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Original Article

Bryostatin I treatment induces apoptosis and enhances phosphorylation of p65NF-Kb in OHS-4 osteoblastic osteosarcoma cells

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Abstract: The present study was aimed to investigate the role of bryostatin I in inducing apoptosis in OHS-4 cells. WST-1 colorimetric assay was used for determination of cytotoxicity in OHS-4 cells on treatment with bryostatin I. The results showed 67% reduction in the survival of OHS-4 cells on treatment with 50 μM concentration of bryostatin I for 48 h compared to untreated cells. Bryostatin I treatment induced apoptosis and enhanced *PTEN, FasL*, and *FasR* mRNA expression in OHS-4 cells. Western blot analysis revealed increase in the phosphorylation level of NF-κB on treatment with bryostatin I and activation of NF-κB followed by its translocation from the cytosol into nucleus after bryostatin I treatment. Degradation of IκBα by bryostatin I treatment was very slow compared to that induced by TNF-α. Therefore, bryostatin I can be of therapeutic value for the treatment of osteosarcoma.

Keywords: Osteosarcoma, bryostatin I, cytosol, cytotoxicity, therapy

Introduction

Osteosarcoma, the most commonly observed primary malignant tumor of bones in children and adolescents is found to affect more than 400 cases each year in the USA alone [1]. The treatment strategies involve chemotherapy and surgical excision but the results obtained are very poor. Therefore, chemists and biologists are screening novel molecules for the development of new therapeutic methods for osteosarcoma treatment [2]. The commonly used agents for the treatment of osteosarcoma patients include high-dose methotrexate, doxorubicin, cisplatin, ifosfamide and etoposide [3].

Apoptosis, the process of programmed cell death plays an important role in the removal of toxic cells, treatment of tumors, in embryogenesis and carcinogenesis [4, 5]. Phosphorylation of apoptosis regulating factors participating actively in cell apoptosis has great impact in apoptotic processes [6]. The expression of genes for the control of cellular processes including growth, differentiation, inflammation, and neoplastic transformation is mediated by nuclear factor-kappa B (NF-κB) [7-9]. NF-κB has a dual role in the process of regulation of cell apoptosis either by causing its promotion or inhibition [10-14]. In the cytoplasm, NF-κB is present in the form of its precursors, p50-p65 complex and p65-p105 complexes. Phosphorylation of IκBα and then its degradation by proteasomes results in activation and translocation of NF-κB to nucleus from the cytoplasm. Inside the nucleus, NF-κB induces expression of target genes which then mediate various activities. However, activation of NF-κB is also facilitated by various enzymes like kinases and phosphatases [15-17].

Bryostatins are the 20 macrocyclic lactones present abundantly in Bugulaneritina and many other marine bryozoa. Earlier, bryostatins were reported to exhibit potential activity against lymphocyte leukemia cell lines [18]. One of the compounds from this family, bryostatin I has already has entered phase II clinical trials for the treatment of melanoma, non-Hodgkin's lymphoma, renal cancer and colorectal cancer [19-21]. The other members of this family are constantly being evaluated against various carcinomas. It has also been shown that bryostatin I enhances the growth of bone marrow progeni-

tor cells in a normal way. This provides advantage over normally lethal doses of ionizing radiations [22].

Materials and methods

Reagents

Bryostatin 1 and TNF-α were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bryostatin 1 was dissolved in dimethyl sulfoxide to prepare stock solution which was stored under dark conditions. Fetal bovine serum (FBS) and Dulbecco's modified Eagle's minimum essential medium (DMEM) were obtained from Gibco BRL (Gaithersburg, MD, USA).

Cell line and cultures

OHS-4 human osteosarcoma cell line was purchased from the American Type Culture Collection (Rockville, MD, USA) and were cultured in DMEM supplemented with 10% (v/v) FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/mL streptomycin. The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The medium was replenished every 3 days.

