BIOCHEMICAL PHARMACOLOGY 73 (2007) 1528–1536

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

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ARTICLE INFO

Article history:
Received 27 October 2006
Accepted 16 January 2007

Keywords:
Cell cycle arrest
G2/M
Cdc2
Cyclin B1
Glioblastoma
Geldanamycin

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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doi:10.1016/j.bcp.2007.01.022

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.

2.7. Inhibition of proteasome activity by MG132

We examined whether Cdc2 and cyclin B1 were degraded by proteasomes in GA-treated cells, as has been described previously for several receptor tyrosine kinases [17,18]. We firstly monitored the cellular level of Cdc2 and cyclin B1 in U87MG glioblastoma cells exposed to 2 μM MG132, a specific proteasome inhibitor [19]. MG132 was added to the culture medium 1 h prior to GA (50 or 500 nM) treatment. Then, the cells were treated with GA alone, or a combination of GA and MG132 for 24 h.

2.8. Western blot analysis

Total cells were harvested from each culture condition after the appropriate time interval, and washed with ice-cold PBS, and then protein was extracted using a lysis buffer containing 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 5 μg/ml phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 5 μg/ml leupeptin, and 2 μg/ml pepstatin. The samples were centrifuged at 14000 rpm for 30 min at 4 °C. Extracts were stored at −80 °C until use. Protein concentrations were determined using the BCA assay (Pierce, Rockford, IL).

For Western blot analysis, equal amounts of protein (30 μg) were electrophoresed on 10% SDS-PAGE gels, transferred to nitrocellulose membranes (Trans-Blot Transfer Medium 0.45 μm, Bio-Rad, Hercules, CA) by electroblotting at 4 °C for 4 h at 60 V and stained with Ponceau S (Sigma). After confirmation of protein transfer, proteins were detected with specific antibodies. All primary antibodies were used at 1:1000 dilution. Actin protein was detected as a control with mouse anti-human β-actin monoclonal antibody (Chemicon, Temecula, CA) used at 1:10000 dilution. Sheep anti-mouse IgG or donkey anti-rabbit IgG horseradish peroxidase-linked secondary antibodies (Amersham, Piscataway, NJ) at 1:4000 dilution were used for 1 h at room temperature. Protein detection was performed using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) and visualized using Hyperfilm ECL (Amersham).

2.9. Immunoprecipitation

Immunoprecipitation was performed as described previously [16]. Firstly, protein G-Sepharose or protein A-Sepharose (Pharmacia Biotech, Uppsala, Sweden) was preincubated with anti-Cdc2 (mouse anti-Cdc2 monoclonal, #17, Santa Cruz Biotechnology) or anti-cyclin B1 (rabbit anti-cyclin B1 polyclonal, H-433, Santa Cruz Biotechnology) antibody, respectively. Then cell lysate (750 μg) was incubated with protein A- or G-Sepharose at 4 °C with constant rotation. The beads were washed seven times with lysis buffer, and then boiled at 95 °C for 5 min. The sample buffer containing immunoprecipitated protein was electrophoresed on an 8% SDS-PAGE gel and the protein was transferred to a nitrocellulose membrane by electroblotting. After confirmation of protein transfer, specific proteins were detected with mouse anti-ubiquitin monoclonal (#13-1600, Zymed, South San Francisco, CA), mouse anti-Hsp90 monoclonal (SPA-830, Stressgen, Ann Arbor, MI), rabbit anti-cyclin B1 polyclonal (H433, Santa Cruz Biotechnology) or mouse anti-Cdc2 monoclonal (#17, Santa Cruz Biotechnology) antibody used at 1:1000 dilution.

2.10. Statistical analysis

All experiments were performed at least three times. The data are expressed as the mean ± standard error of the mean (S.E.M.). Probability (p) was calculated using a Student's *t*-test. *p*-Values lower than 0.05 were considered significant.

3. Results

3.1. GA inhibited proliferation of glioblastoma cells

Firstly, we investigated the effects of GA on the proliferation of glioblastoma cells. The cells were treated with GA for 24–72 h

