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Gambogic acid inhibits the growth of osteosarcoma cells *in vitro* by inducing apoptosis and cell cycle arrest

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Abstract. The natural product gambogic acid (GA) has been demonstrated to be a promising chemotherapeutic drug for some cancers because of its ability to induce apoptosis and cell cycle arrest. Until now, no studies have looked at the role of GA in osteosarcoma. In this study, we observed the effects of GA on the growth and apoptosis of osteosarcoma cells *in vitro*. We found that GA treatment inhibits the proliferation of osteosarcoma cells by inducing cell cycle arrest. Moreover, we found that GA induces apoptosis in MG63, HOS and U2OS cells. Furthermore, we showed that GA treatment elevates the Bax/Bcl-2 ratio. GA mediated the G0/G1 phase arrest in U2OS cells; this arrest was associated with a decrease in phospho-GSK3-ß (Ser9) and the expression of cyclin D1. Similarly, in MG63 cells, GA mediated G2/M cell cycle arrest, which was associated with a decrease in phospho-cdc2 (Thr 161) and cdc25B. Overall, our findings suggest that GA may be an effective anti-osteosarcoma drug because of its capability to inhibit proliferation and induce apoptosis of osteosarcoma cells.

Introduction

Osteosarcoma is the most common type of primary malignant bone tumor and is defined by the presence of malignant mesenchymal cells that produce osteoid or immature bone (1). Currently, the treatment for osteosarcoma is a multi-modal approach that consists of preoperative systemic polychemotherapy, local surgical therapy and postoperative chemotherapy (2). Traditional chemotherapeutics have some toxicities and side-effects, including myelosuppression, hearing loss, mucositis, impaired renal function and gonadal dysfunction (3-6). Recent studies suggest that osteosarcomas acquire resistance to traditional chemotherapeutics over time. This is one reason for the failure of current multimodal therapeutic programs (7,8).

Gambogic acid (GA, $C_{38}H_{44}O_8$) is the main active ingredient of gamboge, a brownish or orange resin that is obtained from the *Garcinia* hanburyi tree (genus *Garcinia* of family *Guttiferae*). In recent years, several studies have shown that GA can induce apoptosis and suppresses the proliferation of a variety of malignant cells, including hepatoma SMMC-7721 cells (9), gastric carcinoma MGC-803 cells (10), BGC-823 cells (11) and breast carcinoma MCF-7 cells (12). GA mediates these responses by inhibiting tumor angiogenesis (13), acquiring chemotherapeutic resistance (14), and selectively binding tumor cells (9), among other mechanisms. Several studies also confirmed that GA was equally effective at suppressing the growth of transplanted tumors in animal models (15-17). Moreover, it was also reported that GA exhibits low toxicity against normal tissues (18,19).

Although several studies suggested that GA could be a promising novel chemotherapeutic agent for some cancers, the effects of GA on osteosarcoma remains to be elucidated. In this study, we observed the effects of GA on the proliferation and apoptosis of osteosarcoma cells *in vitro*, and our results demonstrated that GA suppresses the proliferation and facilitates the apoptosis of three human osteosarcoma cell lines (MG63, HOS and U2OS cells). Furthermore, our study suggested that GA inhibits the proliferation and induces apoptosis by altering the expression of cell cycle and apoptosis regulators, such as Bax, Bcl-2, GSK3-ß, cyclin D1, cdc2 and cdc25B.

Materials and methods

Materials. GA was purchased from Sigma Company (USA) with a purity of >97%. It was dissolved in dimethyl sulfoxide (DMSO) and stored at -20˚C at a concentration of 5 mg/ml, the

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Abbreviations: GA, gambogic acid; DMSO, dimethyl sulfoxide; CCK-8, cell counting kit-8; PI, propidium iodide; PBS, phosphate-buffered saline

Key words: osteosarcoma, gambogic acid, apoptosis, cycle arrest

final concentration of DMSO was <0.1%. The cell counting kit-8 (CCK-8) was purchased from Dojindo Laboratories (Japan), while the Annexin V-FITC detection kit was purchased from Biosea Company (China). Propidium iodide (PI) and RNase were purchased from Sigma Company (USA). The primary antibodies against Bax, Bcl-2, caspase-3, cyclin D1, GAPDH and secondary antibodies were purchased from Santa Cruz Biotechnology (USA). The anti-cdc25B and -cdc2 (phospho Thr161) antibodies were purchased from Bioworld (USA). Lastly, the GSK3-ß (phospho Ser9) antibody was purchased from Abcam (USA).

