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ORIGINAL ARTICLE

Arsenic trioxide sensitizes cancer stem cells to chemoradiotherapy. A new approach in the treatment of inoperable glioblastoma multiforme

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Summary

Purpose: *Glioblastoma multiforme (GBM) still bears a very dismal prognosis even with complete resection followed by adjuvant chemoradiation. The aim of the current study was to evaluate in vitro the antitumor efficacy of arsenic trioxide (ATO) in combination with ionizing radiation plus temozolomide and bevacizumab against cultured glioblastoma stem-like cells, as possible way to increase the therapeutic index in patients diagnosed with recurrent, therapy-refractory GBM.*

Methods: *Stem-like tumor cells isolated from a GBM biopsy were established by cell proliferation assays and up-regulation of stem cell markers, as proven by reverse transcription – polymerase chain reaction (RT-PCR). Low concentrations of ATO were added prior to temozolomide, bevacizumab and ionizing irradiation.*

Results: *Molecular analysis showed that cells expressed CXCR4, Oct-3/4 and GAPDH when compared to placental mesenchymal stem cells, as well as nestin, GFAP and neurofilament protein. Low concentrations of ATO led to morphologic differentiation, with fewer stem cells in Go state and differentiation-associated cytochemical features, like increased sensitivity to cytostatic drugs and radiotherapy.*

Conclusion: *ATO exposure before conventional post-operative chemoradiotherapy for GBM might increase treatment efficacy. Further in vivo experiments on laboratory animals and analysis of absorption rate and side effects are required.*

Key words: arsenic trioxide, glioblastoma multiforme, *in vitro* model

Introduction

GBM is the most common malignant primary brain tumor. Standard initial treatment for this cancer is surgical resection and radiotherapy plus concomitant adjuvant chemotherapy [1]. This WHO grade IV tumor can derive either *de novo* with no prior evidence of a lower-grade tumor, called primary glioblastoma, or through malignant progression from a lower grade malignancy, called secondary glioblastoma [2,3]. At the populational level, most glioblastomas are primary tumors, with a mean period from the first symptoms to pathological diagnosis of 6 months and a median survival of 6-8 months with few patients surviving for more than 2 years, despite the latest salvage chemoradiotherapy regimens. A major cause is the diffuse local growth pattern related to high neoangiogenesis capabilities of glioblastoma's stem cells and a low radio- and chemo-sensitivity

The past two decades have seen only limited progress in basic research for GBM because previously established glioma cell lines are composed of morphologically and functionally diverse cells that express a variety of neural lineage markers and because it was thought that this type of cancer arises by the transformation of an intraparenchymal glial cell that invades the surrounding tissue and develops as a result of stepwise accumulation of multiple genetic alterations. It is now accepted that these previously established concepts do not replicate the major biological features of GBM, and particularly cancer stem cells (CSC).

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High grade gliomas contain subpopulations of cancer cells with stem-like characteristics, including self-renewal and multidrug resistance, thought to be responsible for clinical relapse despite surgery, radiation, chemotherapy and immunotherapy [4]. Due to the urgent need for new and clinically relevant *in vitro* model systems for studying tumor biology and conducting preclinical screening of drugs, the American Association for Cancer Research suggests that modern anticancer therapies should target CSC by completely eliminating them via inhibition of the self-renewing stem cell or by inducing differentiation of brain CSC into non-tumorigenic cells, more sensitive to conventional therapy [5].

The differentiation therapy hypothesis has recently been investigated by Ito et al. and Kobayashi et al. by using the inorganic arsenite arsenic trioxide As2O3 (ATO) in the treatment of leukemia [6,7], in which case the treatment significantly decreased the number of quiescent leukemia stem cells without inducing apoptosis. Because acute promyelocytic leukemia can be considered a model disease for anticancer research [8], we investigated the *in vitro* effects of low concentrations of ATO on CSC isolated from GBM.

