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Original Research Article

Bryostatin inhibits proliferation of ependymoma cells by suppressing expressions of cyclooxygenase-2 and interleukin-8

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Abstract

Purpose: *To investigate the effect of bryostatin on the proliferation of ependymoma cells, and the underlying mechanism(s).*
Methods: *Ependymoma cell lines (SC-EPN1 and SC-EPN2) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and streptomycin (10 mg/ml) in a humidified incubator at 37 °C and 5 % CO_2 atmosphere. Rhe cells were randomly assigned to six groups: control group and five bryostatin groups treated with increasing concentrations of bryostatin (10 - 50 µM). Cell proliferation was determined by MTT assay, while real-time quantitative polymerase chain reaction (qRT-PCR) was used to determine the levels of expressions of apoptosis-related genes. Expressions of cyclooxygenase-2 (COX-2), interleukin-8 (IL-8), Bcl-2, Bax and P-glycoprotein were determined using Western blotting.*
Results: *Treatment with bryostatin significantly and concentration-dependently down-regulated COX-2 and IL-8 mRNAs expressions ($p < 0.05$). On the other hand, proliferation of SC-EPN1 and SC-EPN2 cells were significantly and concentration-dependently inhibited by bryostatin, relative to control group ($p < 0.05$). After 72 h of treatment with bryostatin (50 µM), the extent of apoptosis was significantly higher in SC-EPN1 (57.43 %) and SC-EPN2 cells (52.29 %) than in control group (2.37 %, $p < 0.05$). The results of Western blotting showed that treatment with bryostatin significantly reduced the expressions of Bcl-2 in ependymoma cells, relative to the control group ($p < 0.05$). However, there were no significant differences in the expression of Bax among the groups ($p > 0.05$). P-glycoprotein expression was significantly higher in bryostatin groups than in control group ($p < 0.05$). The results of flow cytometric analysis of rhodamine-123 (rh123) fluorescence showed that after 72 h of treatment with bryostatin (50 µM), rhl23 fluorescence significantly decreased in SC-EPN1 (8.10 %) and SC-EPN2 cells (10.11 %), relative to control group (20.83 %, $p < 0.05$).*
Conclusion: *Bryostatin exerts anti-proliferative and apoptotic effects on ependymoma cells by suppressing COX-2 and IL-8 expressions. Thus, the inhibition of COX-2 expression may constitute an effective chemotherapeutic strategy for ependymoma treatment.*

Keywords: *Bryostatin, Ependymoma cells, Proliferation, Apoptosis, Expression*

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INTRODUCTION

Ependymoma is the third most common childhood tumor, and it accounts for 15 % of brain tumors in children [1,2]. It is an intracranial tumor characterized by poor prognosis and 5 year-survival of 40 to 65 % [3,4]. Surgical resection is the most commonly used method for treatment of ependymoma [3]. However, the use of chemo- and craniospinal radiotherapy for the treatment of this tumor is just beginning to gain relevance [3]. The expression of multi-drug-resistant (MDR) gene against commonly used drugs is the major setback in the treatment of ependymoma [2]. In most brain tumors, there are marked increases in the secretion of prostaglandins [5-7]. Prostaglandins are synthesized from arachidonic acid via the catalytic action of COX-2 which expression is induced by pro-inflammatory cytokines [8]. It is reported that the level of COX-2 in tumors is markedly elevated and its down-regulation inhibits tumor growth and progression [9-12]. High expression of this enzyme in glioma cells is associated with short period of survival, and its inhibition by NS-398 suppresses proliferation and invasion of the cells [9,12]. Cyclooxygenase 2-inhibiting drugs slow the growth of neuro-epithelial tumor cells through induction of apoptosis [11]. Studies have shown that inhibition of COX-2 plays an important role in the treatment of ependymoma. The aim of this study was to investigate the effect of bryostatin on the proliferation of ependymoma cells, and the underlying mechanism(s).

EXPERIMENTAL

Materials and reagents

SC-EPN1 and SC-EPN2 cells were obtained from Seoul National University; FBS was purchased from Hyclon (USA), while DMEM was a product of Gibco BRL (USA). EL800 universal microplate reader was purchased from Bio-Tek Instruments Inc. (USA), and 96-well plates were purchased from Falcon (USA). Annexin V/FITC kit and flow cytometer were products of BD Biosciences (USA); protein extraction and bicinchoninic acid (BCA) assay kits were products of Sangon Biotech Co., Ltd., while bovine serum albumin (BSA) and Trizol reagent were obtained from Thermo Fisher Scientific Inc. (USA). P-glycoprotein, COX-2, Bcl-2, Bax, and β-actin primers were obtained from Santa Cruz Biotechnologies (USA). Horseradish perox-idase-conjugated goat anti-rabbit IgG was a product of Jackson Immuno Research Laboratories Inc. (USA), while chemiluminescence liquid and autoradiography film were purchased from Bio-Rad Laboratories Inc. (USA). Rhodamine 123 from Sigma (USA).

