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Arsenic trioxide-mediated Notch pathway inhibition depletes the cancer stem-like cell population in gliomas

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ABSTRACT

Cancer stem-like cells (CSLCs) are potential targets for treatment of glioblastoma multiforme (GBM) due to their role in tumorigenesis and recurrence. In this study, we investigated the inhibitory effect of arsenic trioxide (As_2O_3) on CSLCs of GBM in human glioma cell lines (U87MG, U251MG and U373MG) *in vivo* and *in vitro*. Immunofluorescence staining and flow cytometry revealed that the percentage of Nestin-positive cells in the aforementioned cell lines was diminished by 12%, 14% and 7%, respectively, after treatment with 2 μM As_2O_3. Furthermore, we used soft-agar in U87MG and tumor xenografts in nude mice to demonstrate the ability of As_2O_3 to inhibit the formation of tumor in the three cell lines. These results indicate the negative regulation of CSLCs by As_2O_3. In addition, a Western blot analysis revealed decreased levels of Notch1 and Hes1 proteins due to As_2O_3 treatment. We conclude that As_2O_3 has a remarkable inhibitory effect on CSLCs in glioma cell lines *in vivo* and *in vitro*; in addition, we determined that the mechanism of CSLC inhibition involves the deregulation of Notch activation.

1. Introduction

GBM is notorious for its resistance to treatment and high frequency of recurrence. However, the potential to understand the mechanisms of treatment resistance and recurrence has increased with the identification of rare populations of cancer stem-like cells in GBM [1,2]. A novel GBM combined chemo- and radiotherapy strategy that targets CSLCs has received extensive attention. Despite recent reports of CSLC resistance to chemo- and radiotherapy [3,4], some agents which regulate signaling pathways have been shown to inhibit CSLC growth in GBM. For example, cyclopamine, which blocks the Hedgehog pathway, can reduce the CSLC population in GBM [5].

Because CSLCs share common properties with normal stem cells, it is plausible that they have overlapping regulation mechanisms. Indeed, one of the most pressing questions concerning the biology of stem cells involves the mechanism of how stem cells maintain self-renewal properties and continue to proliferate. Several studies have demonstrated a key role for genes associated with chromatin regulation and the cell cycle [6–8]. Meanwhile, there is significant evidence that the deregulation of some pathways inhibits tumorigenesis. CSLC signaling pathways, such as the Notch signaling pathway, may be required for the survival and growth of CSLCs in GBM [9]. Notch promotes the survival and proliferation of non-neoplastic neural stem cells and inhibits their differentiation [10,11]. Notch1 is an important transmembrane receptor in the family of Notch receptors. Notch signaling is initiated by ligand binding, which is followed by intramembranous proteolytic cleavage of the Notch1 receptor to generate a Notch intracellular domain (NICD). The NICD subsequently translocates to the nucleus to act as a transcriptional activator [12]. The proliferation of CSLCs could be inhibited by a blockade of the Notch pathway due to the deletion of

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Notch1 protein. Hairy-enhancer of split1 (Hes1), which is independent of Notch activation, is an important regulator of the effect of the Notch pathway on transcription. The deregulation of Hes1 could also lead to the inhibition of CSLC growth.

As of yet, few agents have been shown to target the CSLC subpopulation in GBM. Inorganic arsenite, including arsenic trioxide (As_2O_3), has been used as a therapeutic agent for centuries [13]. The dramatic ability of As_2O_3 to cure acute promyelocytic leukemia (APL) was reported in the mid-1990s [14–16], and arsenic can also inhibit the growth of APL-derived stem cells [17]. Recently, treatment with As_2O_3 has been utilized for many solid tumors including glioblastoma because of its effect on patients with APL [18]. As a novel anti-tumor agent, As_2O_3 induces apoptosis and enhances the radiation-induced killing of GBM cells [19]. However, few studies have addressed the ability of As_2O_3 to target cancer stem cells in solid tumors. We therefore examined the inhibitory effect of As_2O_3 on CSLCs in glioma cell lines. Our results suggested that a low dose of As_2O_3 inhibits the growth of CSLCs in glioma cell lines. Moreover, we analyzed the probable mechanisms underlying the effects of As_2O_3 and observed that the down-regulation of the Notch signaling pathway is an important aspect of the inhibition effect of As_2O_3 on CSLCs. Our data indicate that As_2O_3 can inhibit the proliferation of CSLCs in glioma cell lines by the deregulation of Notch signaling.