Methods

Drug preparation

ATO was kept at 4° C as a stock solution and dilution was made with bidistilled water to a final concentration of 0.5 μM. Temozolomide and bevacizumab were later added in concentrations of 25 μg/ml, exactly as administered in the clinic [9,10].

Radiation treatment

Cells were trypsinized when near confluence of 70-80% and resuspended before being irradiated with 2 Gy as used in the clinic [1], at the Department of Radiotherapy, using a high megavoltage source (Theratron 1000 ^{60}Co source). The dose rate was 0.65 Gy/min. Cells were irradiated in suspension instead of monolayer to avoid subjecting irradiated cells to further stress of manipulation such as trypsinization, which might have interfered with cell recovery. During the irradiation time, control samples were kept outside the ^{60}Co unit at the same temperature as the irradiated cultures. To prevent DNA repair immediately after irradiation, all cell samples were kept on ice and quickly transported to the Department of Radiobiology. Both control and irradiated cell populations were further cultured in stem cell medium, in exactly the same conditions.

Cell culture

CSCs were isolated from a GBM biopsy, as previously described by our team [4]. CSC, already proven to be CD133+, Oct ¾+, CD 90+ and Nanog +, were maintained in Ham's F-12 and Dulbecco's Modified Essential Medium at 1:1 ratio, supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin and 100 μg/mL streptomycin (all from Sigma Aldrich, St Louis, MO, USA) in a 37° C humidified incubator with a mixture of 95% air and 5% carbon dioxide. All experiments were performed on exponentially growing cells, with a doubling time of approximately 24 to 36 h.

RNA isolation and RT-PCR analysis

Total RNA was isolated from glioma stem-like cells and from placental mesenchymal stem cells which were used as control [11], being already known to express the genes of interest. RNA isolation was performed from sub-confluent monolayers of adherent cells plated on 6-well dishes, using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. 1 μg of total RNA was used for reverse transcription with the ImProm Reverse Transcription System (Promega, Madison, WI, USA). Only mRNA was transformed into cDNA by using oligo-dT primers in the reaction mixture, together with: AMV reverse-transcriptase 15 u/μg; buffer solution (10 mM Tris-HCl, pH=9.0; 50 mM KCl; 0.1% Triton X-100); dNTP solution, 1 mM each; $MgCl_2$ 5mM; recombinant ribonuclease inhibitor 1 u/μl; ultrapure nuclease-free water. The cDNA was amplified using GoTaq PCR Core System II (Promega). The primers used were designed according to the corresponding human genes: CXCR4 (sense, 5'-attcctttgcctcttttgcagatata-3'; antisense, 5'-atggccaggtagcggtccagactgatgaa-3'), Oct-3/4 (sense, 5'-aggagtcccaggacatcaaag-3'; antisense, 5'-tcgtttggctgaataccttc-3'), nestin (sense, 5'-aaacagggcctacagagccagatc-3'; antisense, 5'-aattcttggttcttaagaaag-3'), glial fibrillary acid protein-GFAP (sense, 5'-tatagacaggaagcagatgaag-3'; antisense, 5'-agactccaggtcgcaggtcaag-3'), neurofilament protein-NF (sense, 5'-tgaagatggctttggatattgagat-3'; antisense, 5'-tcttcttttgcttcttcagactct-3'), and human glyceraldehyde 3-phosphate dehydrogenase-GAPDH (sense, 5'-acaactttggtatcgtggaa-3', antisense, 5'-aaattcgttgtcataccagg-3') and were used as internal control for PCR. Amplification reactions were performed on a Techne TC3000 thermal cycler (Bibby Scientific Ltd, Staffordshire, United Kingdom) at 95° C for 30 sec, t °C for 1 min (t=56° C for Oct-3/4, nestin, GFAP, NF, 50° C for GAPDH and 61° C for CXCR4), and 72° C for 2 min for 40 cycles. The PCR products were then separated by

electrophoresis on 2% agarose gels and photographed with a UV trans-illuminator.

