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Geldanamycin induces G2 arrest in U87MG glioblastoma cells through downregulation of Cdc2 and cyclin B1

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ABSTRACT

Cell cycle progression requires precise expression and activation of several cyclins and cyclin-dependent kinases. Geldanamycin (GA) affects cell cycle progression in various kinds of cells. We analyzed GA-induced cell cycle regulation in glioblastoma cells. GA-induced G2 or M arrest in glioblastoma cells in a cell line-dependent manner. GA decreased the expression of Cdc2 and cyclin B1 in U87MG cells. And phosphorylated Cdc2 decreased along with Cdc2 in the GA-treated cells. This cell line showed G2 arrest after GA treatment. In contrast, GA failed to down-regulate these cell cycle regulators in U251MG cells. In U251MG cells, the cell cycle was arrested at M phase in addition to G2 by GA. Next, we analyzed the mechanism of the GA-induced regulation of Cdc2 and cyclin B1 in U87MG cells. Cdc2 and cyclin B1 were ubiquitinated by GA. MG132 abrogated the GA-induced decrease of Cdc2 and cyclin B1 indicating that these proteins were degraded by proteasomes. In conclusion, GA controls the stability of Cdc2 and cyclin B1 in glioblastomas cell species-dependently. Cdc2 and cyclin B1 might be responsible for the different responses of glioblastoma cell lines to GA.

1. Introduction

The cell cycle is a highly regulated process involving cyclins, cyclin-dependent kinases, and other regulatory proteins. In normal cells, Cdc2 is responsible for triggering mitosis, and Cdc2 kinase must be tightly regulated to ensure proper timing of the G2/M transition, since Cdc2 induces cells to enter M phase from G2 phase. The regulation of Cdc2 is achieved either by the association of Cdc2 with cyclin B1, or by phosphorylation/dephosphorylation of Cdc2 during the cell cycle [1].

Heat shock protein 90 (Hsp90) acts as a molecular chaperone by stabilizing intracellular proteins in normally growing cells. A number of cell cycle regulators have been demonstrated to use Hsp90 chaperone complex [2]. Cdc2 interacts with Hsp90 in yeast [3]. In avian cells, reduction of Hsp90α caused instability of Cdc2 and resulted in mainly G2 arrest under stress conditions [4].

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Abbreviations: Hsp90, heat shock protein 90; GA, geldanamycin; CHX, cycloheximide; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle medium; PI, propidium iodide; FACS, fluorescence-activated cell sorter; Tyr 15, tyrosine 15; Thr 14, threonine 14

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A benzoquinone ansamycin antibiotic, geldanamycin (GA), binds strongly to Hsp90 and specifically disrupts its chaperone function for several transcription factors and protein kinases [5,6]. Disruption of Hsp90 function results in reduced stability and increased degradation of proteins by proteasome-based mechanisms, resulting in growth inhibition and apoptosis of cells [7]. Previous reports indicated that Hsp90 is expressed in both high and low grade gliomas [8,9]. Its expression is higher in tumors than in normal tissues [10]. Furthermore, GA is reported to possess potent anti-tumor activity and pass through the blood brain barrier [11]. Therefore, it is an ideal anti-tumor drug for glioblastoma.

Recently, we showed that GA induces growth arrest at G2/M phase and mitotic catastrophe followed by cell death in T98G glioblastoma cells [12]. GA treatment at S phase enhances cell cycle arrest and apoptosis in T98G glioblastoma cells, indicating that the effect of GA is cell cycle-specific. In our previous research, we found that GA-induced cell cycle arrest at G2 or M phase depending on cell line. How GA causes G2/M arrest remains unclear, although previous studies have defined an action of GA in G1 arrest [13]. Therefore, we investigated the mechanisms of GA-induced G2 or M arrest in human glioblastoma cells, focusing on the regulation of cell cycle regulators at the G2/M boundary.

Here we show that GA induces G2 or M arrest in glioblastoma cells in a cell line-dependent manner. Differences of Cdc2 and cyclin B1 expression are responsible for the different responses of the two glioblastoma cell lines to GA. It is likely that the different response to GA may be dependent on the genetic background of cell lines examined, and may involve a number of regulatory cell cycle mechanisms [14]. In GA-sensitive cells treated with GA, not only cyclin B1 but also Cdc2 is degraded via the ubiquitin-proteasome pathway.