Cytotoxicity assays

For determination of bryostatin I induced cytotoxicity in the OHS-4 cell line WST-1 colorimetric assay was used. Briefly, the cells were distributed at a density of 2.5×10^4 cells per well onto 96-well culture plates and incubated with various concentrations of bryostatin I for the indicated time. Following incubation, the cells were again incubated according to manufacturer's instructions for 5 h at room temperature with WST-1. Victor3 microplate reader was used to measure the absorbance at 450 nm which was directly proportional to the number of surviving cells in present in the culture. For each assay readings were carried out three times and compared with DMSO treated cells used as control.

Hoechst fragmentation assay

The cells (5×10^6) after washing with PBS were lysed by adding 400 µl of DNA fragmentation lysis buffer (0.1% Triton X-100, 5 Mm Tris-HCl, pH 8.0, 20 mM EDTA). After addition of PEG and NaCl the samples were put on ice for 20 min followed by centrifugation for 40 min at 12000 g. The supernatants were harvested to determine the concentration of DNA using an equal volume of Hoechst dye solution (0.2 µg/mL Hoechst 33258 in PBS, pH 7.4). Following incubation for 30 min at 37°C, the fluorescence was measured at 360 nm using a Ratio-2 System Fluorometer (Optical Technologies Devices Inc., Elmsford, NY).

DNA isolation and agarose gel electrophoresis

The cells after washing with PBS three times were lysed in cold buffer 10 mM Tris-HCl buffer (pH 7.5), 10 mM EDTA, and 0.5% Triton X-100. The lysates were centrifuged at 15,000 g for 45 min to remove the cell debris followed by addition of DNase-free RNase and then incubation for 1 h at 37°C. Then proteinase K at was added over 1 h at 37°C and the DNA was precipitated at -20°C using 50% 2-propanol and 0.5 M NaCl overnight. The centrifugation and drying was followed by dissolution of DNA in TE-buffer (10 mM Tris, pH 8.0, containing 1 mM EDTA). DNA was subjected to electrophoresis through 2.0% agarose gel in the same gels DNA markers (100 bp) (New England BioLabs, Beverly, MA, USA) were also run. Staining of the gels for 15 min using ethidium bromide was followed by visualization of the apoptotic changes in DNA integrity. UV transilluminator (Vilber-Lourmat, MarnelaVallee, France) was used to examine the DNA bands and Polaroid DS-300 camera for taking Photographs.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

The cells were distributed at a density of 2×10^8 cells per dish onto 6 cm plates overnight. The medium was replaced and the cells were incubated in the absence or presence of various concentrations of bryostatin I for indicated time. Following incubation, the cells were collected by a cell scraper (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) and total-RNA was purified using an RNeasy plus mini kit (Qiagen, CA, USA). For the removal of contaminated DNA QIA shredder (Qiagen) was used according to the manufacturer's instructions and stored at -80°C. ReverTra-Plus RT-PCR kit (Toyobo, Osaka, Japan) was used for the purpose of RT-PCR where as GoTaq Master mix (Promega, WI, USA) in a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, CA, USA) was used for the PCR amplification according to