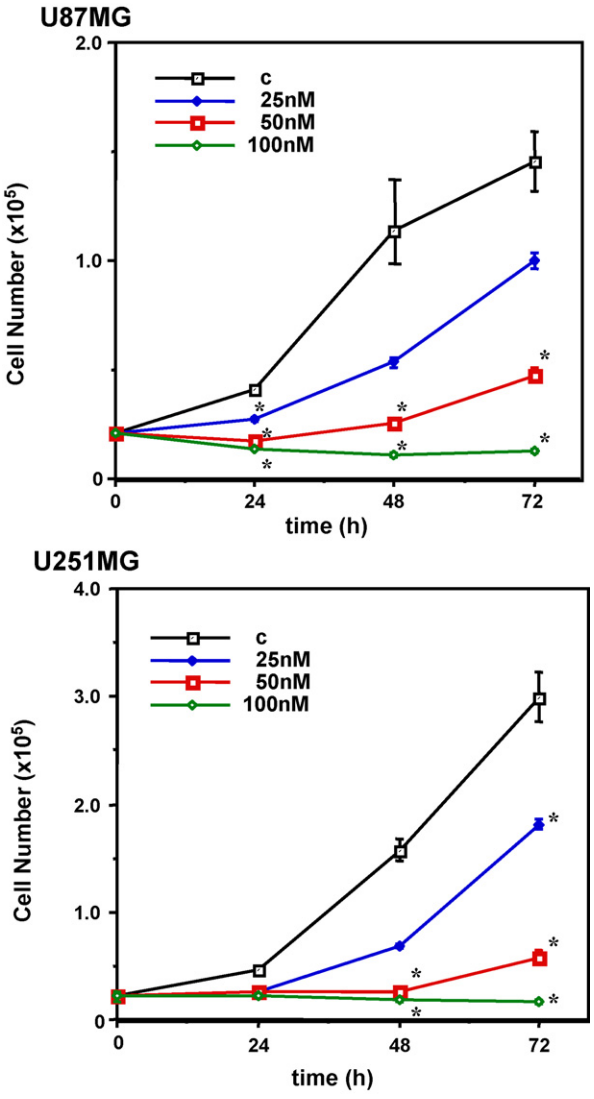

Fig. 1 – Effects of GA on proliferation of glioblastoma cells. GA inhibited proliferation of both U87MG (upper) and U251MG (lower panel) glioblastoma cells. The growth-inhibitory effect of GA was dose-dependent. (*): $p < 0.05$, compared to the control.

at concentrations ranging from 25–100 nM or with vehicle, and the number of viable cells was counted. As shown in Fig. 1, GA showed inhibitory effects on the proliferation of both U87MG and U251MG glioblastoma cells. The growth-inhibitory effect of GA was dose-dependent. A concentration as low as 25 nM was sufficient to decrease proliferation at 48 h. When the cells were treated with GA at doses of 50 nM or greater, cell proliferation was markedly inhibited.

3.2. GA induced G2/M arrest in glioblastoma cells

We next examined the effect of GA on the cell cycle progression of glioblastoma cells. The cells were treated with 50 nM GA for 12–48 h. We chose the concentration of 50 nM for these experiments because it caused a significant reduction of cell proliferation by 48 h. As shown in Fig. 2, the U87MG glioblastoma cells treated with GA began to accumulate at G2/M phase after 12 h (35%) and the G2/M population peaked at 24 h (41%). This arrest was sustained until 48 h (39%). In U251MG glioblastoma cells, the G2/M population increased to 23% at 12 h and finally to 54% at 48 h. On the other hand, in the control cultures of both cell lines, the G2/M population remained around 10% throughout the time course. On the other hand, the cell populations in G0/G1 phase at 48 h in GA-treated U87MG and U251MG cells were 54 and 27%, respectively. These data implied that GA might have a potential to arrest U87MG cells in both G0/G1 and G2/M phases.

