LY294002 INDUCES DIFFERENTIATION AND INHIBITS INVASION OF GLIOBLASTOMA CELLS BY TARGETING GSK-3β AND MMP

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ABSTRACT

Glioblastomas are the most common and devastating primary tumors of the central nervous system, with high proliferative capacity, aggressive invasion, and resistance to conventional therapies. Differentiation therapy has emerged as a promising candidate modality. Here we show that the traditional phosphatidylinositol 3 kinase (PI3K) inhibitor LY294002 is capable of inducing differentiation of C6 glioblastoma cells characterized by morphological changes to astrocytic phenotype, increase in differentiation marker protein glial fibrillary acidic protein and inhibition of proliferation. Small interfering RNA against glycogen synthase kinase-3β (GSK-3β) suppresses the induced-differentiation and invasiveness in C6 cells. LY294002 also inhibits MMP-9 expression and invasion of C6 cells, assembling the role of metalloproteinase (MMP) inhibitor AG3340. Taken together, these findings suggest differentiation-inducing and invasion-inhibitory effectiveness of LY294002 in glioblastomas, most likely involving inhibition of GSK-3β and MMP respectively.

Keywords: LY294002, glioblastoma, differentiation, invasion, GSK-3β, MMP

INTRODUCTION

Glioblastoma is the most frequent and most malignant human brain tumor (DeAngelis, 2001). Similar to many malignant tumors, glioblastoma has a characteristically high proliferation index; however, the most devastating and thus far therapeutically intractable aspect of its biology is its highly invasive nature, which prevents complete tumor resection and causes significant neurological morbidity and, ultimately, mortality (Giese et al., 2003; Keles and Berger, 2004). The prognosis of these tumors is extremely poor, and the majority of patients die within 9 to 12 months after diagnosis (Maher et al., 2001; Stupp et al., 2007). Therefore, the treatment of glioblastoma requires novel strategies that take the presently incurable nature of the disease into consideration.

The process of neoplastic cell growth represents a dysfunctional balance between control of cell proliferation and terminal differentiation (Scott, 1997). The aberrantly
differentiated cells show abnormal growth characteristics and distinct invasive and metastatic properties (Sell, 2004). Treating malignant tumor through the induction of cell differentiation has been successful for the treatment of acute promyelocytic leukemia (Huang et al., 1988), showing promise in treating malignant tumors (Leszczyniecka et al., 2001).

LY294002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] is a flavonoid-based synthetic compound that inhibits phosphatidylinositol 3 kinase (PI3K), competing for the occupancy of the ATP pocket of the catalytic subunit of PI3K (Vlahos et al., 1994). LY294002 has been shown to exert antitumorigenic effect in vivo and in vitro by induction of apoptosis and cell growth arrest (Poh and Pervaiz, 2005; Cheng et al., 2008). Moreover, tumor cell invasion and migration could be significantly attenuated by LY294002 treatment in several tumor models (Matsushita et al., 2007; Liu et al., 2008; Meng et al., 2009). Until now, however, little is known about its possible role involved in the process of cancer cell differentiation.

In the present study, we demonstrated that LY294002 induces cellular differentiation and inhibits invasion of malignant glioblastoma cells. GSK-3β suppression via siRNA-triggered gene silencing inhibits the induced differentiation; metalloprotease (MMP) inhibitor AG3340 decreased the inhibited invasiveness, indicating that differentiation and invasion-inhibition triggered by LY294002 is affected through GSK-3β and MMP respectively. It also provides novel evidence that GSK-3β is a potential target in the differentiation therapy of human glioblastoma multiforme.

**MATERIALS AND METHODS**

**Cell culture and drug treatment**

C6 cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco’s modified Eagle’s Medium (DMEM, Invitrogen, Grand Island, NY) supplemented with 10% FBS (Invitrogen) in a humidified atmosphere of 5% CO2 at 37 °C. Cell differentiation was induced with 20 μM LY294002 (Sigma, St. Louis, MO) in DMEM containing 1% FBS. Control was treated with an equivalent volume of DMEM containing 0.1% DMSO.

**Morphological evaluation**

The morphologies of cells were studied using an Olympus (Melville, NY) IX71 inverted microscope along with a Olympus DP Controller software.