Cell lines and grouping

The SC-EPN1 and SC-EPN2 cells were cultured at 37 °C in DMEM supplemented with 10 % FBS and streptomycin (10 mg/ml) in a humidified incubator containing 5 % CO2. Cells in exponential growth phase were used for this study. In each group of cancer cell line, the cells were randomly assigned to six groups: control group and five bryostatin groups which were treated with increasing concentrations of bryostatin (10 - 50 µM).

MTT assay

Cells (5 x $l0^5$ cells/well) at exponential growth phase were seeded into 96-well plates containing DMEM. After 24 h of incubation, the cells were treated with varied concentrations of bryostatin (10 - 50 µM) and cultured for 72 h. This was followed by the addition of 20 ml of 0.5 % MTT solution within 4 h, after which the culture medium was changed. Dimethylsulfoxide (DMSO, (150 µl) was added in drops to each well, and the wells were placed on an oscillator for 10 min to completely dissolve the formazan crystals. The control wells contained culture medium, MTT solution and DMSO only, and were treated same way as the sample wells. Each well was incubated in the dark for 2 h and absorbance was measured at 570 nm using an EL800 universal microplate reader. The procedure was performed in triplicate and cell proliferation (P) was calculated as in Eq 1.

$$P\ (\%) = \{(1 - As)/Ac\}100$$

where *As* and *Ac are the* absorbance of sample and control, respectively.

Apoptosis assay

The extent of apoptosis in SC-EPN1 and SC-EPN2 cells treated with bryostatin (40 and 50 µM), and control cells was determined using a flow cytometer. The cells were incubated for 72 h, harvested and subsequently treated with HEPES binding buffer containing Annexin V-FITC and PI. The cells were then placed on a flow cytometer and read. The measurements were performed in triplicates and the mean taken.

Western blotting

The expressions of COX-2, Bcl-2, Bax, and P-glycoprotein in SC-EPN1 and SC-EPN2 cells

treated with bryostatin (40 and 50 µM) were determined using Western blotting. Ice-cold cell plates were treated with phenylmethylsulfonyl fluoride and phosphatase, and the protein content was extracted using protein extraction kit. The cell suspension was centrifuged at 12, 000 g for 45 min, and the protein concentration of the supernatant was determined using BCA assay. A portion of total cell protein (50 µg) from each sample was separated on a 12 % sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to a fixed polyvinylidene fluoride membrane at 110 V and 90 ° C for 120 min. Subsequently, 3 % BSA in Tris-buffered saline containing 0.2 % Tween-20 (TBS-T) was added with gentle shaking at room temperature and incubated to block non-specific binding of the blot. Thereafter, the blot was incubated with primary antibody (1: 500) at 4 °C overnight.

The membrane was washed thrice with TBS-T and further incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. The blot was developed using an X-ray film. Grayscale analysis of the bands was performed using ImageJ analysis software. Respective protein expression levels were normalized to that of β-actin which was used as a standard reference.

Quantitative RT-PCR

The treated and control cells were lysed using radio-immunoprecipitation assay (RIPA) buffer and their total RNAs were extracted using Trizol reagent. The cells were treated with trichloromethane ($CHCl_3$) and centrifuged at 16,000 g at 4˚C for 20 min to obtain supernatant. The supernatant was then treated with isopropyl alcohol and dispersed to isolate the RNAs. Following centrifugation, the RNAs were maintained in diethyl pyrocarbonate (DEPC) water, and their concentrations determined spectrophotometrically. The RNAs were reverse-transcribed to cDNAs, and the reaction was carried out at 37 ˚C for 20 min and 85 ˚C for 6 sec. The PCR conditions were: pre-denaturation at 95 ℃ for 30 sec, denaturation at 95 ℃ for 3 sec, annealing at 60 ℃ for 34 sec, and 40 cycles. The procedure was performed in triplicates and the mean taken. Relative expression was quantified using $2^{\Delta\Delta}$Cq method, and β-actin gene was used as internal reference.