Survival assay

Cell survival was assessed using the MTT assay, as previously described [12]. For 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, CSC in monolayer culture were irradiated, incubated in DMEM media supplemented with 10% FCS, 100 U/mL penicillin and 100 μg/mL streptomycin before being washed twice with phosphate buffer solution (PBS). Cells were then incubated with trypsin-EDTA, resuspended in culture medium with FCS, counted and plated in 100 μL media at 15×10^3 cells/well in 96-well microtiter plates. After 24 h, cells were washed and treated with a concentration of 0.5 μM ATO before adding bevacizumab and temozolomide. For combination treatment, cells were treated with ATO at 12, 6, 4 or 0.5 h before and after being exposed to a single dose of γ-irradiation of 2 Gy and then cultured for an additional 24 and 48 h. Absorbance of the MTT was measured at 492 nm in a BioTek Synergy 2 fluorescence plate reader (Winooski, VT, USA).

Statistical analysis

Statistical analysis was done using Prism 5.0 statistics program for Windows (GraphPad, San Diego, USA). Data were analyzed using one-way ANOVA with the Bonferroni multiple comparison test (Kruskal-Wallis as nonparametric). Statistical significance was set at $p < 0.05$ and all experiments were performed in triplicate.

Results

PCR RNA analysis from glioblastoma stem-like cells is shown in Figure 1.

When adding ATO prior to treatment, the results showed a reduced survival of tumor cells in a dose-dependent manner (Figure 2). Statistical analysis showed no significant survival difference in the case of CSC vs. CSC + temozolomide (95% CI from -0.06786 to 0.6812), CSC vs. CSC + temozolomide + bevacizumab (95% CI from -0.02786 to 0.7212), CSC vs. CSC + ATO (95% CI from -0.3712 to 0.3779), CSC + temozolomide vs. CSC + temozolomide + bevacizumab (95% CI from -0.3345 to 0.4145), CSC + temozolomide vs. CSC + radiotherapy (95% CI from -0.2445 to 0.5045), CSC +

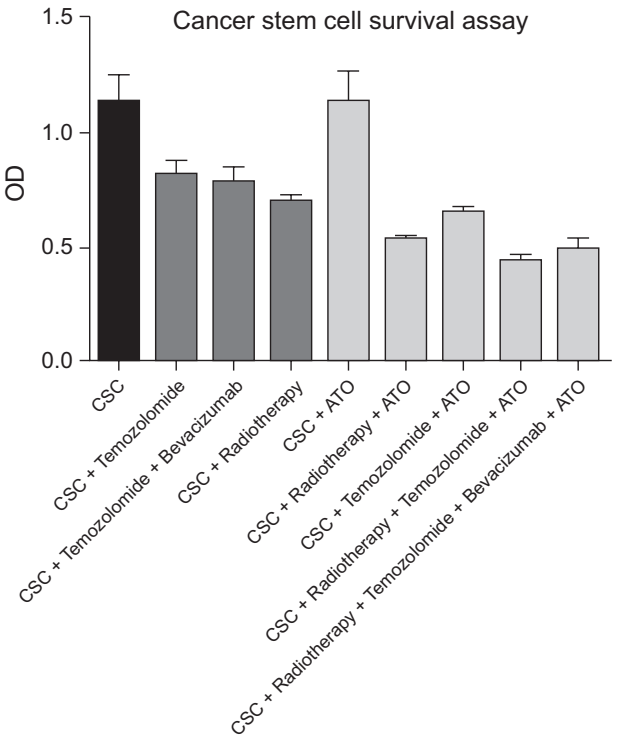

Figure 2. Even if the tumor cells with stem-like characteristics were initially resistant to conventional therapy, when combined with ATO the survival curves shifted downward dramatically. OD: optical density. For other abbreviations see text.