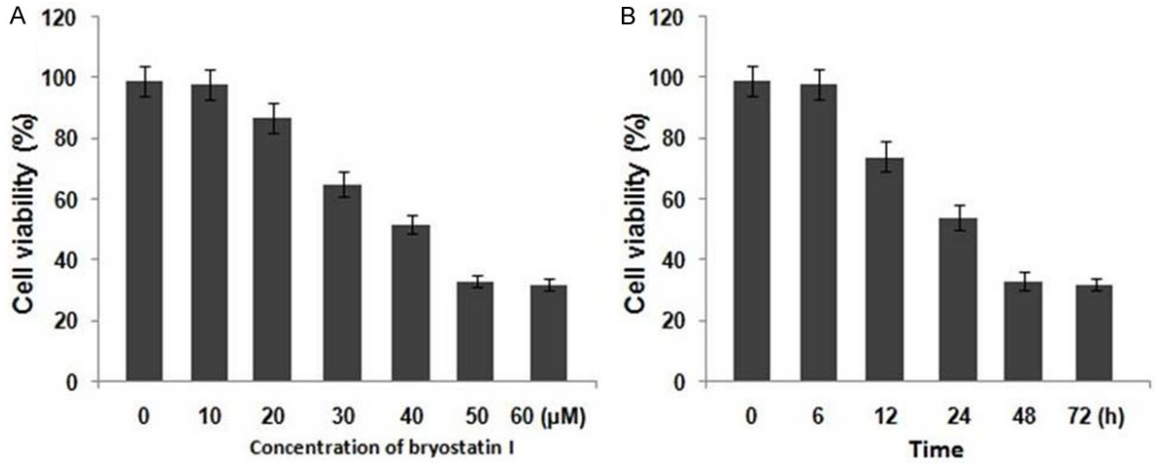

Figure 1. Effect of bryostatin on the viability of OHS-4 cells. Bryostatin I treatment reduces the viability of OHS-4 cells in (A) dose- and (B) time dependent manner.

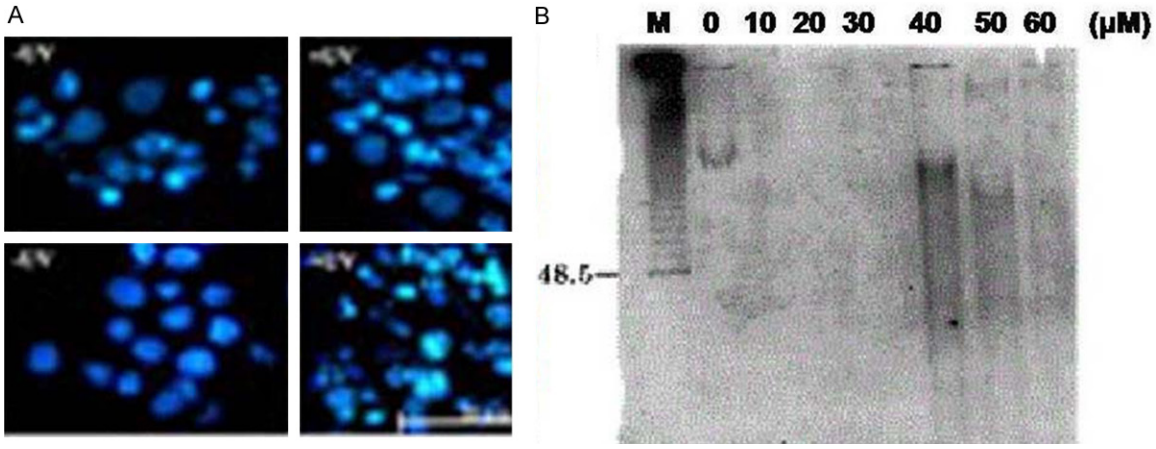

Figure 2. Bryostatin I treatment induces (A) nuclear fragmentation and (B) DNA ladder formation in OHS-4 cells. (A) Nuclear morphology of OHS-4 cells treated with 50 μM bryostatin I for 48 h. (B) DNA ladder formation in OHS-4 cells on treatment with 50 μM bryostatin for 48 h.

the manufacturer's instructions. Here, total RNA (500 ng) was reverse transcribed to cDNA using ReverTra Ace reverse transcriptase and oligo(dt)20. The 2% agarose gel electrophoresis in 1X Tris-acetate-EDTA buffer was used to resolve the PCR products using ethidium bromide staining. For detection and quantification of bands MultiGauge (Version 3.0, FujiFilm, Tokyo, Japan) was used.