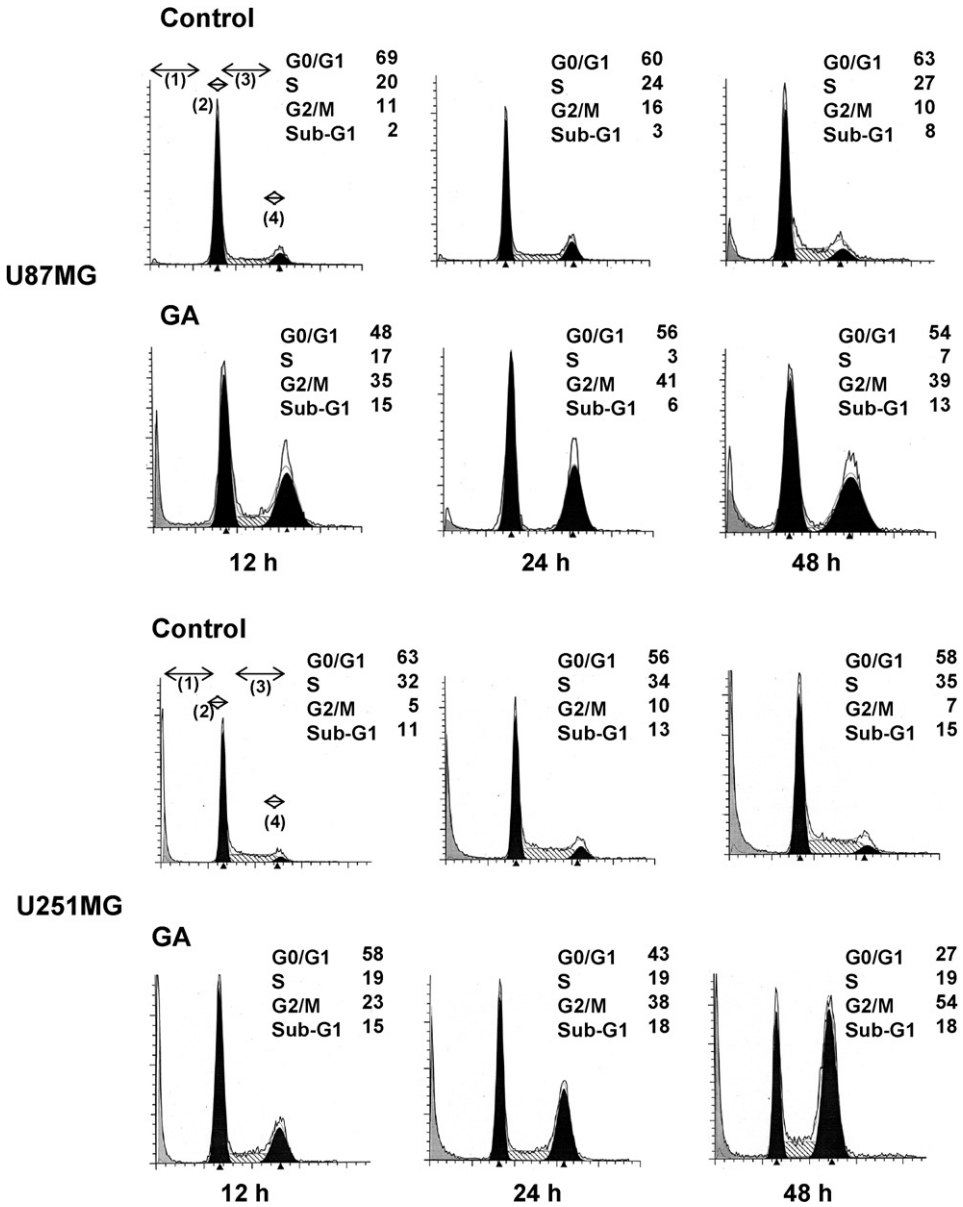

Fig. 2 – Effects of GA on cell cycle distribution of glioblastoma cells. U87MG (upper) and U251MG (lower panel) cells were treated with 50 nM GA for 12–48 h. The cell-cycle distribution at each time point is indicated. The cells treated with GA began to accumulate at the G2/M phase after 12 h and this accumulation was sustained for at least 48 h in both cell lines. The figure shows representative results of three independent experiments. The four values in each panel indicate the percentage of (1) sub-G1, (2) G0/G1, (3) S and (4) G2/M phase.