Flow cytometric analysis of rh123 fluorescence

The SC-EPN1 and SC-EPN2 cells (3 x $l0^4$ cells/well) were seeded into 96-well plates and incubated for 24 h. The cells were then treated with HEPES buffer containing sodium chloride (130 mM), potassium chloride (6 mM), magnesium sulphate (1 mM), sodium potassium phosphate (1.4 mM), sodium acetate (10 mM), HEPES (6 mM), calcium chloride (2 mM) and glucose (8 mM) at 37 °C. The cells were further incubated in HEPES-buffered medium supplemented with 1 µM rh123. They were thereafter treated with bryostatin (40 and 50 µM) or DMSO (control group) for 72 h, and incubated with calcium ion-free phosphate buffer containing trypsin. Trysinization was stopped by the addition of ice-cold HEPES buffer (pH 7.4) containing rhl23 (1 uM) and FBS. The resultant digest was centrifuged at 1200 g (4 °C) for 10 min, and the cell pellets were dissolved in ice-cold HEPES-buffered solution (2 ml). The rhl23 fluorescence was measured using a flow cytometer fitted with a 553/35 nm optical band-pass filter.

Statistical analysis

Data are expressed as mean ± SEM, and the statistical analysis was performed using SPSS (17.0). Groups were compared using Student *t*-test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Expressions levels of COX-2 and IL-8 in ependymoma cells

Table 1 shows that treatment with bryostatin significantly and dose-dependently down-regulated COX-2 and IL-8 mRNAs expressions after 72 h of incubation ($p < 0.05$).

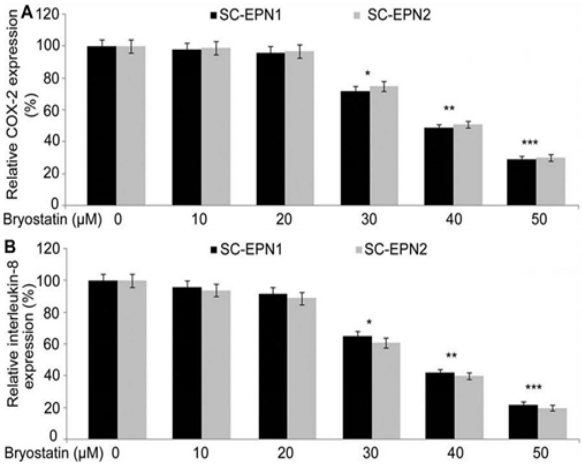

Figure 1: Effect of bryostatin on expressions of COX-2 and IL-8. A: Expression of COX-2; and B: Expression of IL-8; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared to control group

Effect of bryostatin on cell proliferation

The proliferations of SC-EPN1 and SC-EPN2 cells were significantly and dose-dependently inhibited by bryostatin, relative to control group ($p < 0.05$). In SC-EPN1 cells, at 10, 20, 30, 40 and 50 µM of bryostatin the percentages of cell viability were 96, 81, 63, 42 and 31 %, respectively. However, in SC-EPN2 cells the percentages of cell viability were 98, 91, 73, 54 and 39 % at 10, 20, 30, 40 and 50 µM of bryostatin, respectively. These results are shown in Figure 2.

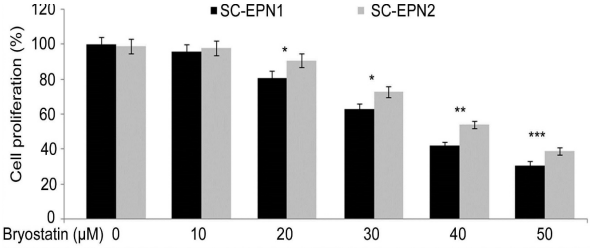

Figure 2: Effect of bryostatin on the proliferation of ependymoma cells. $^{*}P < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ when compared to the control group

Effect of bryostatin on cell apoptosis

As shown in Figure 3, the extent of apoptosis was significantly higher in SC-EPN1 (57.43 %) and SC-EPN2 cells (52.29 %) than in control group (2.37 %, $p < 0.05$).

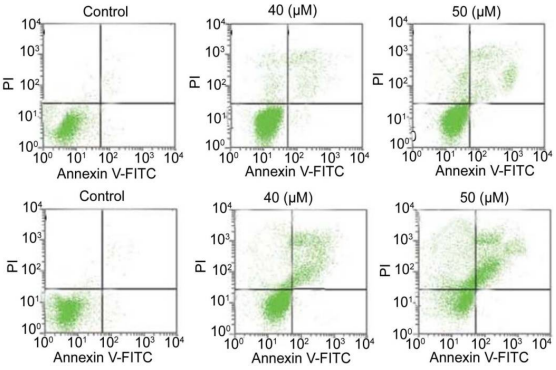

Figure 3: Effect of bryostatin on apoptosis of ependymoma cells

Expressions of Bcl-2 and Bax

Results from Western blotting showed that treatment with bryostatin significantly reduced the expressions of Bcl-2 in SC-EPN1 and SC-EPN2 cells when compared with the control group ($p < 0.05$). However, there was no significant difference in the expression of Bax among the groups ($p > 0.05$). The results are shown in Figures 4 A and 4 B).

