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Chronic D609 treatment interferes with cell cycle and targets the expression of Olig2 in Glioma Stem like Cells

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ABSTRACT

Glioma Stem-like Cells (GSCs) isolated from patient derived tumors have high metabolic activity and survive in the absence of exogenous growth factors. We recently demonstrated that acute D609 (Tricyclodecan-9-yl-xanthogenate), a PC-PLC inhibitor with anti-oxidative property, can decrease the ATP content & GADD45β protein in GSCs cultured without growth factors, but not in the presence of growth factors. In this study we examined the effect of chronic D609 treatment on GSCs cultured in complete medium containing growth factors. Our results show that chronic exposure of GSCs to D609 decreased the ATP content and reduced the expression of GADD45β protein. Furthermore, cyclin D1 content and the phosphorylation of retinoblastoma protein also diminished, resulting in the arrest of cells in G1 phase of cell cycle after D609 treatment. In addition, the expression of Olig2, a protein responsible for the progression of glioblastoma was reduced by D609. Together these results indicate that chronic D609 treatment can inhibit the growth of glioma cells by arresting cells in G1 phase of cell cycle and/or reducing Olig2 expression.

1. Introduction

D609 (Tricyclodecan-9-yl-xanthogenate) is a small anti-oxidative (Ansari et al., 2006) molecule that inhibits phosphatidylcholine specific phospholipase C (PC-PLC) (Cecchetti et al., 2015) and sphingomyelin synthase (SMS) (Burns et al., 2013) thereby affecting the survival and proliferation of many cell types (Abalsamo et al., 2012; Kalluri and Dempsey, 2010; Spadaro et al., 2008). However the effects of D609 on the proliferation of patient derived glioma stem like cells (GSC) is not clear. We recently showed that acute D609 decreased the ATP content and GADD45β protein expression in GSCs cultured in growth factor free medium, but not in complete medium (Kalluri et al., 2016). In addition, the effect of D609 was shown to be diminished by increasing the concentration of exogenous growth supplements in the medium (Kalluri et al., 2013). Together these studies indicate that the consequences of D609 depends on its concentration, the medium composition and cell type.

Several studies have shown that GADD45β (Growth Arrest & DNA Damage) protein plays a major role in the survival (Papa et al., 2008), apoptosis (Kim et al., 2010; Ou et al., 2010) and proliferation of cells (Higgs et al., 2010; Ma et al., 2009). Basing on these contradictory reports it was suggested that over-expression of GADD45β may promote tumorigenesis while its basal expression may have anti-apoptotic activity (Engelmann et al., 2008). Furthermore, the proliferative response of GADD45β was shown to be mediated by the induction of trophic factors thus promoting neurogenesis (Ma et al., 2009). It is also proposed that the expression of GADD45β may protect cells against UV irradiation or certain anticancer drugs (Gupta et al., 2005). We previously showed the GSCs are resistant to cell death upon the withdrawal of growth factors (Clark et al., 2012) and acute D609 exposure promoted cell death in growth factor free conditions (Kalluri et al., 2016). These studies indicate a potential role for GADD45β in the survival and proliferation of glioma stem like cells. In this study we examined the effect of chronic D609 treatment on the proliferation of glioma stem like cells.

2. Materials and methods

2.1. Materials

Neurobasal medium, DMEM, Hams F12 medium, B27 (without retinoic acid), recombinant human FGF-2, EGF and antibiotic mixture were purchased from Thermofisher Scientific (Waltham, MA, USA). Cell Titer Glow reagent (CTG) from Promega Inc. (Madison, WI, USA); Accutase, heparin, anti-actin antibodies and glutamine were from Sigma chemical company (St. Louis MO, USA); D609 from Enzo life

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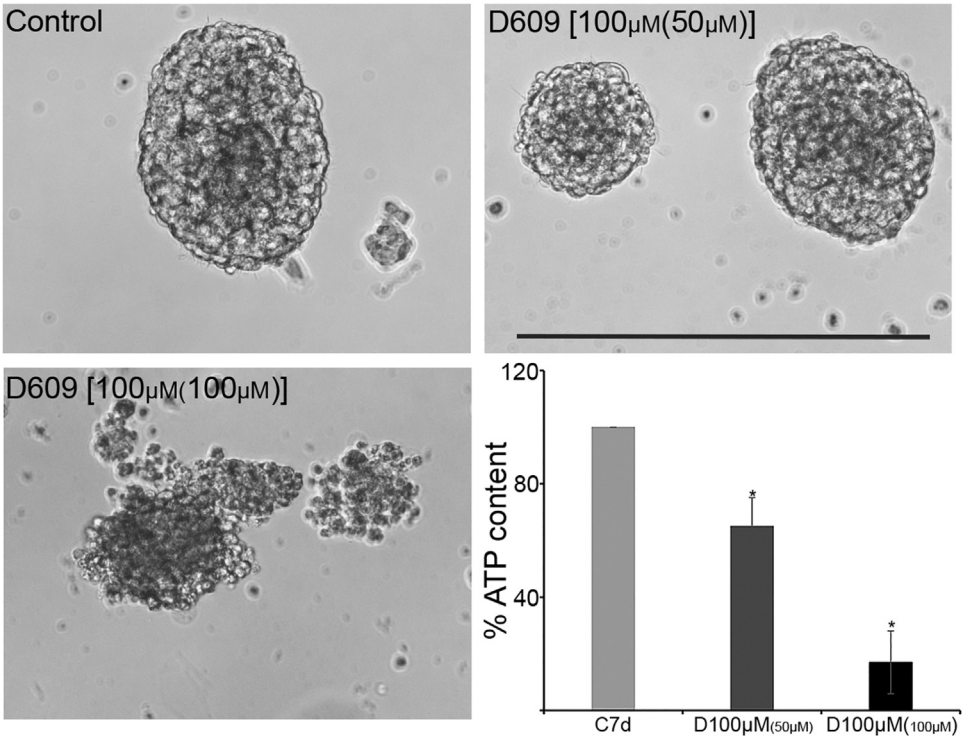