2. Materials and methods

2.1. Reagents

As_2O_3 was prepared by the Department of Pharmacy in the First Affiliated Hospital of Harbin Medical University (Harbin, PR China). Stock solutions of As_2O_3 were diluted in PBS at five different doses (1, 2, 4, 8 and 16 μM) for the *in vitro* and *in vivo* experiments.

2.2. Cell culture and cell viability

The cell lines U87MG, U251MG and U373MG were cultured in Dulbecco's Modified Eagle's Medium (DMEM) plus 10% FBS. A growth inhibition assay was performed to determine the growth inhibition effect of As_2O_3 using a cell-counting kit (CT02, CHEMICON). This cell proliferation assay was modified from the MTT assay, in which WST-8, a tetrazolium salt, is used as the substrate [20]. Briefly, 1×10^4 cells were plated per well in 100 μl of medium in 96-well microtiter plates. The cells were grown for 4 h, and various concentrations of As_2O_3 were added. After an additional 24–72 h of incubation, the medium was aspirated and WST-8 solution was added. After incubation at 37 °C for 30 min, the absorbance of each well was determined in a microplate reader by absorbance spectrophotometry at a wavelength of 570 nm. The assay gave an absorbance that linearly correlated with the number of cells and was not affected by As_2O_3 itself. Cell growth was expressed as a percentage of the absorbance in the vehicle-treated control wells. Dose–response curves were drawn, and the IC_{50}, the concentration that inhibits 50% of the growth of control cells, was calculated. The experiments were performed in triplicate.

2.3. Flow cytometry

Nestin-FITC staining for the detection of CLSCs in glioma cell lines (U87MG, U251MG and U373MG) treated by As_2O_3 for 72 h was performed with Nestin-FITC antibody (R&D System) according to the manufacturer instructions. The cells stained with Nestin-FITC were analyzed by FACS (BD Cor). Flow cytometric data was analyzed using WinMDI 2.8[7]and the results were confirmed using ModFit LT 3.1(Verity).

2.4. Immunofluorescence staining

After incubation with 2 and 4 μM As_2O_3 for 72 h in a chamber slide, cells were fixed for 15 min in 4% formaldehyde, treated for 10 min with 0.2% Triton X-100 in PBS for permeability, blocked for 5 min with Protein Blocking Agent (Thermo), and incubated at 4 °C overnight with mouse monoclonal anti-Nestin (1:200 dilution) (MAB5326, CHEMICON). Subsequently, the cells were incubated at room temperature for 1 h with FITC (DakoCytomation) – conjugated secondary antibody (1:20 dilution). The cells were mounted in an antifade solution with DAPI (Sigma). Labeled cells were analyzed by laser confocal microscopy.

In the apoptotic test, the cells were treated by 2 and 4 μM As_2O_3 for 24 h. The primary antibodies of mouse monoclonal anti-Nestin (1:200 dilution) (CHEMICON) and rabbit polyclonal anti-caspase-3 (1:40 dilution) (CHEMICON) were used. The secondary antibodies of goat anti-mouse FITC (CHEMICON) for detecting Nestin and goat anti-rabbit PE (SIGMA) for detecting caspase-3 were used.

2.5. Colony formation assay

Exponentially growing U87MG cells (3×10^3 cells/well) in DMEM – 0.3% agar containing various concentrations of As_2O_3 (2, 4 and 8 μM) were layered in 6-well culture dishes on top of a basal 0.7% agar layer containing the same concentrations of As_2O_3. Triplicate tests were performed for each concentration. Colonies larger than 60 μM were counted under a phase-contrast microscope, and the results were expressed as even numbers of colony each field.

2.6. In vivo tumor formation assays

The therapeutic effect of As_2O_3 to tumor formation was evaluated in an orthotopic inoculation mouse model as reported previously [19]. Ten-week-old male SCID mice (BALB/c; Charles River Japan Inc., Tokyo, Japan) were maintained in a specific, pathogen-free environment and handled in accordance with the Guidelines for Animal Experiments of Kansai Medical University. Cell lines U87MG, U251MG and U3737MG (1.0×10^6 in 20 ml of serum-free DMEM), which were infected with As_2O_3 for 72 h, was subcutaneously inoculated into the right flank of 10-week-old male SCID mice as described. Mice were divided into the following three subgroups according to the As_2O_3