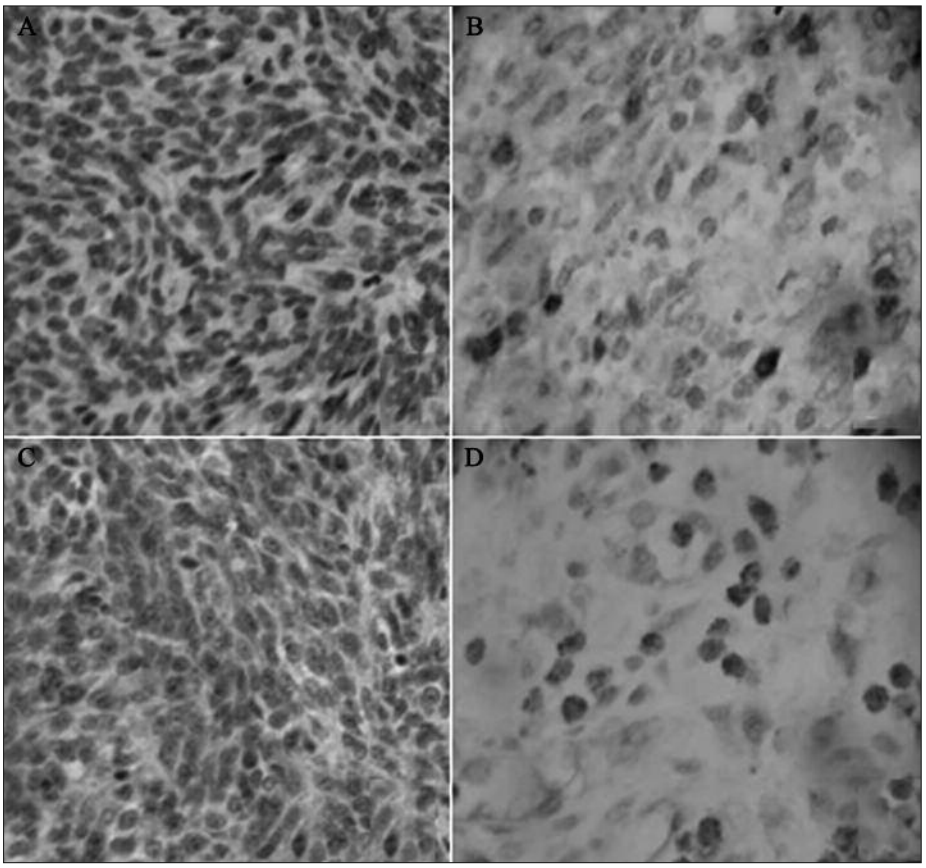

Figure 2. Immunohistochemistry for protein expression in implanted tumor from the control group and As_2O_3-PLGA group. (***A, B,*** Bcl-2; ***C, D,*** Caspase-3). Cells in which the cytoplasm was stained yellow were defined as positive cells.

a technique that has been developed in recent years, provides a new way to improve the prognosis of neurogliocytoma[12]. The features of this type of therapy include a prolonged action period; delivery of a higher local concentration; direct action on the brain cancer cells, avoiding the blood-brain barrier (BBB); and a very low drug concentration in the blood and adjacent brain tissue. PLGA, which was used in this study, is a highly biodegradable material with long-term safety that is used in pharmaceutical and in clini-

Table II. Percentages of Bcl-2- and caspase-3-positive cells at week 3 after treatment (n=8, mean ± SD, %).

Group	Bcl-2 positive	Caspase-3 positive
Control	77.14 ± 13.25	26.72 ± 6.99
PLGA	76.68 ± 9.39*	28.93 ± 5.88•
As_2O_3-PLGA	22.69 ± 4.61#	82.12 ± 10.09#

Note: Compared with the control group, $^{*}p = 0.9251 > 0.05$; compared with the control group, $^{\bullet}p = 0.9251 > 0.05$; compared with the control group, $^{\#}p < 0.0001$.