Fig. 1. Effect of chronic D609 on the ATP content. GSCs were cultured in complete medium containing B27/EGF/FGF2 containing glutamine and heparin ± D609 for 7 days. Cells were treated with D609 (100 μM) on first day followed by either 50 μM (low dose) or 100 μM (high dose) on every alternate day for 3 days. Photomicrographs of cells Control; D609 [100 μM (50 μM)] (Low dose); D609 [100 μM (100 μM)] (High dose). Chronic D609 decreased the content of ATP in glioma stem like cells. The data is analyzed by ANOVA supplemented by Neuman Keuls (SNK) test and represented as percentage decrease as compared to control. n = Control (10) Low dose (10) High dose (7). * $P < 0.05$. Note the loss of neurosphere integrity at high dose D609 [100 μM (100 μM)] along with a drastic decline in ATP content (~ 75%) indicating cell death. Therefore we used low dose for all the experiments. Scale bar = 400 μ.

sciences; PVDF membranes (Bio-Rad Inc. Hercules, CA, USA); Stripping buffer & ECL reagent (Pierce, Rockford, IL, USA); anti-GADD45β, Olig-2 (Santa Cruz Biotech), phospho-p38 $^{(Thr180Tyr182)}$, phopho-Akt $^{(ser473)}$, phospho-ERK $^{(Thr\ 202/Tyr204)}$, phospho-Rb $^{(ser780)}$, p38, Akt, ERK, Cyclin-D1, HRP-coupled anti-mouse IgG and anti-rabbit IgG (Cell Signal Tech. MA, USA), RNA Isolation kit (Qiagen, CA, USA).

2.2. Cell culture

Glioma Stem-like Cells (GSC) derived from human gliomas were isolated and cultured previously (Clark et al., 2012; Zorniak et al., 2012) following approval from the Institutional Review Board of the University of Wisconsin-Madison with informed consent obtained from patients. The GSCs isolated from recurrent glioblastoma were cultured as neurospheres and maintained in DMEM/F12 (70:30) medium containing 2% B27 (without retinoic acid), EGF (20 ng/ml), FGF-2, (20 ng/ml) as previously described by us (Kalluri et al., 2016). All the experiments (Passage#20–30) were performed in neurobasal medium supplemented with 2% B27, EGF/FGF2 (each 20 ng/ml), heparin (5 μg/ml), glutamine (2 mM) and antibiotics ± D609 (chronic) for 7 days and used for protein and RNA analysis.

2.3. Chronic D609 treatment

The effect of D609 on glioma stem-like cells was studied by treating cells with D609 (100 μM) on first day followed by D609 (50 μM) or D609 (100 μM) on every alternate days for 3 days. The cells were used for analysis after 7 days.

2.4. Electrophoresis and immunoblotting

Proteins were separated by SDS-PAGE and transferred to PVDF membrane and probed with either GADD45β, phospho-p38, phospho-ERK, phospho-Akt, phospho-Rb, p38, ERK, Akt, Cyclin-D1, Olig-2 or actin antibodies as described earlier (Kalluri et al., 2016). Antigen-specific antibodies were identified using peroxidase-coupled secondary antibody (anti-mouse IgG/anti-rabbit IgG) and super signal detection reagents (Pierce, Rockford, IL). The blots were normalized after stripping & reprobing with anti-actin antibodies, quantified using Image J software and represented as the ratio of protein to actin.

2.5. ATP content

Total cellular ATP content was measured by using Cell Titer-Glow assay kit (Promega) as described by us previously (Kalluri et al., 2016). Glioma stem-like cells (1×10^4/100 μl) were cultured in complete medium in an opaque 96 well micro-titer plate for 7 days and treated with chronic D609 on alternate days as described in Methods 2.3. After 7 days in culture, the cells were equilibrated at room temperature (RT) for 30 min and mixed with equal volume of Cell Titer-Glow reagent on a shaker for 2 min and incubated for another 10 min at RT. The luminescence generated following the hydrolysis of ATP was measured in a luminometer (Veritas) using promega protocol and expressed as percentage of ATP content as compared to control. Each individual experiment was repeated 7–10 times in triplicate. Triplicate wells without cells were used as blank. The volume of D609 or CTG reagent added was determined by measuring the volume of medium in blank wells before the addition of compound. This method was used to account for the evaporation of medium from wells over 7d period due to small volume.

2.6. Cell cycle analysis

Cell cycle analysis was performed as described by us (Kalluri et al., 2013). Glioma stem-like cells were cultured in neurobasal medium containing B27, EGF/FGF2 in the absence and presence of chronic D609 as described in treatment protocol (Section 2.3.). At the end of experimental period (7days) neurospheres were dissociated & cells were suspended in PBS ($1–2 \times 10^6$ cells/ml) followed by overnight fixation in 3 volumes of ethanol (100%) at − 20 °C. The fixed cells were washed in a solution of 1% BSA containing 1 mM EDTA and suspended in propidium iodide (PI) solution [PI (50 μg/ml) +RNase (1 mg/ml) + Triton X-100 (0.5%)] for 30 min in dark at 37 °C. The stained cells were kept in dark overnight at 4 °C and analyzed using flow cytometer (BD