**All-trans Retinoic Acid Modulates Cancer Stem Cells of Glioblastoma Multiforme in an MAPK-dependent Manner**

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**Abstract.** Glioblastoma multiforme (GBM), a grade IV glioma, appears to harbor therapy-resistant cancer stem cells (CSCs) that are the major cause of recurrence. All-trans retinoic acid (ATRA), a derivative of retinoid, is capable of differentiating a variety of stem cells, as well as normal neural progenitor cells, and down-regulates expression of the stem cell marker nestin. This study investigated the effects of ATRA on differentiation, proliferation, self-renewal, and signaling pathways of CSCs in GBM. CSCs differentiated into glial and neuronal lineages at low concentrations of ATRA (10 μM). Proliferation and self renewal of neurospheres were reduced following ATRA, although ATRA induced apoptosis at higher (40 μM) concentrations. Analysis of mitogen-activated protein kinase signaling pathways, specifically extracellular signal-regulated kinases (ERK1/2), showed that ATRA-induced alterations in ERK1/2 were associated with regulation of differentiation, proliferation and apoptosis. These results emphasize that low doses of ATRA may have therapeutic potential by differentiating GBM CSCs and rendering them sensitive to targeted therapy.

Glioblastoma multiforme (GBM), a WHO grade IV astrocytoma, is the most aggressive and common primary brain tumor in adults. Despite modern treatment modalities involving surgery, chemotherapy and radiation therapy, the median survival for patients with GBM remains approximately 1 year (1). Distinct molecular subtypes of GBM have been identified conferring unique susceptibility to therapeutic agents and prognostic significance (2, 3). The cell of origin of GBM are cancer stem cells (CSCs) arising from neural stem cells (NSCs) (4). GBM cells expressing CD133 (surface marker for NSC) are capable of forming tumors of the same genotypic origin as the parental tumor (5-7). Many characteristics of NSCs are associated with CSCs including: high motility, capability of forming multiple lineages, proliferative potential, association with blood vessels and white-matter tracts, as well as stem cell-like expression profiles (8). Further studies demonstrated the capability of CD133+ cells to form in vitro CSC-containing neurospheres which could be serially recloned, showed tumorigenicity in vivo, and expressed various NSC-related markers (CD133, musashi-1, Sox2, Bmi-1) (6). Serial orthotopic intracranial injection of CSCs into SCID/BG mice showed features of GBM including nuclear atypia, high mitotic index, and invasive cell migration, with the same histomorphology as primary tumors, as well as an unabated ability to form neuronal, astrocytic and oligodendroglial lineages (5). While current therapies are directed towards eradicating GBM, their inability to target CSCs may result in the failure to develop a definitive cure.

All-trans retinoic acid (ATRA) is a derivative of the naturally occurring compound, retinoic acid, commonly known as vitamin A (9). Currently, ATRA is being explored in a variety of hematological malignancies, including myelodysplastic syndromes, multiple myeloma and chronic myelogenous leukemia (10-12). Alone or in combination with other therapies, ATRA is also being investigated in a variety of solid tumors including prostate cancer, breast cancer and glioma (13-15). A combination of ATRA and interferon γ (IFNγ) showed the ability to induce both differentiation and apoptosis of GBM cells independent of phosphates and tensin homolog on chromosome ten (PTEN) status by up-regulating multihistocompatibility molecules and cell cycle inhibitors while down-regulating cyclin-dependent kinase 2 (CDK2), NF-κB, and nitric oxide production (16-18). The concomitant use of ATRA with cytosine arabinoside resulted in therapeutic effect in patients with recurrent malignant glioma (19). Retinoic acid and its derivatives activate retinoic acid receptors and retinoid X receptor (RAR-RXR) complexes and induce neural differentiation of NSCs (20).
In this study, we used ATRA as a means of differentiating CSCs of GBM which then can be targeted for therapy.

We observed that ATRA was able to differentiate CSCs into astrocytic and neuronal lineages, significantly decreased CSC proliferation in a dose-dependent manner, and cause apoptosis at high doses. Furthermore, the apoptotic dose (40 μM) caused up-regulation of ERK1/2 activity and differentiating concentrations (10 μM) of ATRA caused suppression of pERK1/2.