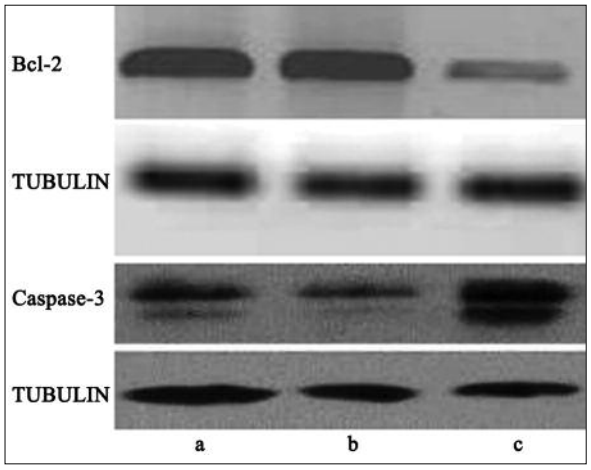

Figure 3. Western blot analysis. There was no significant difference in the expression level of Bcl-2 in the implanted tumor tissues between the control group and the PLGA group. The expression level of Bcl-2 in the implanted tumor tissue in the control group was statistically significantly lower than that in the As2O3-PLGA group. There was no significant difference in the expression level of caspase-3 protein in the implanted tumor tissue between the control group and in the PLGA group. The expression level of caspase-3 in the implanted tumor tissue in the control group was statistically significantly higher than that in the As2O3-PLGA group. ***a,*** Control group; ***b,*** PLGA group; ***c,*** AS2O3-PLGA group.

cal settings[13]. In addition, this polymer has favorable biocompatibility. One As_2O_3-PLGA sustained release tablet was implanted in the neurogliocytoma of each nude mouse in the experimental group in this study. The dose of As_2O_3 was 1.6 mg (1/5 LD50 ×7), far greater than LD50. This dose had no effects on the growth of the nude mice but significantly inhibited the growth of tumor, demonstrating once again that PLGA has great utility when it is used as a material for sustained drug release.

As_2O_3 has achieved favorable clinical efficacy when used for the treatment of leucocythemia and lymphoma, and domestic and international researchers have begun to further investigate the therapeutic efficacy of As_2O_3 and its mechanism of action in various solid tumors in recent years[14-16]. In human glioblastoma cells, As_2O_3 has the effects of changing the gene expression of metallothioneins and involving in arsenic-related induction of type II cell death[17]. At both normoxia and hypoxia condition, neuroblastoma cells are responsive to arsenic trioxide and supporting its as a potential candidate drug as a complement to conventional treatments for high-risk neuroblastoma patients[18]. Further research[19] found that the mechanisms of As_2O_3 induced apoptosis was via activation of caspase-3. In another research[20], synergistic effects of arsenic trioxide and silibinin on apoptosis was also observed. Our previous *in vitro* study also confirmed that As_2O_3 can inhibit the proliferation of tumor cells by inducing the apoptosis of neurogliocytoma cells[21]. The sustained release material PLGA was used as a carrier for As_2O_3 in this study. The results demonstrate that As_2O_3 can induce the apoptosis of neurogliocytoma cells *in vivo*, as determined by histopathological examinations and the TUNEL assay.

Apoptosis involves a series of active death processes that are activated in a cascade after various death signals are received. Apoptosis is regulated by multiple genes closely related to the Bcl-2 family[22,23]. Caspase is the abbreviation for cysteinyl aspartate-specific proteinase, and caspases play an important role in the apoptotic pathway, especially caspase-3, which is a key link in the apoptotic pathway. Caspase-3 is the "core" proteinase that induces apoptotic proteinase cascade reactions[24], and changes in the caspase-3 activity level can significantly affect the apoptotic capacity of tumor cells. Bcl-2 is an oncogene that was isolated from follicular lymphoma by Tsujimoto in 1984. The function of the protein is to block the apoptosis[25]. Our study demonstrates that in the process of As_2O_3-induced apoptosis, the expression of Bcl-2 is downregulated and the enzyme activity of caspase-3 is upregulated.

Conclusions

As_2O_3 has a significant inhibitory effect on the growth of implanted C6 neurogliocytomas in nude mice and can induce the apoptosis of tumor cells. The molecular mechanisms by which apoptosis is induced probably involve the downregulation of the expression of the Bcl-2 protein and the upregulation of the expression of the caspase-3 protein. As_2O_3-PLGA may be a potential formulation that can be used to treat neurogliocytoma with As_2O_3.

Acknowledgements

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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