**Proliferation assay**

For cell proliferation assay, a 5-bromo-2-deoxyuridine (BrdU) labeling and detection kit (Roche Diagnostics, Mannheim, Germany) was used according to the manufacturer’s instructions. Cells seeded in 96-well plates at 2 × 10³ cells/well with triplicate wells for each condition were labeled with BrdU for 4 h, then anti-BrdU-POD Fab fragments and substrate were added in sequence. The OD was determined at 405 nm using an EXL800 microimmunoanalyzer (Bio-Tek Instruments, Burlington, VT).

**Cell cycle analysis**

A flow cytometry analysis of DNA content of cells was performed to assess the cell-cycle phase redistributions as described (Roz et al., 2002). In brief, the cells were collected by trypsinization, washed in PBS and fixed in 70% ethanol for 30 min at 4 °C. After washing with PBS, cells were incubated with the DNA-binding dye propidium iodide (PI, 50 mg/ml), RNase (4 Ku/ml), NaF (0.3 mg/ml) and sodium citrate (1 mg/ml) for 30 min at 37 °C in the dark. Finally, red fluorescence from 488 nm laser-excited PI in every cells was analyzed by EPICS ALTRA flow cytometer (Beckman Coulter, Fullerton, CA) using a peak fluorescence gate to discriminate aggregates. The percentage of cells in G0/G1, S and G2/M was determined from DNA content histograms by Multicycle for windows (Phoenix Flow Systems, San Diego, CA).
Cell invasion assay

Cell invasion was assayed in a cell culture chamber (BD Biosciences, Bedford, MA) with 8 μm pore size polycarbonate membrane filters. The filters were pre-coated with 50 μl Matrigel (1.25 mg/ml). Cells were harvested and seeded in upper chambers at a density of 1×10⁴ cells/well with DMEM containing 1% FBS. Meanwhile, 0.6 ml complete medium with 10% FBS and 20 μM LY294002 was placed in bottom chambers. At the same time, moreover, equal cells were plated to 96-well plates for cell number assay (MTT). The chamber was incubated at 37 °C for 24 h and then the Matrigel was removed with a cotton bud. The invaded cells were fixed with 4% paraform, stained with hematoxylin, photographed and counted. The invasiveness of C6 cells was determined by the percentage-of-invasion score (invaded cell number / total cell number × 100%).

Western Blot analysis

After lysis of cells and measurement of protein concentration, the cells were dissolved in SDS sample buffer [62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, and 0.1% bromophenol blue]. Equal amount of proteins were analyzed by SDS-PAGE on 12% poly-acrylamide gels. Proteins were electroblotted on a nitrocellulose membrane. Membranes were incubated in 5% nonfat dry milk in TBST (Tris-buffered saline, 0.05% Tween-20) and then overnight at 4 °C with antibodies against GFAP, p-Akt, Akt, p-GSK-3β, GSK-3β (1:1000, Cell Signaling Technology, Beverly, MA), PCNA (1:10000, Cell Signaling Technology) MMP-2, MMP-9 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA) and GAPDH (1:2000, Cell Signaling Technology) respectively. After incubation with horseradish peroxidase-labelled secondary antibody (1:1000, Cell Signaling Technology), visualization was achieved with enhanced chemiluminescence (Pierce, Rockford, USA) using a GeneGnome chemiluminescence imaging and analysis system (Syngene Bio Imaging, Cambridge, UK).

RNA silencing of GSK-3β

Rat GSK-3β siRNA and scrambled siRNA (control) were purchased from Invitrogen. C6 cells with 30–50% confluence were transfected using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer’s protocol. Inhibition of GSK-3β protein expression was assessed by immunoblot analysis 48 h after transfection. 36 h post-transfection, cells were incubated with LY294002 for additional 48 h for further analysis.

Statistical analysis

Data are presented as mean ± standard deviation (SD) of three separate experiments. Statistical significance was determined by Student’s t-test. A result with a p-value of less than 0.05 is considered statistically significant.