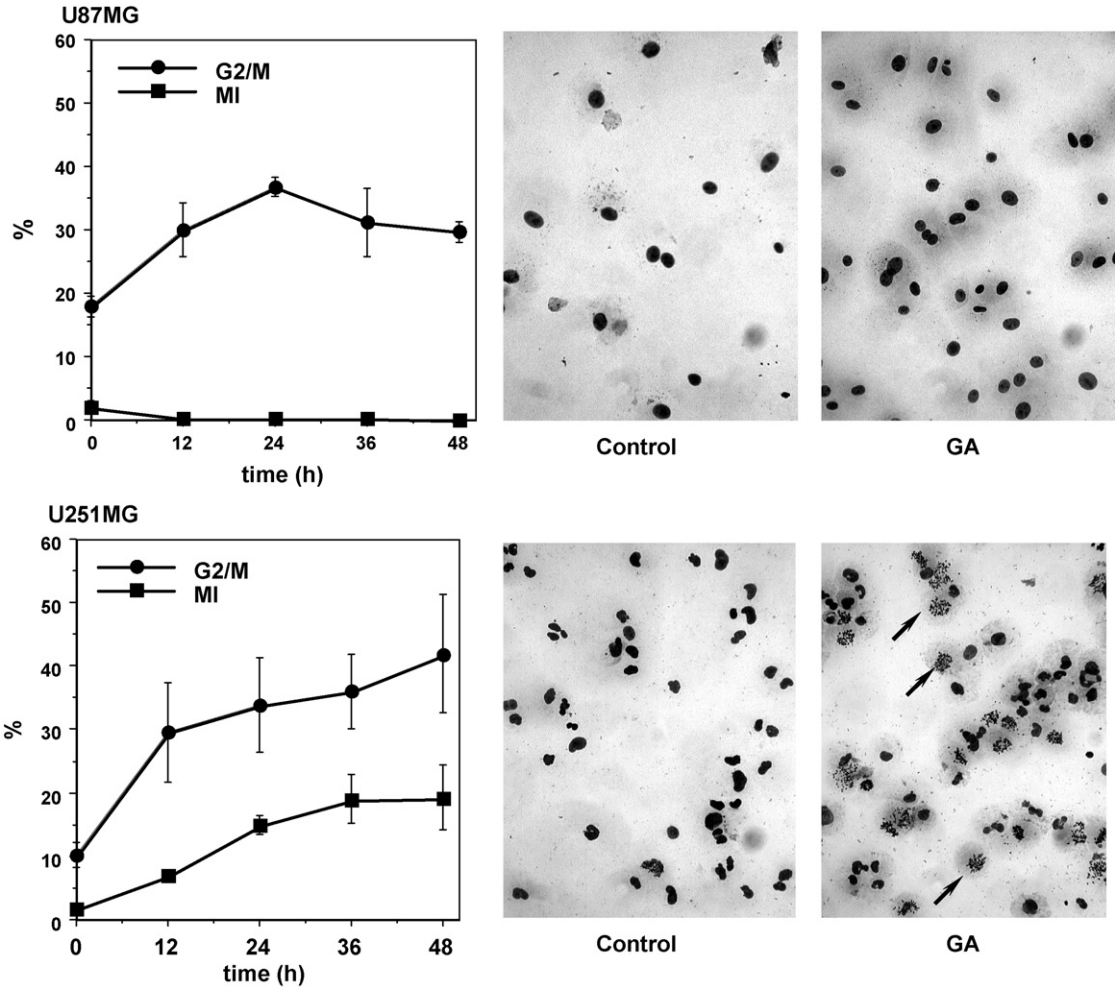

Fig. 3 – Cell population in G2/M phase and mitotic index in glioblastoma cells. The graphs show the mean ratio of G2/M population and the mitotic index of the glioblastoma cells. Microphotographs show representative cell populations at 24 h stained with Giemsa Grunwald stain. When U87MG (upper left panel) glioblastoma cells were treated with GA for 12 h, The mitotic index decreased to 0%. Mitotic figures could not be detected (upper right). On the other hand, in U251MG (lower left) glioblastoma cells, the cell population in mitosis gradually increased after exposure to GA, indicating M phase arrest. Cells in mitosis (arrows) were apparent after treatment with GA for 24 h. G2/M; mean percentage of cell population in G2/M phase, MI; mitotic index.

3.3. *Induction of G2 or M arrest by GA was cell line-dependent*

FACS analysis could not distinguish between cell populations in G2 and M phase. Therefore, the mitotic index was calculated to determine whether GA induced a high percentage of M-phase population in the two glioblastoma cell lines. As indicated in Fig. 3, mitotic index decreased from 2% to 0% at 12 h in U87MG glioblastoma cells, indicating that mitotic cells disappeared. Cells in mitosis were not observed up to 48 h. In contrast, the mitotic cell population began to increase at 12 h and further increased up to 18% at 48 h in U251MG glioblastoma cells. As indicated in the microphotograph, a large number of mitotic figures were observed in U251MG glioblastoma cells treated with GA (arrows), consistent with an increase in M phase of the cell cycle. These results indicated that GA induced G2 arrest in U87MG glioblastoma cells. On the other hand, GA induced M arrest in about 20% of the cells in addition to G2 arrest in U251MG cells.

3.4. *GA reduced Cdc2 and cyclin B1 proteins in U87MG*

To understand the mechanisms of the GA-induced G2 and M arrest in glioblastoma cells, we performed Western blot analysis to examine the expression of cell-cycle regulatory proteins at the G2/M boundary, Cdc2, cyclin B1 and phosphorylated form of Cdc2. As shown in Fig. 4, the levels of Cdc2 and phosphorylated Cdc2 at tyrosine 15 (Tyr15) started to decrease after GA treatment for 8 h and almost diminished after 24 h in U87MG glioblastoma cells. Cyclin B1 started to decrease at 12 h and diminished after 36 h.