Cell culture. All cells were obtained from the Cell Bank of the Shanghai Institute of Biochemistry & Cell Biology, Chinese Academy of Sciences (China), and cells were maintained in MEM medium (Hyclone) supplemented with 10% heat-inactivated fetal bovine Serum (Sijiqing Company, Ltd.), 1% penicillin-streptomycin (Hyclone, USA) and 1% NEAA (Hyclone, USA). The cells were incubated in a stable environment with 5% CO_2 at 37˚C in a humidified incubator (Heal Force HF90).

Cell viability detection. A total of $4x10^3$ cells per well were seeded into 96-well plates and cultured overnight. The cells were treated with varying concentrations of GA for 24 h. Then, the medium was replaced with 100 μl of fresh culture medium and 10 μl of CCK-8 solution. The cells were subsequently incubated for an addition 4 h at 37˚C. The cell suspension was placed on a micro-vibrator for 5 min, and the absorbance (A) was measured at 490 nm by a Microplate Reader (Bio-Rad550, USA). The percentage of inhibition was calculated as follows: inhibition (%) = (1-A of experimental well)/A of control well x 100%). The IC_{50} was the concentration of GA that caused 50% inhibition of cell viability.

Cell cycle analysis. For cell cycle analysis, A total of $15x10^4$ cells per well were seeded into 6-well plates and cultured overnight. Then treated with various concentrations of GA for 24 h, collected and washed twice with cold phosphate-buffered saline (PBS). Next, the cells were fixed with ice-cold 70% ethanol overnight at 4˚C. The cells were then incubated with 100 mg/l RNase A, 50 mg/l PI, and 0.1% Triton X-100 in the dark. After 30 min, the cells were analyzed by a flow cytometer (BD FACSCalibur System).

Apoptosis assay. To compare the extent of apoptosis in GA-treated cells, the cells were treated with GA for 24 h as described above. Next, the cells were harvested, washed twice and resuspended with cold PBS. After that, the cells were resuspended in 300 μl of PBS and were treated with 5 μl of Annexin V-fluorescein isothiocyanate and 10 μl of PI, and incubated in dark for 15 min. The stained cells were analyzed using a flow cytometer (BD FACSCalibur System) and were directly observed using a confocal laser microscope (LSM510, Carl Zeiss).

Western-blot assay. For Western blot analysis, the cells were similarly exposed to various concentrations of GA for 24 h. Harvested cells were then lysed with RIPA lysis buffer (Beyotime, China) containing PhosStop (Roche, Switzerland) and incubated on ice for 1 h. Then, the cells were pelleted at 12,000 x g for 5 min at 4˚C. The supernatants were subsequently collected, and the protein concentration was determined by measuring the absorbance at 540 nm by using a Microplate Reader (Bio-Rad550). The proteins were then separated with an 8-12% SDS-polyacrylamide gel (SDS-PAGE) and transferred onto an NC membrane. The membrane was incubated overnight with the appropriate primary antibody [Bax, Bcl-2, caspase 3, GSK3-ß (phospho Ser9), cyclin D1, cdc25B, cdc2 (phospho Thr161) and GAPDH] (1:1,000). After that, the membrane was incubated with a secondary antibody (anti-mouse or anti-rabbit) (1:10,000), visualized by using a chemiluminescence substrate kit, and exposed to medical X-ray film.

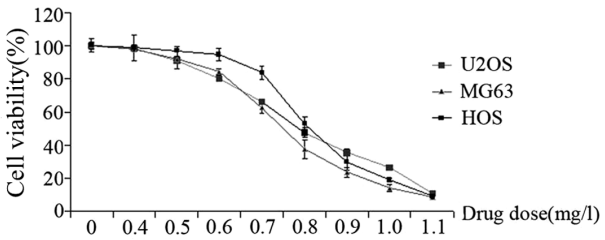

Figure 1. Inhibitory effect of GA on the proliferation of MG63, HOS and U2OS cells. A dose-dependent inhibition of proliferation was observed in MG63, HOS and U2OS cells after treatment with GA for 24 h ($p<0.05$). Each value is the mean ± SD of three experiments.

Statistical analysis. Data were expressed as means ± SD. Statistical comparisons of the results were performed using analysis of variance (ANOVA). Significant differences ($p<0.05$) between the control and GA-treated cells were analyzed by Dunnett's test.