Materials and Methods

Cell culture and treatments. The GBM cell line U87 MG (ATCC, Manassas, VA, USA) was maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Mediatech, Manassas, VA, USA) with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 1% penicillin/streptomycin/amphotericin supplement (Mediatech) in a humidified incubator with 5% CO2 at 37˚C. Neurospheres derived from U87 MG glioblastoma cell line were incubated in serum-free media for 24 h prior to treatment with ATRA (5, 10, 20 and 40 μM; Sigma-Aldrich, St. Louis, MO, USA) for 1, 3 and 7 days. In a separate study, MAP kinase 1 and 2 (MEK1/2) inhibitor U0126 (10 μM; Sigma-Aldrich) was given prior to treatment with ATRA. Phase-contrast photomicroscopy was performed at ×10 using an Axiovert 100M (Zeiss, Thornwood, NY, USA).

Differentiation analysis. Neurospheres were dispersed using 0.05% trypsin/0.53 mM EDTA in Hank’s buffered salt solution (HBSS) (Mediatech), plated and starved in serum-free media for 24 h prior to treatment with ATRA (10 μM) for 24 h. Cells were fixed for 30 minutes in 4% paraformaldehyde, permeabilized in phosphate buffered saline (PBS)/0.1% Tween, and blocked with 10% serum. Cells were incubated overnight with nestin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), washed and reincubated overnight with either glial fibrillary acidic protein (GFAP; Sigma-Aldrich), or class III beta-tubulin (TUJ1; R&D Systems, Minneapolis, MN, USA). Incubation with appropriate secondary antibodies, rhodamine red or fluorescein isothiocyanate (FITC) green (Jackson ImmunoResearch, West Grove, PA, USA), was performed followed by 4’6-diamidino-2-phenylindole (DAPI) counterstaining (Sigma-Aldrich). After fixation (Dako, Carpenteria, CA, USA), visualization was performed with an Axiovert 100M at ×40 (Zeiss).

Immunoblotting. Protein extraction was performed using whole lysis buffer containing 1% Triton X-100, 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, phosphatase and protease inhibitors (Sigma-Aldrich). Protein concentrations were determined by the modified Lowry Method (Bio-Rad Laboratory, Hercules, CA, USA). Equal amounts of protein (20-50 μg) were resolved on a 10% sodium dodecyl sulfate polyacrylamide gel and then electrotransferred onto a nitrocellulose membrane. A routine procedure utilized primary antibodies for nestin and activated extracellular regulated kinase 1 and 2 (ERK1/2; Santa Cruz Biotechnology) followed by detection by chemiluminescence (Millipore, Billerica, MA, USA). Blots were stripped (Pierce, Rockford, IL, USA) and re-probed with actin or total antibody (Santa Cruz Biotechnology) to ensure equal loading. Densitometric analysis was performed using ImageJ (NIH, Bethesda, MD, USA).

Neurosphere formation (1-2 weeks) and Neurosphere self-renewal assay (Day 0-2). Figure 1. A schematic representation shows the neurosphere proliferation and self-renewal assay used in this study.

Neurosphere proliferation assay. Serum-starved neurospheres were treated with 5, 10, 20 or 40 μM of ATRA for up to 48 h. Serial images of treated cells were taken at ×2.5 using an Axiovert 100 M (Zeiss) where field coordinates were maintained. Neurosphere diameters were measured (AxioVision software; Zeiss) across all visibly identifiable neurospheres by a single blinded observer, while diameters across ellipsoid neurospheres were measured diagonally across all treatments to ensure uniformity (Figure 1). Each dish contained neurospheres ranging from 50 to 300 μm. Neurosphere diameters were normalized to day zero for each treatment.

Statistical analysis. Values are presented as the mean. One-way ANOVA was carried out for multiple comparisons and two-tailed t-tests were used for single comparisons. A p-value of <0.05 was considered significant. All experiments were repeated at least three times.