RESULTS

LY294002 induces differentiation of C6 glioblastoma cells

Differentiation of rat C6 glioblastoma cells towards astrocyte type is characterized by morphological transformation from flat polygonal appearance to spindle-shape with processes (Li et al., 2007). Microscopic observation of C6 glioblastoma cells treated with 20 μM in our study revealed major alterations in their morphology. Unlike the mainly polygonal morphology of control, the shape of LY294002 treated C6 cells was similar to that of mature astrocytes, with smaller round cell bodies and much longer, fine, tapering processes (Figure 1A). This indicates that LY294002 has the ability to induce glioblastoma cells differentiating into maturation process of astrocytes.
Figure 1: LY294002 induces differentiation of C6 glioblastoma cells. (A) Morphological transformation induced by 20 μM LY294002 for 48 h (Scale bar: 50 μM). (B) Immunoblot of GFAP and PCNA expression. Cells were treated with 0, 5, 10 and 20 μM LY294002 for 48 h. (C) Cell cycle distributions in cells treated with 20 μM LY294002 for the time indicated. (D) Proliferating cells determined by measuring the amount of BrdU incorporation. Cells were treated with 20 μM LY294002 for the time indicated. Results are means ± SD (n=3). Statistical differences compared with the controls are given as *p < 0.05 and **p < 0.01.

We further examined the expressions of glial fibrillary acid protein (GFAP), a well-established marker of mature astrocytes (Roymans et al., 2001) and PCNA, the reliable marker of proliferating cells (Krishna et al., 1994). As indicated in Figure 1B, Western blotting analysis confirmed a significant up-regulation of GFAP protein expression in LY294002 treated cells compared with the controls in a concentration-dependent manner with a maximal induction effect at 20 μM, while the expression of PCNA was notably restrained by LY294002 in a similar concentration dependent manner. Flow cytometry analysis also showed that LY294002 led to a time-dependent C6 accumulation in G1 phase. Concomitantly, there was a striking decrease in the S fraction (Figure 1C), indicating that a subset of cells actually exits from the cell cycle and then differentiates. BrdU incorporation assay was next used to examine the effect of LY294002 on growth of C6 cells (Figure 1D). Treatment of C6 cells for 48 h with 20 μM LY294002 caused marked inhibition of growth in a time dependent manner. As seen in Figure 1D, LY294002 (20 μM) diminished the proliferation of C6 cells to 78.7 % of the controls at 24 h (p < 0.05).

LY294002 inhibits invasion of C6 glioblastoma cells

Brain invasion is a biological hallmark of glioblastoma that contributes to its aggressiveness. We thus tested whether LY294002 may also affect the invasiveness of C6 cells. Matrigel invasion analysis confirmed that LY294002 led to a remarkable reduction of invading cells. As seen in Figure 2A and B, LY294002 (20 μM) diminished the invasion ability of the C6 cells to 52.2 % of the controls at 24 h (p < 0.05). Thus, the results indicate that LY294002 can regulate the invasion of glioblastoma cells.
Figure 2: LY294002 inhibits the invasion of C6 glioblastoma cells. (A) Matrigel invasion assay of C6 cells treated with 20 μM LY294002 after 48 h (Scale bar = 50 μM). (B) Statistical analysis of three independent experiments. The data are mean ± SD (n=3). * p <0.05, ** p <0.01 compared with control.

Activation of GSK-3β in glioblastoma cells

We first confirmed the PI3K-Akt inhibitory activity of LY294002 at concentrations used in the current study. Glioblastoma cells were treated with 20 μM LY294002 and the effect on Akt activation was assessed by Western blot analysis. As shown in Figure 3, LY294002 treatment effectively inhibited Akt phosphorylation following 6 h drug treatment.

To determine whether LY294002 affects activity of GSK-3β, the downstream kinase of Akt, GSK-3β phosphorylation at the Ser-9 residue, which represents the inactive form of GSK-3β kinase (Cross et al., 1995), was determined. As shown in Figure 3, GSK-3βSer-9 protein level was significantly downregulated in C6 cells after 6 h incubation. The most robust GSK-3βSer-9 protein reduction appeared at 6 h treatment and then maintain at a low level. Total Akt and total GSK-3β remains stable throughout. These data revealed that LY294002 inhibits PI3K/Akt pathway and activates its downstream kinase GSK-3β.