Results

GA inhibits the growth of osteosarcoma cells in vitro. To investigate the potential role of GA on the growth of human osteosarcoma cells, we examined the effect of GA on cell viability in MG63, HOS and U2OS cells by using the CCK-8 assay. The results showed that the growth of the three cell lines was inhibited by GA after treatment for 24 h (Fig. 1). The IC_{50} values were 0.75, 0.77 and 0.81 mg/l for the MG63, HOS and U2OS cells, respectively.

GA inhibits cell cycle progression of MG63 and U2OS. Since GA inhibits the viability of osteosarcoma cells, we hypothesized that GA may have an effect the cell cycle of these cells. To determine the effects of GA on the cell cycle of osteosarcoma cells, we compared the cell cycle distribution of various concentrations of GA (0.6, 0.7 and 0.8 mg/l) treated osteosarcoma cells and untreated cells by measuring the intracellular DNA content 24 h after treatment.

The results demonstrated that GA dramatically arrested cells at the G2/M in MG63 cells ($p<0.01$) and G0/G1 phase in U2OS cells ($p<0.01$), but there was no effect on HOS cells (Fig. 2). These findings suggested that GA might selectively inhibit the proliferation of osteosarcoma cells by inhibiting cell cycle progression.

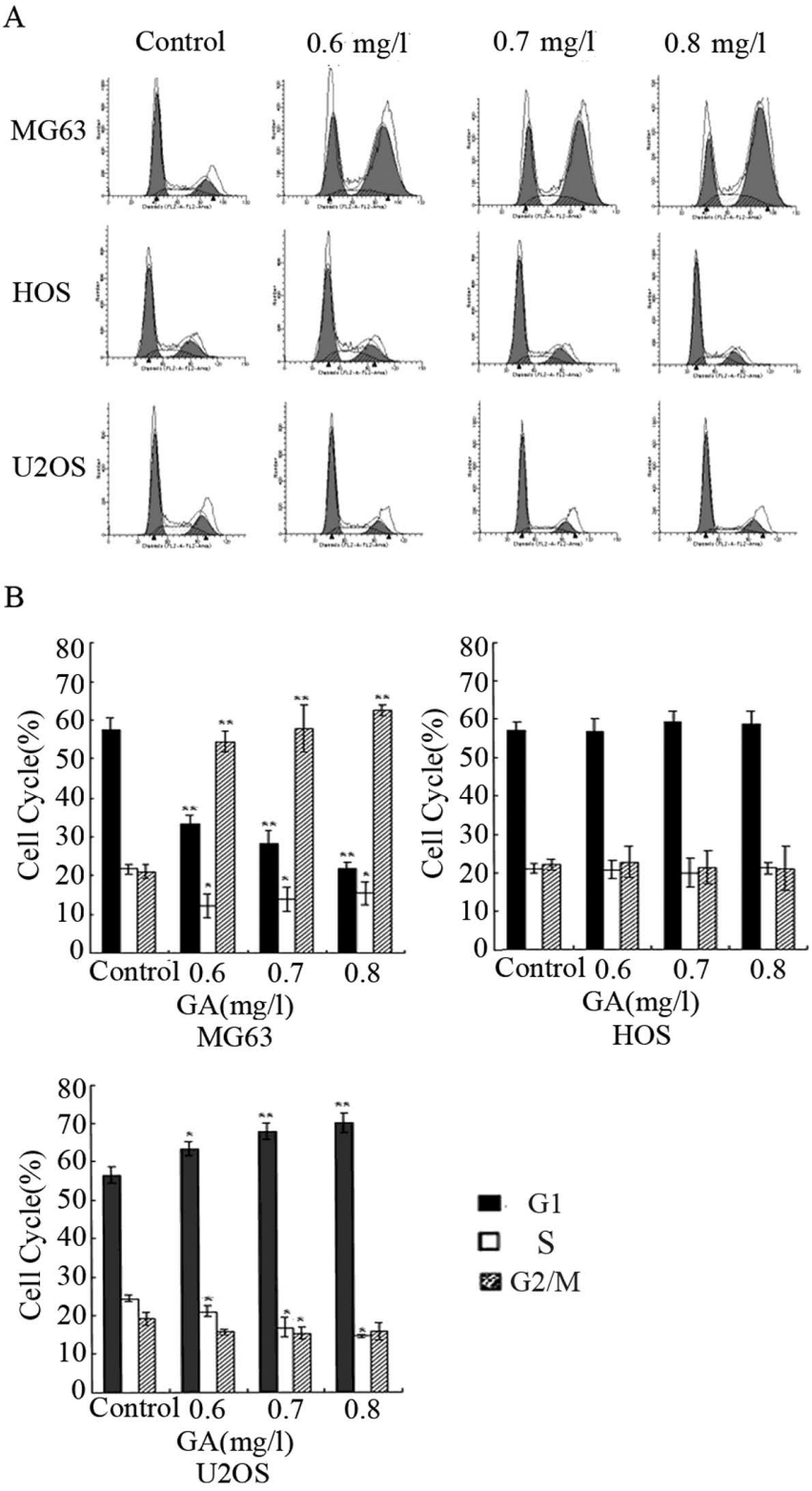

Figure 2. Different effects on the cell cycle distribution of MG63, HOS and U2OS cells after treatment with GA. MG63, HOS and U2OS cells were incubated with different concentrations of GA (0.6, 0.7 and 0.8 mg/l) for 24 h. Untreated cells served as control. $^{*}p<0.05$; $^{**}p<0.01$.

GA promotes apoptosis of osteosarcoma cells. Apoptosis is another factor of cell viability. To investigate the effect of GA on the apoptosis of osteosarcoma cells, we compared the PI-Annexin V-FITC staining of GA-treated cells 24 h after treatment.

The flow cytometry results showed that apoptosis was significantly elevated in all three GA-treated cell lines with a dose-dependent manner. This function of GA was more notable in MG63 and HOS cells. Overall, GA (0.8 mg/l) induced early and late apoptosis all in 32.33, 29.91 and 17.39%