Results

Differentiation of GBM stem cells by ATRA. Neurospheres from GBM were treated with 10 μM ATRA for 24 h and markers of stem cells, astrocytes and neurons were probed. Expressions of NSC marker (nestin), astrocytic marker (GFAP) and neuronal marker (TUJ1) were quantified (Figure 2). Vehicle-treated cells showed an abundance of nestin-expressing cells and only a limited number of GFAP-expressing cells (Figure 2A). Exclusively DAPI-staining cells were present in about 40% of cells. Treatment with ATRA caused a significant increase in cells expressing GFAP, while reducing nestin-expressing cells (t-test, p<0.05). ATRA treatment enhanced the proportion of cells expressing TUJ1 as compared to control, and showed reduction of nestin-expressing cells (Figure 2B, t-test, p<0.05). A small population of GFAP+nestin+ and TUJ1+/nestin+ co-expressing cells remained after treatment with ATRA. High doses of ATRA (40 μM) resulted in widespread apoptosis (data not shown), precluding immunofluorescence analysis.

ATRA causes dose-dependent suppression of neurosphere proliferation. An in vitro neurosphere assay was used to study the effects of ATRA on CSC proliferation (Figure 1). Representative phase-contrast images showed the effects of ATRA treatments where low doses (10 μM) demonstrated signs of differentiation and high doses (40 μM) showed signs of apoptosis.
of apoptosis (neurosphere fragmentation) at day 1 and 2 (Figure 3A). Treatment with 5, 10, 20 and 40 μM of ATRA revealed a dose- and time-dependent alteration in mean neurosphere diameter (Figure 3B). The results presented are relative to day 0 for respective treatments. The data demonstrated that by day 1 and 2, neurosphere diameter increased for controls but ATRA (10, 20 and 40 μM) suppressed neurosphere diameter by day 2 (one-way ANOVA, \( p<0.05 \)). The highest ATRA dose (40 μM) suppressed the mean neurosphere diameter at day 1.

ATRA-mediated differentiation and apoptosis involves activation of ERK1/2. U87 MG cells and neurospheres treated with ATRA (1, 5 and 10 μM) showed a reduction in pERK1/2 expression (Figure 4A, B). U87 MG cells at 20 μM and 40 μM, as well as neurospheres at 10 μM, showed higher pERK1/2 expression relative to cells treated at lower doses. While ATRA caused a dose-dependent suppression of nestin expression in neurospheres, it had the opposite effect in U87 MG cells, namely, an increase in nestin expression in a dose-dependent manner (Figure 4C).

To investigate whether the effect of ERK1/2 is regulated by its upstream signaling pathway following ATRA treatment, we pre-treated cells with MEK1/2 inhibitor, U0126, and observed that ATRA combined with U0126 did not alter pERK1/2 expression; U0126 completely abolished the activity of ERK1/2 (Figure 4D).

Discussion

The results of this study demonstrated that GBM stem cells were differentiated into multiple lineages, including astrocytic as well as neuronal cell types, following ATRA treatment. In addition, ATRA caused a dose-dependent decrease in proliferation, and apoptosis at a higher dose. ATRA-induced expression of ERK1/2 indicated that differentiating concentrations (5, 10 μM) reduced pERK1/2, whereas at higher concentrations (20, 40 μM) increased pERK1/2 expression. This response of pERK1/2 to ATRA was noticeably different in U87 MG cells as compared to CSCs (neurospheres). Significantly, nestin levels were suppressed in CSCs of GBM upon ATRA treatment.

Our observations extend the findings of others showing an ability of ATRA to induce differentiation of GBM cells (16, 20) and have quantified cell differentiation from neurosphere-forming CSCs into neuronal or astrocytic lineages in GBM (Figure 2A and B). The significance of this finding lies in the fact that these markers were associated with dissipation of NSC nestin. It is important to note that some cells continued to coexpress neuronal (TUJ1) or astrocytic (GFAP) and nestin, suggesting these cells represent a process whereby loss of stemness accompanies differentiation.

Low doses of ATRA were able to cause the differentiation of CSCs, while at high doses we observed that ATRA significantly caused apoptosis as evidenced by fragmentation of neurospheres (Figure 3A). This is consistent with numerous other studies where ATRA has been shown to cause apoptosis at higher doses (16-18, 21-23).