On the other hand, GA did not affect the protein levels of Cdc2 or phosphorylated Cdc2 in U251MG glioblastoma cells. A slight increase of cyclin B1 expression was observed 4 h after

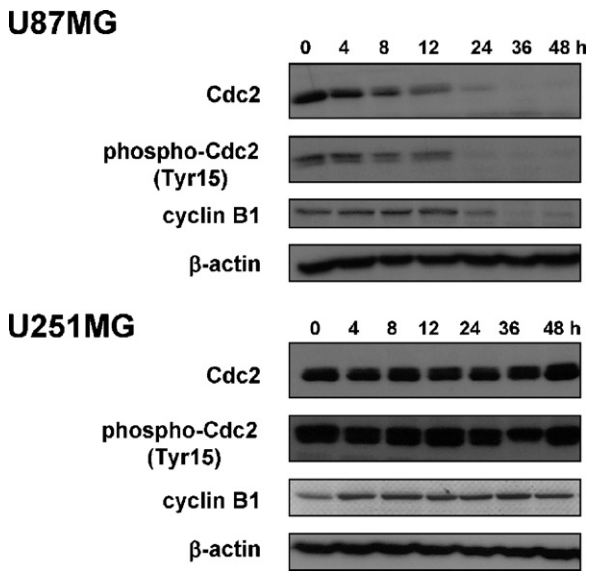

Fig. 4 – Effects of GA on expression of Cdc2 and cyclin B1 and on the level of phosphorylated Cdc2. U87MG (upper) and U251MG (lower panel) cells were treated with 50 nM GA. At each time point, expressions of Cdc2, cyclin B1 and phosphorylated Cdc2 were analyzed by Western analysis. The upper panel (U87MG) shows that the levels of Cdc2 and phosphorylated Cdc2 began to decrease after GA treatment for 8 h and almost disappeared at 24 h. Cyclin B1 started to decrease at 12 h and diminished after 36 h. On the other hand, GA up-regulated cyclin B1 protein at 4 h, and had no effect on the expression of Cdc2 and phosphorylated Cdc2 in U251MG glioblastoma cells (lower panel).

GA treatment. These data suggested that GA induced different responses of these proteins depending on the cell line, and consequently GA induced different kinds of cell cycle arrest in different cell lines.

3.5. *GA reduced the half-lives of Cdc2 and cyclin B1 proteins in U87MG*

To analyze the mechanism of the regulation of Cdc2 and cyclin B1 in U87MG glioblastoma cells, the cells were pretreated with 500 nM GA for 1 h, and protein synthesis was subsequently inhibited with CHX. We chose the concentration of 500 nM to be able to observe the significant effect of GA on the protein half-lives [16]. As shown in Fig. 5, the Cdc2 level in U87MG glioblastoma cells treated with GA and CHX declined significantly, indicating a protein half-life of 10 h. On the other hand, only a slight decrease of Cdc2 expression was observed in the cells treated with CHX alone. GA also reduced the half-life of cyclin B1 to about 7 h, and the amount of the protein decreased to 40% of the control value. These data indicated that the existing Cdc2 and cyclin B1 proteins in U87MG glioblastoma cells were degraded as a result of GA treatment.

3.6. *Cdc2 and cyclinB1 were degraded by proteasomes after GA treatment in U87MG*

We hypothesized that Cdc2 is degraded by proteasomes in GA-treated cells, as has been described previously for several receptor tyrosine kinases. To test this hypothesis, we monitored the cellular levels of Cdc2 and cyclin B1 in U87MG glioblastoma cells exposed to a specific proteasome inhibitor, MG132. MG132 was added to the culture medium to