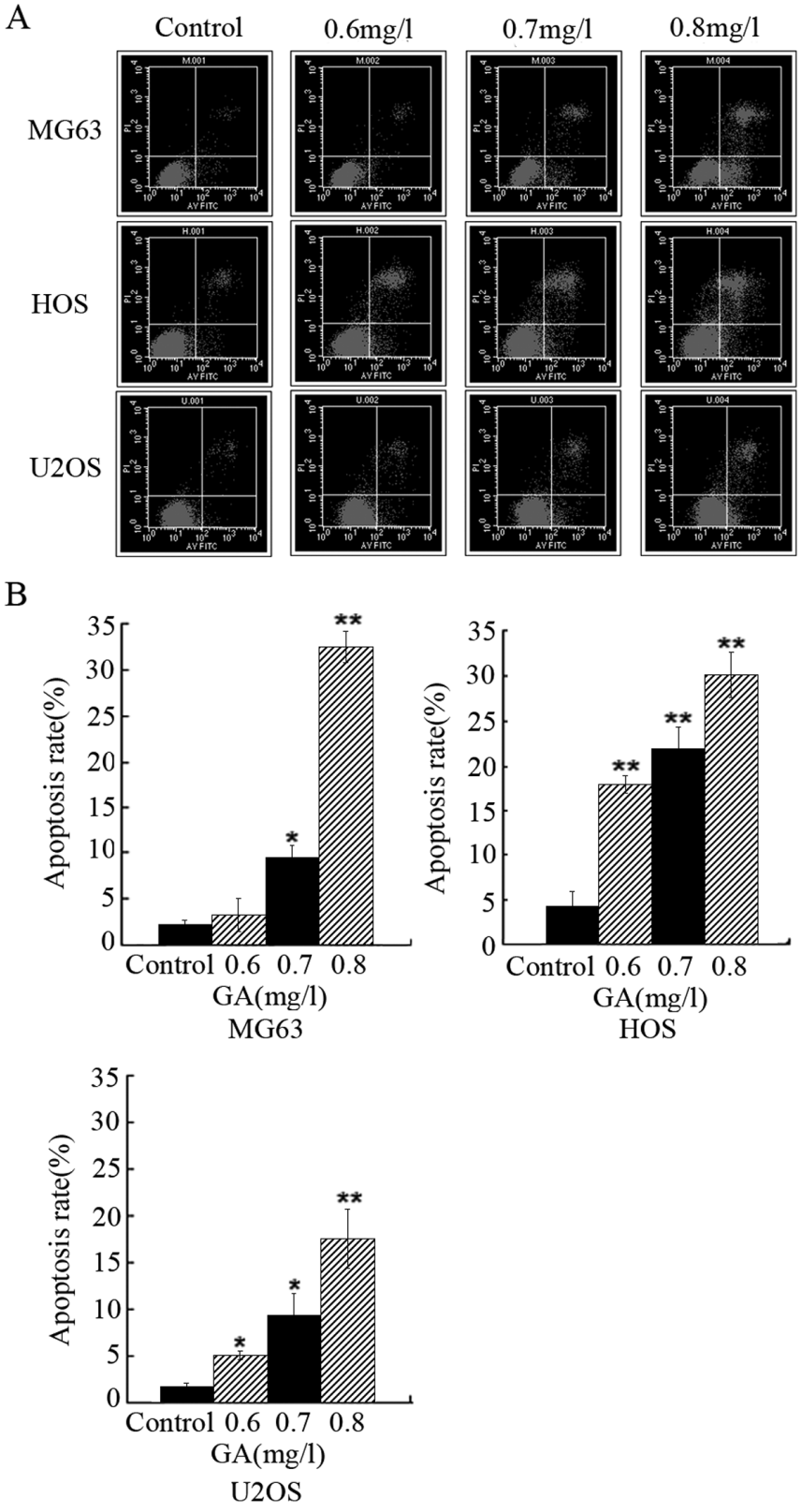

Figure 3. The apoptosis effects of GA in MG63, HOS and U2OS cells. Cell cultures untreated and treated with different concentrations of GA for 24 h. Apoptotic cells after staining with Annexin-V-FITC as well as propidium iodide analyzed by using a flow cytometer. $^{*}p<0.05$; $^{**}p<0.01$

of MG63, HOS and U2OS cells, respectively (Fig. 3). On the other hand, using a confocal laser microscope we found the apoptotic cells induced by GA (0.8 mg/l) were stained by Annexin-V-FITC and propidium iodide (Fig. 4).