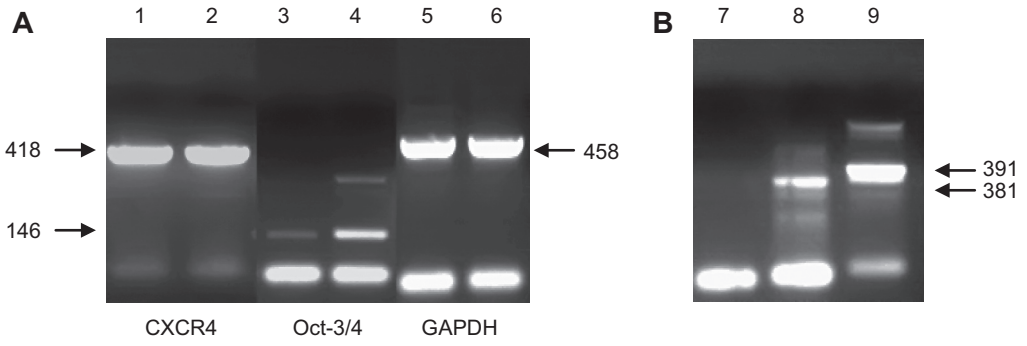

Figure 1. A: Expression levels of CXCR4, Oct-3/4 and GAPDH as determined by RT-PCR in stem-like cells from glioblastoma (lanes 1, 3 and 5) compared to placenta mesenchymal stem cells (lanes 2, 4 and 6); **B:** Expression of nestin (lane 7), GFAP (lane 8) and neurofilament protein (lane 9) in glioblastoma. The numbers near the arrows represent the size in bp of the PCR amplification products corresponding to the studied genes.

temozolomide + bevacizumab vs. CSC + radiotherapy (95% CI from -0.2845 to 0.4645) and in the case of CSC + radiotherapy + ATO vs. CSC + temozolomide + ATO (95% CI from -0.3979 to 0.3512). Using Bonferroni's multiple comparison test, statistically significant survival data ($p<0.05$) were noticed between CSC vs. CSC + radiotherapy (95% CI from 0.06214 to 0.8112), CSC vs. CSC + radiotherapy + ATO (95% CI from 0.5221 to 1.271), CSC vs. CSC + temozolomide + ATO (95% CI from 0.4988 to 1.248), CSC vs. CSC + radiotherapy + temozolomide (95% CI from 0.5555 to 1.305), CSC + temozolomide vs. CSC + temozolomide + ATO (95% CI from 0.1921 to 0.9412), CSC + temozolomide + bevacizumab vs. CSC + temozolomide + ATO (95% CI from 0.1521 to 0.9012), CSC + radiotherapy vs. CSC + radiotherapy + ATO (95% CI from 0.08547 to 0.8345), CSC + ATO vs. CSC + radiotherapy + ATO (95% CI from 0.5188 to 1.268) and CSC + ATO vs. CSC + temozolomide + ATO (95% CI from 0.4955 to 1.245).

Regarding the sequence of ATO administration in combination with chemoradiotherapy on glioblastoma cancer stem cell survival, the survival rates showed that the administration of ATO 4h before standard therapy was correlated with the lowest survival level (Figure 3).

Discussion

Chemoradiation starting 4-6 weeks after maximal tumor resection is today's standard therapy for GBM [13,14]. Clinical studies indicating that temozolomide administered during irradiation provides survival advantage suggest that this DNA methylating agent acts to enhance the radiosensitivity of cancer cells, achieving a significantly longer median time to progression (6.9 vs. 5.0 months) and a longer median survival (14.6 vs. 12.1 months) [15].

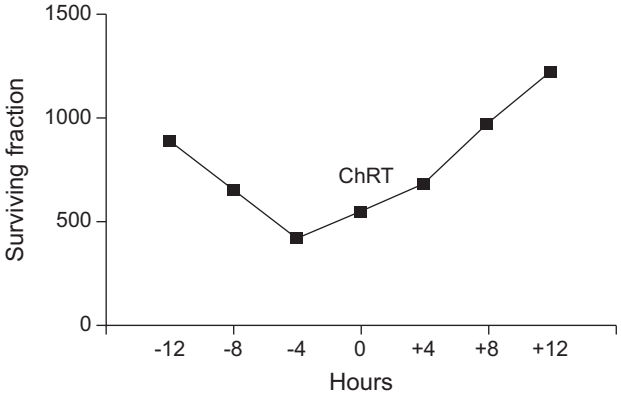

Figure 3. Tumor cell survival according to the timing of arsenic trioxide administration and standard therapy (chemoradiotherapy/ChRT).