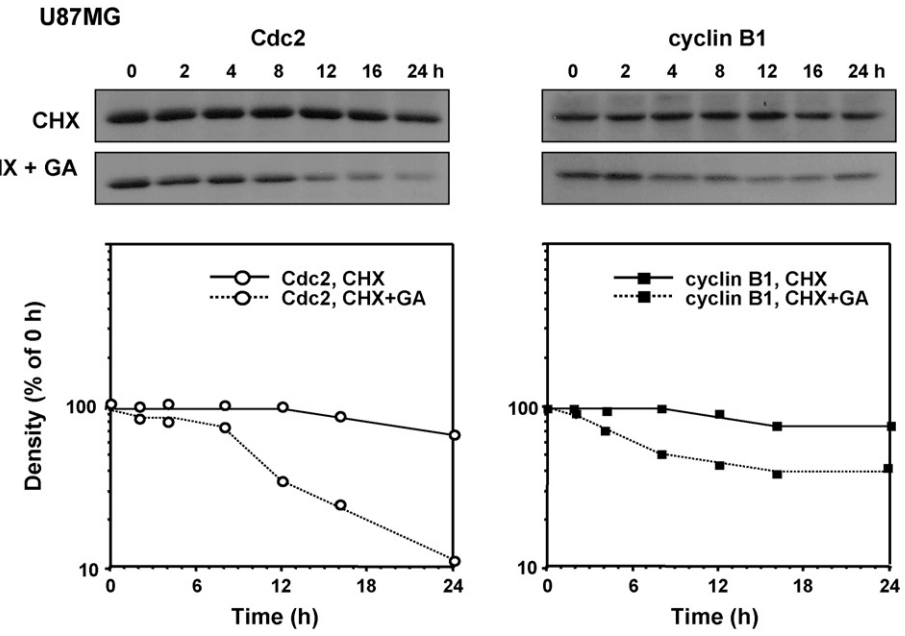

Fig. 5 – Effect of GA and/or CHX on Cdc2 and cyclin B1 expression. The figures (upper, Western blot analysis; lower, densitometric analysis) show that the Cdc2 and cyclin B1 levels in U87MG glioblastoma cells treated with GA and CHX declined rapidly (protein half-lives of 10 and 7 h, respectively). On the other hand, no significant change was observed in Cdc2 or cyclin B1 expression in the cells treated with CHX alone.

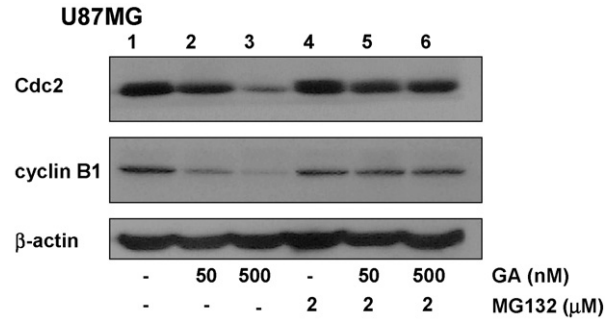

Fig. 6 – Proteasome degradation of Cdc2 and cyclin B1 after GA treatment. MG132 treatment (lane 4) caused no change in Cdc2 or cyclin B1 protein expression. The presence of MG132 significantly attenuated the GA-induced decreases of Cdc2 and cyclin B1 expression (lanes 5 and 6).

the concentration of 2 μM 1 h before treatment of the cells with GA. As indicated in Fig. 6, Western blot analysis of the total cell lysate with an anti-Cdc2 antibody revealed that MG132 alone (lane 4) caused no change in the Cdc2 protein level compared to that in the control (lane 1). As shown in lanes 5 and 6, the addition of MG132 significantly attenuated the GA-induced decrease of Cdc2 expression in both 50 and

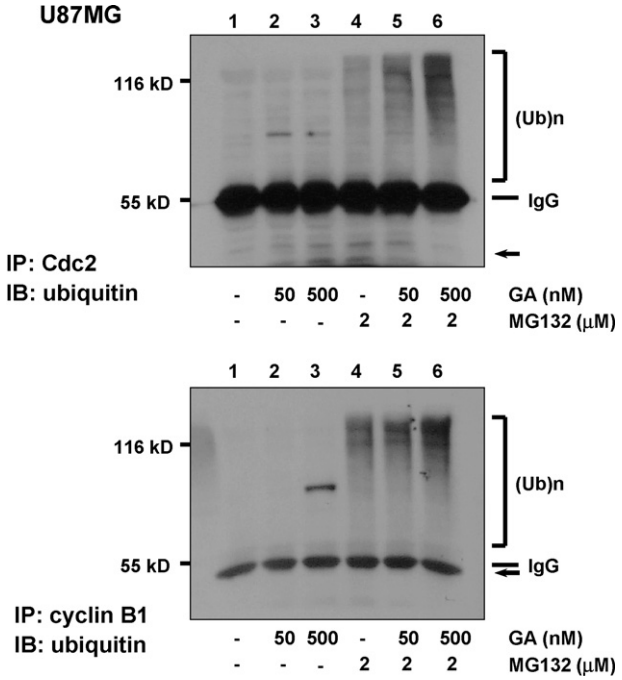

Fig. 7 – GA induces ubiquitination of Cdc2 and cyclin B1. GA alone did not induce an accumulation of ubiquitinated Cdc2 (upper) or cyclin B1 (lower panel) in U87MG glioblastoma cells (lanes 2 and 3). Treatment with MG132 alone induced a slight increase of ubiquitinated proteins (lane 4). In the cells treated with both GA and MG132, a significant amount of ubiquitinated protein was observed (lanes 5 and 6). The arrow in each panel indicates the location at which Cdc2 and cyclin B1 should be detected. IgG indicates that equal amount of proteins are loaded.