Figure 3: Akt inhibition and GSK-3β activation induced by LY294002 in C6 glioblastoma cells. Immunoblot of p-AktSer473, Akt, p-GSK-3βser9 and GSK-3β levels in C6 cells treated with 20 μM LY294002 for the indicated time.

GSK-3β gene knockdown inhibits differentiation induced by LY294002 in C6 cells

To further confirm the role of GSK-3β in glioblastoma cell differentiation, we examined the effects of GSK-3β inhibition. To selectively down-regulate expression of GSK-3β proteins, we evaluated the effect of isoform-specific GSK-3β gene silencing mediated by siRNAs. As shown in Figure 4A, cells transfected with GSK-3β siRNA resulted in dramatically knockdown of the protein levels compared with those transfected with scrambled control siRNA. In addition, morphological transformation and elevation of GFAP during C6 cell differentiation were blocked by 10 nM GSK-3β specific siRNA (Figure 4B and 4C). These results suggest the requisite role of GSK-3β in the differentiation of glioblastoma cells and suggest that GSK-3β positively regulates the differentiation induced by LY294002.
Silencing GSK-3β blocks differentiation induced by LY294002 in C6 cells. (A) Immunoblot of the GSK-3β protein levels after transfection with 10 nM scrambled (Scram) or GSK-3β siRNAs (siGSK-3β) for 36 h. (B-C) Morphology (Scale bar = 50 μM). (B) and immunoblot of the GFAP and PCNA levels (C) in GSK-3β knockdown cells subsequently stimulated with 20 μM LY294002 for 48 h.

GSK-3β gene knockdown inhibits invasion of C6 glioblastoma cells

We further examine the role of GSK-3β in glioblastoma invasion. Matrigel-based invasion assay indicate that GSK-3β gene knockdown obviously suppress the invasion of C6 cells (Figure 5A and 5B), indicating LY294002 inhibit invasion independent its PI3K/Akt inhibition and the following GSK-3β activation activity.

MMP inhibitor suppresses differentiation of C6 glioblastoma cells

Cellular invasion requires the participation of several types of hydrolytic enzyme systems, including plasminogen activators and the MMP, such as gelatinase (MMP-2 and MMP-9). MMP-2 and MMP-9 expression in C6 glioblastoma cells was then assessed by Western blot analysis. As shown in Figure 6A, LY294002 treatment markedly decreased MMP-9 protein level, while the protein level of MMP-2 remained stable. In addition, by using AG3340 which specifically inhibits MMPs, we noted a significant reduction in C6 invasion through a Matrigel barrier at doses of 100 μM (Figure 6B), which had no effect on cell growth (data not shown). The invasiveness was reduced more than 50 % (p < 0.01) by AG3340 (Figure 6C). The action of LY294002 on MMP may account for its drastic effect on the inhibition of the invasiveness of glioblastoma cells.
Figure 6: AG3340 impaired invasion of C6 glioblastoma cells. (A) Immunoblots of the MMP-2 and MMP-9 protein levels of cells treated with 20 μM LY294002 for 48 h. (B) Matrigel invasion assay of C6 cells pretreated with 100 μM AG3340 for 24 h (Scale bar = 50 μM). (C) Statistical analysis of three independent experiments in panel B. The data are mean ± SD (n=3). * p < 0.05, ** p < 0.01 compared with control.

DISCUSSION

Rat C6 glioblastoma cells are a rapidly proliferating cell line with an undifferentiated phenotype and oligodendrocytic, astrocytic and neuronal properties. It has been used as a model to study glial differentiation. GFAP is important in maintaining normal morphology and regulating aspects of the growth of glioblastoma cells. Synthesis of GFAP seems to be required for the formation of stable astrocytic processes (Chen and Liem, 1994). An increase in GFAP expression in cultured glioma cells associated with morphological changes indicates cellular differentiation (Rutka et al., 1994; Takanaga et al., 2004; Li et al., 2007). Here we showed that exposure of C6 glioblastoma cells to LY294002 resulted in their morphological changes to astrocytic phenotype, increase in astrocytic differentiation marker protein GFAP and decrease in proliferation, indicating the differentiation-inducing effectiveness of LY294002.