GA alters the expression of proteins that are associated with cell cycle regulation and apoptosis. To identify the molecular mechanism for the GA-mediated inhibition of proliferation and promotion of apoptosis in osteosarcoma cells, we investigated

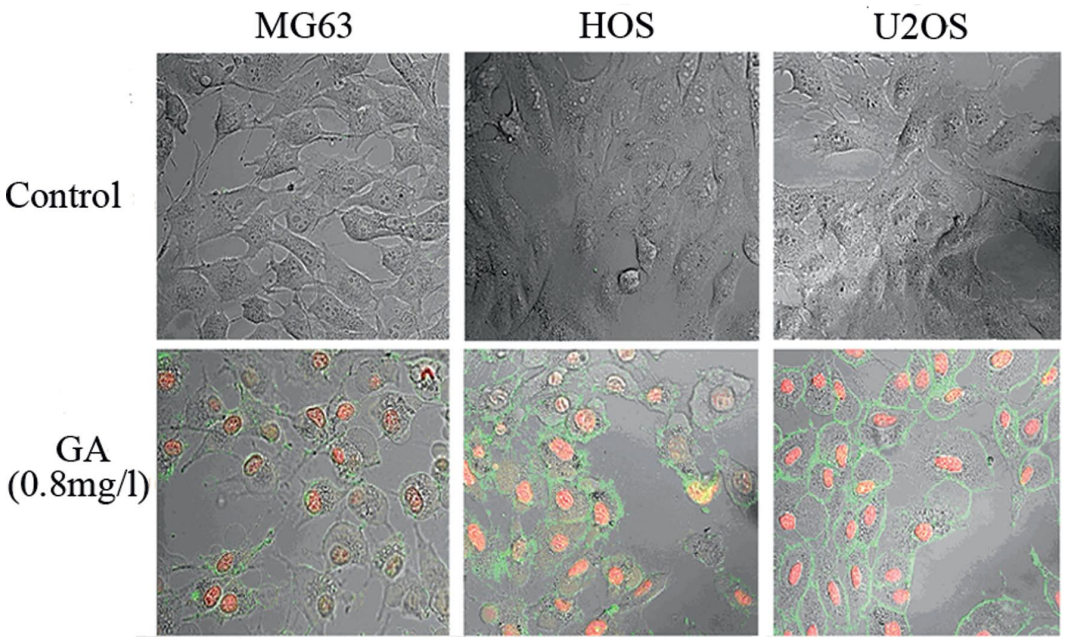

Figure 4. Apoptotic cells after staining with Annexin-V-FITC and propidium iodide were observed by confocal laser microscope. Apoptotic cells with red nuclear and blue membrane after staining with Annexin-V-FITC and propidium iodide can be seen in GA (0.8 mg/l) groups. There were no apoptotic cells in control groups.