Western blot analysis

OHS-4 cells after washing three times in cold PBS were treated with the buffer of radioimmunoprecipitation assay [50 mM Tris-HCl, pH 6.8; 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 0.1 mM Na_3VO_4, 1 mM sodium fluoride (NaF), 1% Triton X-100, 1% NP-40, 1 mM dithiothreitol, 1 mM PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin and 1 μg/mL pepstatin A] for cell lysis. The cell lysates were put into 1.5 mL tubes and agitated for 30 min under dark conditions (4°C) followed by centrifugation at 13,000 ×g for 20 min. The supernatant was harvested and subjected to bicinchoninic acid assay (Sigma Aldrich) for the determination of protein concentrations. The protein samples were resolved using electrophoresis on 10% SDS-polyacrylamide gel and then transferred onto polyvinylidenedifluoride membranes. The membrane was blocked for 45 min with 5% skimmed milk in buffer [10 mM Tris-HCl (pH 7.6), 100 mM NaCl and 0.1% (v/v) Tween-20] (25°C). Incubation of the membranes was performed with primary antibodies in cold conditions overnight and then the membranes were washed thrice

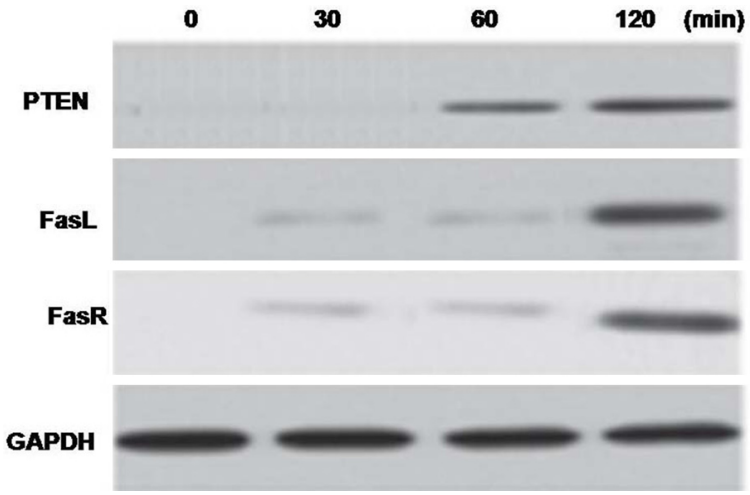

Figure 3. Effect of bryostatin I on the expression of level of *PTEN*, *FasL*, and *FasR* mRNA in OHS-4 cells. Bryostatin I exhibits time dependent inhibitory effect on the expression of *PTEN*, *FasL*, and *FasR* mRNA.

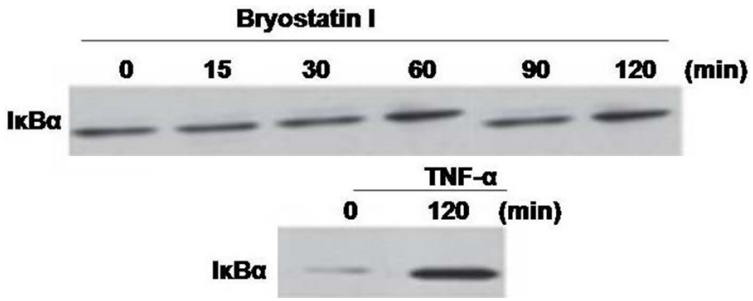

Figure 4. Effect of bryostatin I and TNF-α on the regulation of IκBα protein in OHS-4 cells. OHS-4 cells were treated with 50 μM bryostatin I or 10 ng/mL TNF-α for indicated time and subjected to IκBα expression analysis using Western blotting.

with Tris-buffered saline and Tween-20. The membranes after incubation for 1 h with secondary antibodies were subjected to proteins semi-quantitation using Tanon Gel Imager system (Tanon, Shanghai, China).

Statistical analysis

All the data presented are representative of three independent experiments and were analyzed by student's *t*-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Bryostatin I treatment induces apoptosis in OHS-4 cells

The results from phase-contrast microscopy revealed a concentration and time-dependent effect of bryostatin I on the survival of OHS-4 cells. Treatment of the cells with 50 μM doses of bryostatin I for 24 h led to rounding and shrinking of the cells to a significant extent compared to the untreated cells. Investigation of the results from WST-8 cell viability assay showed 67% reduction in the survival of OHS-4 cells on treatment with 50 μM concentration of bryostatin I compared to untreated cells (**Figure 1**).