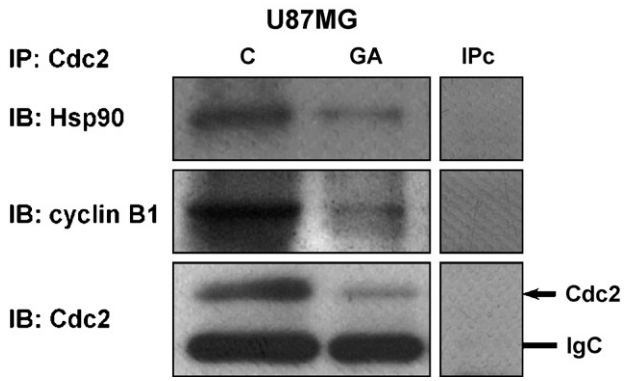

Fig. 8 – Immunoprecipitation of Hsp90 and cyclin B1 by antibody against Cdc2 in whole cell lysates. U87MG glioblastoma cells were treated with 50 nM GA or vehicle for 24 h. The cell lysates were immunoprecipitated (IP) with anti-Cdc2 antibody. After transfer of the proteins, the membranes were probed with antibody for Hsp90, cyclin B1 or Cdc2. IgG indicates that equal amount of proteins are loaded. IPc; immunoprecipitation without antibody.

500 nM concentration (lanes 2 and 3). The same phenomenon was observed for cyclin B1. These data indicated that Cdc2 and cyclin B1 proteins were degraded by proteasomes in GA-treated U87MG glioblastoma cells.

3.7. *GA induced ubiquitination of Cdc2 and cyclin B1*

Previous reports demonstrated that GA-mediated proteasomal degradation of Hsp90 client proteins was preceded by ubiquitination [20,21]. We therefore tested whether Cdc2 and cyclin B1 were ubiquitinated in GA-treated cells.

We immunoprecipitated Cdc2 and cyclin B1 from U87MG whole-cell lysates, and analyzed the immunoprecipitates for ubiquitination by Western blot analysis. As shown in Fig. 7, treatment of cells with GA (50 or 500 nM) alone did not cause an accumulation of ubiquitinated Cdc2 or cyclin B1 (lanes 2 and 3). Co-treatment with GA and 2 μM MG132 increased the levels of the ubiquitinated forms of both proteins (lanes 5 and 6).

These results showed that ubiquitin-conjugated Cdc2 and cyclin B1 were substrates for proteasomes.

3.8. *GA decreased the Cdc2-Hsp90 and Cdc2-cyclin B1 complexes in U87MG*

Hsp90 has been shown to chaperone Cdc2 (3, 4). GA was found to disrupt Hsp90-protein interactions. To investigate the effect of GA on Cdc2–Hsp90 protein complex in U87MG glioblastoma cells, we next carried out immunoprecipitation assay. As shown in Fig. 8 (upper panel), immunoprecipitation of Cdc2 resulted in co-precipitation of Hsp90 in the control, indicating the association of Cdc2 with Hsp90. Treatment with GA significantly reduced the amount of Hsp90 that was co-precipitated with Cdc2.

We next determined the effect of GA on Cdc2-cyclin B1 protein interactions. Cdc2-cyclin B1 is known to be a master

mitotic regulator. Fig. 8 (middle panel) showed that cyclin B1 was co-precipitated with Cdc2 in vehicle-treated cells. GA decreased cyclin B1 expression along with Cdc2 in the cells. These results indicated that Cdc2 bound directly to cyclin B1 and Hsp90 and that these complexes were reduced by GA.