2. Materials and methods

2.1. Reagents and antibodies

GA, MG132 and cycloheximide (CHX) were purchased from Sigma Chemical Co. (St. Louis, MO). GA was prepared as a 1 mM stock in dimethyl sulfoxide (DMSO). MG132 was prepared as a 10 mM stock in DMSO, and stored at −80 °C until use. CHX was dissolved in ethanol as a stock solution at 100 mg/ml. The primary antibodies used for Western analysis were mouse anti-cyclin B1 monoclonal antibody (GNS1, Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-Cdc2 monoclonal antibody (#17, Santa Cruz Biotechnology); and rabbit anti-phospho-Cdc2 (Tyr15) polyclonal antibody (#9111, Cell Signaling Technology, Danvers, MA).

2.2. Cell lines

U87MG and U251MG glioblastoma cells were obtained from American Type Culture Collection (Manassas, VA). The cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Bio Whittaker, Rockland, ME) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine (Gibco BRL, Grand Island, NY) in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C. Cells were split every 3–4 days to ensure logarithmic growth.

2.3. Proliferation and cytotoxicity assay

To determine the effect of GA on glioblastoma cell proliferation and cytotoxicity, cells were plated (3×10^4) in 6-well culture dishes (Becton Dickinson, Franklin Lakes, NJ) in 2 ml of DMEM with 10% FBS. After 24 h, the cells were treated with GA at concentrations of 25–100 nM or with vehicle (DMSO) and the total (attached and floating) cells were harvested at 24, 48, and 72 h, and viable cells were counted using a hemocytometer after trypan blue staining.

2.4. Cell cycle analysis

Cell cycle analysis was done as previously described [12]. Briefly, cells were seeded (2.4×10^5) in 10-cm dishes in 8 ml of medium and total cells were harvested from each culture condition after the appropriate time interval. Cells were washed in ice-cold PBS, resuspended in 400 μl of ice-cold PBS, and diluted by dropwise addition of 1 ml of 100% ethanol. After fixation in 70% (v/v) ethanol, samples were stored at 4 °C for at least 1 h. Samples were then incubated in 500 μl of PBS containing 50 μg/ml propidium iodide (PI) and 0.5 mg/ml RNaseA (Qiagen Inc, Valencia, CA) for 1 h at room temperature. PI-stained nuclei were then analyzed using a Becton Dickinson FACScan (San Jose, CA). The percentage of cells in each phase of the cell cycle was determined using fluorescence-activated cell sorter (FACS) analysis.

2.5. Cytological studies (mitotic index)

Cytological studies were done as previously described [12]. In detail, to analyze the mitotic index of cells, cells were seeded (2.4×10^5) in 10-cm dishes and harvested after the appropriate time interval from each culture condition. Total cells were harvested and centrifuged at 1500 rpm for 3 min. The pelleted cells were treated with hypotonic solution (PBS:water, 1:1) for 10 min. The swollen cells were fixed by the dropwise addition of freshly prepared fixative (methanol:acetic acid, 3:1), centrifuged at 3000 rpm for 5 min, and resuspended in 40 μl of the same fixative. Cells were dropped onto clean microscope slides, dried, and stained with 5% May Giemsa Grunwald stain for 30 min [15]. Cells were observed under a light microscope. Five different fields were randomly selected for counting at least 1000 cells. The percentage of mitotic cells was calculated. Mitotic index was defined as the percentage of cells showing mitosis and chromosome condensation.

2.6. Inhibition of protein synthesis by CHX

To analyze the stability of Cdc2 and cyclin B1 proteins in glioblastoma cells, the cells were treated with 500 nM GA or vehicle for 1 h, and subsequently treated with 100 μg/ml CHX to inhibit protein synthesis [16]. Cellular Cdc2 and cyclin B1 protein levels were determined by Western blot analysis of the total cell lysate. The expression levels of Cdc2 and cyclin B1 proteins in the cells exposed to both GA and CHX were compared with those in the cells exposed to CHX alone.