After 48 h of the bryostatin I treatment, OHS-4 cells showed presence of typical apoptotic nuclei on staining with Hoechst 33342 (**Figure 2A**). The cells treated with bryostatin I for 48 h showed formation of DNA laddering pattern at the concentration of 50 μM (**Figure 2B**).

Effect of bryostatin I on the expression of PTEN, FasL, and FasR mRNA in OHS-4 cells

Bryostatin I treatment caused a concentration dependent enhancement in the expression of *PTEN, FasL*, and *FasR* mRNAs in OHS-4 cells after 48 h (**Figure 3**). Compared to the control cells, the expression of *PTEN, FasL*, and *FasR* mRNAs was significantly higher at 50 μM concentration of bryostatin I.

Effect of bryostatin I on IκBα expression in OHS-4 cells

The results from Western blot analysis showed a significant reduction in the IκBα staining intensity up to 2 h and thereafter it increased up to 4 h (**Figure 4**). However, treatment of the cells with TNF-α led to degeneration of IκBα within 30 min and then its expression was increased after 2 h.

Bryostatin I treatment induced translocation of NF-κB in OHS-4 cells

Treatment of OHS-4 cells with 50 μM bryostatin I for 48 h resulted in a significant increase in staining intensity for p65 NF-κB in both cyto-

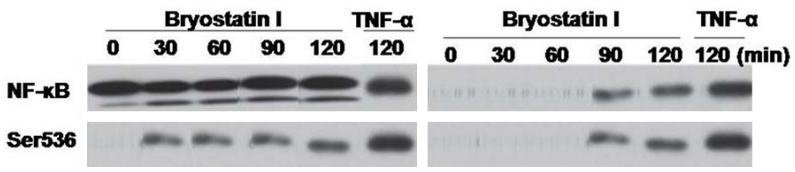

Figure 5. Effect of bryostatin I on nuclear translocation of NF-κB. Treatment of OHS-4 cells with 50 μM bryostatin I for 48 h led to nuclear translocation of NF-κB.

solic and nuclear fractions compared to the untreated cells (**Figure 5**). Treatment of the cells with TNF-α led to reaction of cytosolic and nuclear fraction proteins with anti-phospho-Ser536 p65 NF-κB antibody.

Discussion

The present study demonstrates the role of bryostatin I for inducing apoptosis in human osteoblastic OHS-4 cells. It is reported that various agents induce apoptosis in the human osteoblastic Saos-2 cells and human squamous cell carcinoma SCC-25 cells [23-25]. The results from the present study revealed that bryostatin I treatment induced morphological changes in OHS-4 cells and resulted in loss of cell viability. Use of Hoechst 33342 staining caused significant nuclear condensation and fragmentation into spherical bodies. Bryostatin I treatment also led to DNA ladder formation in OHS-4 cells after 24 h. Therefore, exposure of OHS-4 human osteoblastic cells to bryostatin I induced apoptosis.

It is reported that PTEN, FasR, and FasL play a vital role in the regulation of apoptosis induced by NF-κB [12, 26]. Increase in the expression of factors including PTEN, FasR and FasL by NF-κB has a promising role in the process of cell apoptosis [27, 28]. Our results revealed that bryostatin I treatment caused a marked increase in the expression of *PTEN, FasL*, and *FasR* mRNA in OHS-4 cells. These findings suggest that bryostatin I induced apoptosis in OHS-4 cells may involve increase in the expression of apoptosis inducing factors, *PTEN, FasL*, and *FasR*.

Phosphorylation of IκBα results in damage to proteasomes which then induce translocation of some complexes to nucleus and their binding to κB-response element. Synthesis of the genes, IκBα which acts as the inhibitor for NF-κB plays a vital role in the inhibition of activation of NF-κB [29]. The results from the present study revealed that exposure of OHS-4 cells to TNF-α induced IκBα degeneration and NF-κB translocation into the nucleus instantly. However, in the bryostatin I treated cells degeneration of IκBα was much slowed compared to the TNF-α treated cells. The phosphorylation of p65 NF-κB is a process highly regulated by both cell- and stimulus-dependent activating kinases and phosphatases.

Disclosure of conflict of interest

None.

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