4. Discussion

Cyclins and cyclin-dependent kinases such as cyclin B1 and Cdc2 control cell cycle progression by regulating the phosphorylation or dephosphorylation of proteins [1]. Mitosis in human cells is initiated by the protein kinase Cdc2–cyclin B1 complex, which is activated at the end of G2 phase by dephosphorylation of two inhibitory residues of Cdc2, threonine 14 (Thr14) and Tyr15 [22]. The Cdc2 protein level is constant during cell cycle progression in normally growing cells [23], whereas cyclin B1 is degraded via the ubiquitin-proteasome pathway. Its degradation is necessary in order for the cells to exit M phase [24]. In cancer cells, cell cycle regulators are commonly deregulated, so that normal growth control and checkpoints are evaded [25]. Some drugs, including GA, have been reported to induce G2 or G2/M arrest by down-regulation of Cdc2 in several kinds of cells [26,27]. However, the mechanism of GA-induced G2/M arrest is not completely understood. We showed that GA induced G2/M arrest in U87MG and U251MG glioblastoma cells. Further morphological observation revealed that U87MG glioblastoma cells showed G2 arrest after GA treatment. In this cell line, GA reduced both Cdc2 and cyclin B1. GA also down-regulated the expression of the inhibitory phosphorylated Cdc2. In contrast, GA failed to down-regulate Cdc2 and rather up-regulated cyclin B1 in U251MG glioblastoma cells. And some cells progressed through G2 and arrested at M phase in addition to G2. Our results suggested that a potential mechanism for GA-induced G2 arrest in U87MG glioblastoma cells was through a decrease of the total protein levels of Cdc2 and cyclin B1. The decrease of the expression of Cdc2 and cyclin B1 by GA inhibited these glioblastoma cells from entering M phase. And the GA-induced M arrest in U251MG glioblastoma cells might be due to up-regulated expression of cyclin B1. To our knowledge, this is the first investigation showing that GA induced G2 arrest through down-regulation of Cdc2 and cyclin B1 in glioblastoma cells.

As described above, we found that G2 arrest due to Cdc2 and cyclin B1 reduction was cell line-dependent. Maloney et al. [14] reported that the effects of Hsp90 inhibition were often cell line specific and likely to be dependent on the genetic background and biochemical features of individual cells. A common biological feature of tumor cells is that they contain many mutated proteins that were acquired during tumorigenesis [28,29]. Simizu et al. [30] reported that GA decreased the Plk level in cells expressing a wild-type Plk protein. However, the Plk level did not decrease in any cells expressing a mutant Plk protein, because mutations within the C-terminal region of the Plk protein disturb its interaction with Hsp90. Although the mechanism of the cell line-dependent destabilization of Cdc2 and cyclin B1 induced by GA is not clear, it is possible that different sensitivities to GA may be due to mutations of the genes for these proteins.

As a key regulator of the G2/M transition, Cdc2 phosphorylation is necessary for G2 arrest. Tyrosine phosphorylation is an important inhibitory mechanism for regulation of Cdc2 activity [15]. Dephosphorylation of Cdc2 at Thr14 and Tyr15 activates Cdc2 kinase activity resulting in entry into M phase [31,32]. We examined the phosphorylation status at the Tyr15 residue of the Cdc2 protein in the glioblastoma cells. We expected that GA would up-regulate the phosphorylation of Cdc2 in U87MG glioblastoma cells, because the cell cycle in these cells was arrested at G2 phase. However, contrary to our expectation, phosphorylated Cdc2 decreased along with Cdc2 in the cells treated with GA. Lock et al. [33] reported the accumulation of phosphorylated Cdc2 accompanied by decreased-Cdc2 expression in a radiation-induced G2 arrested model. Nakamizo et al. [34] reported that ACNU increased the phosphorylation of Cdc2 at Tyr15 and decreased cyclin B1 protein, with no change in the total amount of Cdc2 protein in U373MG and U251MG glioblastoma cells. Those cells showed G2/M arrest. Our data indicated that GA-induced G2 arrest in U87MG glioblastoma cells might be due to decreases of Cdc2 and cyclin B1 proteins rather than to an increase of phosphorylated Cdc2.

Finally, we analyzed the mechanism of Cdc2 and cyclin B1 degradation in U87MG glioblastoma cells. GA interferes with signal transduction by inducing ubiquitination and degradation of signaling molecules chaperoned by Hsp90 through a mechanism involving the proteasome [35,36]. Our data indicated that the proteasome might be involved in the pathway of GA-induced degradation of Cdc2 and cyclin B1 proteins, and that this pathway might share common characteristics with the degradation of other Hsp90 client proteins. Inactivation of the Cdc2–cyclin B1 complex at the exit from M phase depends on the specific proteolysis of cyclin B1, whereas the Cdc2 subunit remains present at constant levels throughout the cell cycle [24]. In this study, we found that not only cyclin B1 but also Cdc2 was degraded in GA-treated cells. This was due to the inhibition of Hsp90 by GA.

In conclusion, we showed that GA induced either G2 or M arrest in glioblastoma cells. Reduced protein expression of Cdc2 and cyclin B1 was found to be responsible for the GA-induced G2 arrest in U87MG glioblastoma cells. This is the first report demonstrating that Cdc2 and cyclin B1 are molecular targets affected by GA that cause G2 rather than M arrest in U87MG glioblastoma cells. Further studies should be necessary to clarify whether other mechanisms participate in the arrest of glioblastoma cells in a particular stage of the cell cycle by GA.

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