The phosphatidylinositol 3-kinase (PI3K)-Akt/PKB signaling is a well-characterized pathway involved in the control of cell proliferation, apoptosis, angiogenesis and oncogenesis (Vivanco and Sawyers, 2002; Yamaguchi et al., 2006). Deregulated activation of this pathway, achieved through numerous genetic and epigenetic alterations, has been found in many types of cancer including glioblastomas (Ramos et al., 2005; Tokunaga et al., 2008). LY294002 is a commonly used pharmacologic inhibitor of PI3K, where it acts on the ATP-binding site of the PI3K enzyme, thus selectively inhibiting the PI3K-Akt nexus. Akt is considered to be the principal enzyme that phosphorylates and inhibits GSK-3 activation at an N-terminal serine (serine-9 in GSK-3β) (Cross et al., 1995).

GSK-3 regulates diverse processes, including metabolism, cell fate specification, cell division, and cell death (Frame and Cohen, 2001; Doble and Woodgett, 2003). Two closely related isoforms, GSK-3α and GSK-3β, function in multiple pathways, including Wnt, notch, tyrosine kinase, and G-protein coupled receptor signaling. The role of GSK-3 in cancer cell differentiation is barely studied, and current data are conflicting. For example, it has been suggested Microtubule-associated protein 1B (MAP1B) phosphorylation by a novel isoform of GSK-3 is induced during PC12 cell differentiation (Goold and Gordon-Weeks, 2001). On the other hand, GSK-3β is accumulated in poorly differentiated pancreatic adenocarcinoma and inhibition of GSK-3β arrests pancreatic tumor growth (Ougolkov et al., 2005). Little is known regarding the function and status of GSK-3 in glioblastoma cells. Our data, suggesting that GSK-3β inhibition can block glioblastoma cell differentiation, suggest that GSK-3β is a central point of regulation.
for the cellular differentiation of glioblastoma cells. Identification of pro-differentiation molecules that downstream of GSK-3β is a major part of our ongoing studies in this area.

A major characteristic of glioblastoma is the propensity to invade and become resistant to chemotherapeutic agents. Glioblastoma cells often infiltrate beyond any obviously defined tumor margin and contribute to the high incidence of recurrence (Yanamandra et al., 2004). The invading cells are extremely resistant to radiation and chemotherapy, and currently there are no anti-invasive therapies available (Lefranc et al., 2005). Because local invasion of neoplastic cells into the surrounding brain is perhaps the most important aspect of the biology of glioblastomas precluding successful treatment, pharmacological inhibition of glioblastoma cell migration and brain invasion is considered as a promising strategy for the treatment of glioblastoma (Giese et al., 2003; Kislin et al., 2009; Zhang et al., 2009). The Matrigel invasion assay confirmed that LY294002 led to a remarkable reduction of invading cells in the C6 glioblastoma cells.

The acquisition of invasive potential through proteinase expression is an essential event in tumor progression. MMPs are a family of regulating cell invasion processes through their proteolytic function in normal and pathological states. Among proteinases, MMP-2 and MMP-9 have been shown to be increased in glioblastoma and are thought to play a key role in facilitating the invasion of glioblastoma cells through brain parenchyma (Rao et al., 1993; Forsyth et al., 1998). Our results reveal that the expression of MMP-9 was significantly decreased in the glioblastoma cells while MMP-2 was invariable. MMP inhibitor AG3340 resembled the invasion-inhibition role of LY294002, while GSK-3β siRNA could not attenuate the inhibitory activity of LY294002. These results indicate that LY294002 suppresses glioblastoma cell invasion and migration at least in part involving down-regulation of MMP-2, independent of its PI3K-Akt inhibitory and GSK-3β activating activity. These data provide a novel explanation for the anticancer properties of LY294002 in glioblastoma.

In summary, our present data show that treatment of C6 glioblastoma cells with LY294002 resulted in growth inhibition, mature morphological changes, and increased GFAP expression indicative of cellular differentiation. Intervention in GSK-3β function by siRNA abolished the effectiveness of LY294002 in glioblastoma cells. LY294002 also potentially inhibited invasiveness of glioblastoma cells as the MMP inhibitor. In conclusion, our work suggests that LY294002 may serve as a novel anti-glioblastoma agent, since it is implicated in many areas of tumor progression, including cell growth, cell differentiation and invasion, at least in part by targeting GSK-3β and MMP-9.

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