Cancer recurrences have been attributed to CSC since the pre-Röentgen era [16]. The existence of CSC has profound implications for cancer biology and therapy because it is likely that eradication of these tumor cells is the critical determinant in achieving cure. There are many different ways to overcome the resistance of stem cells to therapy, including antibody-based targeted molecular therapy, but the differentiation and self-renewal signalling pathways are still under investigation [17].

In the clinical trial reported by Estey et al, untreated patients with acute promyelocytic leukemia were given all-trans retinoic acid and gemtuzumab ozogamycin as induction and then randomized to receive low doses of ATO or not as an early consolidation followed by 2 cycles of conventional chemotherapy [18]. Patients on the ATO arm in all risk groups had improved disease-free survival rates when compared to those in the non-ATO arm. Although used medically for over 2400 years (since Hippocrates treated ulcers with realgar), arsenic compounds were recently reported as the treatment of choice in various diseases. ATO interacts with the target cell due to a high binding affinity to proteins containing the vicinal sulfhydryl groups, found in many enzymes. It also activates nicotinamide adenine dinucleotide phosphate oxidase, an enzyme involved in the production of superoxides and other reactive oxygen species [19]. But what is really interesting is its ability to induce stem cell differentiation, followed by apoptosis when used in low doses. When treating NB4 acute promyelocytic leukemia cells with low micromolar concentrations of ATO, Fojo and Bates have reported morphologic differentiation with CD11b expression and differentiation-associated cytochemical features [20]. Cell cycle analysis also revealed that ATO arrests malignant cells either in G2/M or in both G1 or G2/M, sensitizing them to cytotoxic drugs such as paclitaxel [21].

The "differentiation hypothesis" in anticancer research is also confirmed by our study. CSC isolated from a patient diagnosed with GBM, previously resistant to both ionizing radiation and chemotherapy (temozolomide and bevacizumab), were sensitive to conventional treatment after being treated with 0.5 μM of ATO. In our model there was no difference between the surviving fraction of CSC with or without ATO, showing no intrinsic antitumor effect of ATO at the doses we used. The ATO effect is validated only as a radio- or chemosensitizer if given prior irradiation or temozolomide or both. The addition of bevacizumab seemed not to offer additional benefit in this experiment. That seems quite reasonable as bevacizumab does not target the stem cell but the vascular niche, which was not even intended to be reproduced in our *in vitro* study.

We have also shown that cells are most affected by chemoradiotherapy when adding ATO 4h before

irradiation. These results are confirmed by Ning and Know, by using ATO before high-dose radiotherapy, similar to that used in stereotactic radiosurgery [22].

The concentration of 0.5 μM is readily and safely achievable in patients. The administration of ATO before conventional therapy would be no problem because although its distribution in the central nervous system has not been accurately studied, it is possible to achieve therapeutic levels in the cerebrospinal axis due to arsenical penetration of the blood-brain barrier. Arsenicals are used in the treatment of trypanosomiasis, an infection leading to coma and encephalopathy [23], as well as in leukemia with meningeal involvement, with concentration in the cerebrospinal fluid being approximately one third of the corresponding blood level.

Conclusion

To our knowledge, this is the first paper looking at the radio-chemosensitizing effect of ATO for GBM. ATO induces differentiation while inhibiting cell proliferation when combined with both irradiation and temozolomide of a glioblastoma stem-like cell culture. Further studies on animal models will be needed, but if it would be possible to deplete solid tumor stem cells by therapeutically induced cell differentiation, GBM's cure can become a realistic scenario.

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