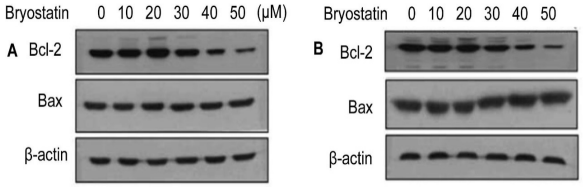

Figure 4: Effect of bryostatin on expressions of Bcl-2 and Bax. A: Expressions of Bcl-2 and Bax in SC-EPN1 cells; and B: Expressions of Bcl-2 and Bax in SC-EPN2 cells

Effect of bryostatin on the expression of P-glycoprotein

In both cancer cells, the expression of P-glycoprotein was significantly higher in bryostatin-treated groups than in control group ($p < 0.05$). The results of flow cytometric analysis of rh123 fluorescence showed that after 72 h of treatment with bryostatin (50 µM), rhl23 fluorescence was significantly reduced in SC-EPN1 (8.10 %) and SC-EPN2 cells (10.11 %), relative to control group (20.83 %, $p < 0.05$). The results are shown in Figure 5.

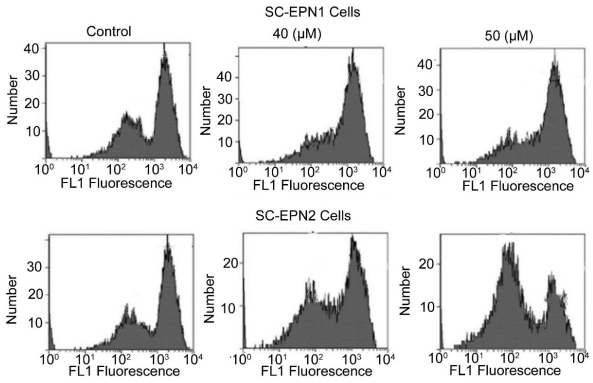

Figure 5: Expression of P-glycoprotein after treatment with bryostatin

DISCUSSION

Ependymoma, the third most commonly diagnosed brain tumor in children is characterized by poor prognosis [3,4]. Cyclooxygenase-2 (COX-2) possesses apoptosis-inhibitory and tumor-angiogenic properties, and so constitutes an important target in the treatment of various types of cancers [11,12]. In the present study, treatment with bryostatin significantly regulated COX-2 and IL-8 mRNA expressions after 72 h of incubation. These results suggest that bryostatin may effectively inhibit the growth and proliferation of ependymoma cells. In most brain tumors, inhibition of growth involves induction of cell apoptosis [9,11].

In this study, treatment with bryostatin led to the induction of apoptosis in ependymoma cells, an indication that bryostatin may induce apoptosis in ependymoma cells by inhibiting the expression of COX-2. High expression of COX-2 has been shown to increase the level of Bcl-2 which is an anti-apoptotic protein [13]. However, in this study, bryostatin suppressed the expression of Bcl-2 in ependymoma cells.

Development of multi-drug resistance is presently the major factor leading to failure of chemotherapy. It has been reported that the mechanism of multi-drug resistance involves the expression of P-glycoprotein on cell membrane [14-16]. P-glycoprotein exerts its effect either by expelling the drug from the cell thereby decreasing its concentration below desired level, or by preventing the drug from entering the cell [17]. There are reports that in ependymoma cells the expression of P-glycoprotein is markedly elevated [18,19]. Studies have shown that expression of COX-2 is associated with the activity of P-glycoprotein [20,21].

Overexpression of COX-2 stimulates the release of prostaglandins from arachidonic acid which in turn promotes the expression of mdrlb gene. However, inhibitors of COX-2 down-regulate the expression of mdrlb gene. In a previous study, the expression of MDR1 and activity of P-glycoprotein in glomerular mesangial cells were significantly higher than their corresponding levels in normal cells [21].

CONCLUSION

The findings of this study show bryostatin exerts anti-proliferative and apoptotic effects on ependymoma cells by suppressing COX-2 and IL-8 expressions and that treatment with bryostatin significantly reduced rh123 fluorescence. Thus, the results appear to suggest that inhibition of COX-2 expression may constitute an effective chemotherapeutic strategy for ependymoma treatment.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Authors' contribution

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Weiyuan Xu performed the experimental work and carried out the literature survey. Chengren Xie designed the study and wrote the paper. Yuchen Feng compiled the data and partly carried out the experimental work. All the authors approved the paper for publishing this work.

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