Signaling pathway analysis suggested that activation of ERK1/2 was suppressed during differentiation, but was enhanced following ATRA treatment with higher doses (20, 40 μM) (Figure 4A and B). These observations corroborated with the findings of others that ATRA-induced apoptosis correlated with ERK1/2 activation (22). We observed that both U87 MG cells and neurospheres showed a reduction in...
ERK1/2 activation at 1, 5 and 10 μM. In order to investigate whether ERK1/2 expression was regulated by upstream MAPK pathway, we pre-treated cells with MEK1/2 inhibitor, U0126, prior to ATRA-treatment. As shown in Figure 4D, pre-treatment with MEK1/2 inhibitor did not alter ATRA-induced activation of ERK1/2, suggesting an alternative pathway may be involved in regulation of ERK1/2 by ATRA. In other studies, ATRA in combination with the mitotic inhibitor paclitaxel has been shown to up-regulate MAPKs, specifically pERK1/2, with little or no change in other MAPKs in GBM, such as pJNK and p-p38 (22). Moreover, the up-regulation of pERK1/2 by ATRA was associated with activation of apoptotic pathways, including tumor necrosis factor-α (TNFα) induction, caspase activation, cytochrome c release, and BID cleavage, implying ATRA may induce apoptosis by up-regulating pERK1/2 (22).

ATRA treatment showed a distinct effect on expression of NSC marker nestin in neurospheres and U87 MG cells (Figure 4C). In neurospheres, the expression of nestin was highest at 1 μM of ATRA and gradually declined in a dose-dependent manner with the lowest levels being observed at 40 μM ATRA, where ATRA caused apoptosis. Conversely, U87 MG cells showed a trend towards increased nestin expression, which was highest at 20 μM.

The neurosphere self-renewal assay showed that ATRA was able to reduce neurosphere size in a dose- and time-dependent manner (Figure 3A and B). It is interesting to note that reduction in neurosphere size can be seen at the low, physiologically achievable dose of 10 μM (22). The reduction in neurosphere diameter was seen at the concentrations of 10, 20 and 40 μM at day 2, however, reduced diameter was seen at day 1 only at the highest concentration of 40 μM ATRA (Figure 3B). At this concentration (40 μM), ATRA treatment caused neurosphere fragmentation (Figure 3A). The use of an endogenous forming sphere line, such as U87 MG, is advantageous due to the limited requirement for growth factor supplementation and may be a more effective neurosphere assay method ensuring uniformity of culture conditions (24). Improvements of the neurosphere self-renewal assay have suggested the creation of microwells to contain neurospheres during treatment protocols, which may be a viable approach (25).

Finally, with ATRA treatment in GBM, a combination of ATRA and paclitaxel was able to synergistically reduce GBM tumor growth in both an in vivo and in vitro model (22). ATRA has shown an ability to suppress transcription of hepatocyte growth factor and its receptor c-Met in order to abrogate a potential autocrine, mitogenic feedback loop in GBM (26). The findings of this study highlight that low doses of ATRA may have therapeutic potential by its ability to differentiate GBM CSCs rendering them sensitive to targeted therapy.

**Acknowledgements**

We gratefully acknowledge The American Research Foundation for its support.
Figure 4. ATRA-mediated differentiation and apoptosis involves activation of ERK1/2. ATRA induced activation of ERK1/2 differently in U87 MG cells versus neurospheres. A: U87 MG cells showed reduced pERK1/2/tERK1/2 expression at low doses of ATRA (1, 5 and 10 μM) after 24 h treatment. An increase in ERK1/2 activation was seen at higher concentrations of ATRA treatment (20, 40 μM) relative to lower concentrations. B: Neurospheres showed reduced pERK1/2/ERK1/2 expression following ATRA treatment (1, 5 μM), which increased at 10, 20 and 40 μM. C: The ratio of nestin:actin was higher following ATRA treatment in a dose-dependent manner in U87 MG cells and lower in neurospheres. D: Expression of pERK1/2 in U87 MG cells was suppressed following ATRA treatment (10 μM) relative to the control. Inhibition of MEK1/2 by U0126 completely abrogated pERK1/2 expression. Combined treatment of ATRA and U0126 did not suppress ERK1/2 activation. Expression of actin and total ERK1/2 were used as loading controls.
References


Received July 14, 2010
Revised November 4, 2010
Accepted November 5, 2010