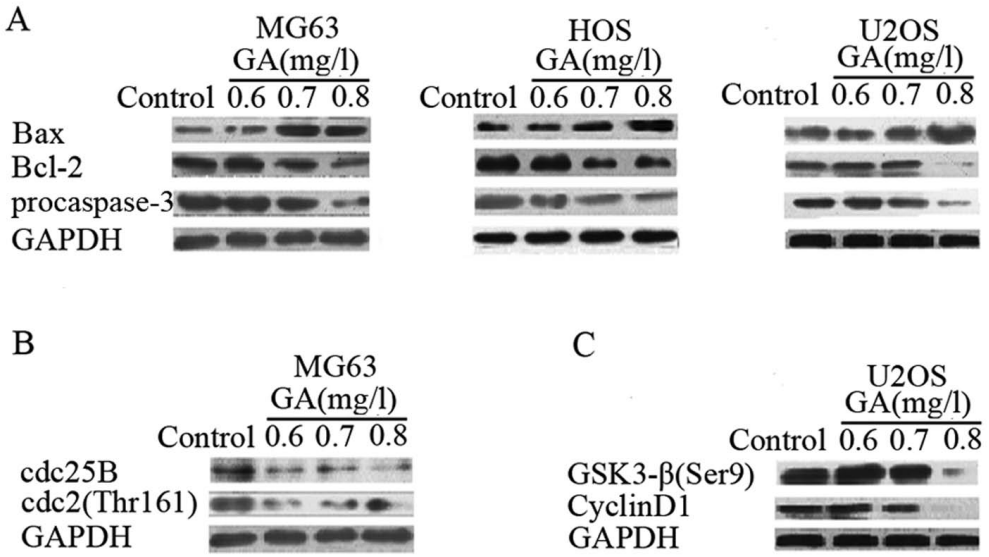

Figure 5. The expression changes of genes associated with the regulation of cell apoptosis and cell cycle. (A) Compared with the control group, the expression of Bax, Bcl-2 and procaspase-3 proteins changed significantly in the GA-treated groups. The expression of Bax was increased, while the expression of Bcl-2 and procaspase-3 was reduced. (B) After 24 h of treatment with various concentration of GA, cdc25B and Thr161 phosphorylated cdc2 were decreased significantly in MG63 cells compared to the control cells. (C) After 24 h of treatment with various concentration of GA, Ser9 phosphorylated GSK3-ß and cyclin D1 were decreased significantly in U2OS cells compared to the control cells.

the expression of several proteins involved in the regulation of the cell cycle and apoptosis after the three cell lines were treated with various concentrations of GA (0.6, 0.7 and 0.8 mg/l) for 24 h. Our results demonstrated that Bcl-2 was down-regulated, while Bax and the active form of procaspase-3 was increased in all three cell lines (Fig. 5A). The G2/M regulatory proteins, cdc25B and Thr161 phosphorylated cdc2 were significantly decreased in MG63 cells (Fig. 5B), while the G0/G1 regulatory proteins, Ser9 phosphorylated GSK3-ß and cyclin D1 were significantly decreased in U2OS cells (Fig. 5C).

Discussion

In response to cancer therapies, apoptosis is one of the major mechanisms of cell death (20). There are three main pathways regulating apoptosis: endoplasmic reticulum pathway, mitochondrial pathway and death receptor pathway. Members of the Bcl-2 family of proteins are critical regulators of the mitochondrial apoptotic pathway that are not regulated by the extrinsic pathway or the cell surface receptor pathway (21-23). The Bcl-2 gene encodes an integral outer mitochondrial

membrane protein that blocks the apoptotic death of some cells, such as lymphocytes. Bax (Bcl-2 antagonist X) was identified by the Korsmeyer group as a Bcl-2-interacting protein that opposed Bcl-2 and promoted apoptotic cell death (24). As an adaptor molecule of the Bcl-2 family, Bax is considered to be a downstream mediator of the mitochondrial apoptotic pathway because its absence abolishes most apoptotic responses (25). When activated, Bax translocates to the outer mitochondrial membrane where it oligomerizes and permeabilizes the outer mitochondrial membrane, consequently freeing pro-apoptogenic factors, such as cytochrome-c, which promote the activation of proteases (caspases). Both Bcl-2 and Bax regulate apoptosis, and when the expression of Bax/Bcl-2 increases, cell death occurs (24).

In the present study, we found that GA can induce apoptosis by increasing the expression of Bax/Bcl-2 in MG63, HOS and U2OS cells. This result is consistent with previous findings that GA induces apoptosis in some other tumor cells by up-regulating the Bax/Bcl-2 ratio (9,10,26,27).

Previous work demonstrated that GA inhibits the proliferation of tumor cells by mediating cell cycle arrest. However, GA is not a phase-specific inhibitor of the cell cycle because it inhibits different transition phases of the cell cycle in different tumor cells. For example, GA induces G0/G1 cell cycle arrest in K562 leukemia cells (28) as well as G2/M cell cycle arrest in gastric carcinoma BGC-823 (29) and breast carcinoma MCF-7 cells (12). In our study, we found that GA also displayed a non-phase-specific cycle arrest. We found that GA inhibited G2/M and G0/G1 in MG63 and U2OS cells, respectively, but it had no effect on HOS cells. Thus, GA is a highly cell specific cycle inhibitor, that was no reported before.

The cell cycle is regulated through the sequential activation and inactivation of cyclin-dependent protein kinases (CDK) that control cell cycle progression, such as the transition from G1 to S and G2 to M. CDK activation requires binding to different cyclins, such as cyclin A, cyclin B, cyclin D and cyclin E, which are expressed throughout the cell cycle. Cyclin D1 is one of the two cyclins that are necessary and rate-limiting for the passage of cells through the G1 phase of the cell cycle. Cyclin D1 levels are low in quiescent (G0) cells, rise throughout G1, and remain elevated for the remainder of the cell cycle. The cytoplasmic serine/threonine protein kinase GSK3 (glycogen synthase kinase 3) was first described in a metabolic pathway for glycogen synthase regulation that is sensitive to insulin-mediated inhibition (30). Several studies report that the ubiquitin-dependent degradation of cyclin D1 requires phosphorylation of a specific threonine residue (Thr-286) located near the protein's carboxyl terminus. This phosphorylation is mediated by GSK-3ß and links processes governing cyclin D1 subcellular localization with its proteasomal degradation (31). In this way, GSK-3ß affects the formation of cyclin D1/CDK complexes, which reduces the subsequent phosphorylation of pRb (32) and results in G1 arrest. However, the activity of GSK3-ß is dependent on the phosphorylation of Ser9 (33). In this study, we found that cyclin D1 and the Ser9-phosphorylated GSK3-ß was decreased after GA treatment for 24 h in U2OS cells. Because we observed G1 arrest in these cells, the findings suggest that GA induced G1 arrest in U2OS cells by inhibiting the phosphorylation at GSK3-ß Ser9, resulting in Thr-286 phosphorylation of cyclin D1 and the degradation of cyclin D1.

Cdc25 phosphatases, such as cdc25A, B and C, are a novel family of CDK phosphatases, which activate CDK/cyclin complexes by dephosphorylating CDKs at different checkpoints of the cell cycle (34,35). Cdc25A is expressed during the late G1 and controls the G1/S transition by activating the CDK2/cyclin E complex (36), while cdc25B and C are essential for the G2 and G2/M transition (37,38). Lammer and co-workers found that the ablation of cdc25B by antibody microinjection blocked cell cycle progression by inhibiting entry into mitosis (38). Furthermore, Karlsson showed that overexpression of cdc25B was more potent than cdc25C in inducing premature entry into mitosis (39). The ultimate target of G2 is the mitotic regulator cyclin B/cdc2, and the Thr161 dephosphorylation of cdc2 was required for a sustained G2 arrest after the activation of the DNA damage checkpoint. In the present study, we demonstrated that GA down-regulates the expression of cdc25B and inactive Thr161 phosphorylated cdc2 in MG63 cells, resulting in G2/M arrest.

In conclusion, our study demonstrated that GA suppresses the proliferation and promotes the apoptosis of osteosarcoma cells by altering the expression of genes that are associated with the regulation of cell cycle and apoptosis, suggesting that GA may represent a novel and promising therapeutic drug for the treatment of osteosarcoma.

Acknowledgments

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