Chinese Journal of Physiology 58(2): 95-103, 2015
DOI: 10.4077/CJP.2015.BAC267

Induction of Survivin Inhibition, G_2/M Cell Cycle Arrest and Autophagic on Cell Death in Human Malignant Glioblastoma Cells

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Abstract

Chemotherapy efficacy is limited by intrinsic and acquired resistance in glioblastoma (GBM); hence, novel tactics are crucial. Survivin has been demonstrated as a key resistant factor in GBM because of its function in inhibiting apoptosis, regulating autophagy, and in promoting G_2/M cell cycle transition. Parthenolide has been reported to be an effective antitumor agent in a variety of tumor cells and decreases survivin level in leukemia cells. But the effect of parthenolide on survivin and the cell death process in GBM is still unknown. The aim of this study was to examine whether parthenolide had the potential to inhibit cell proliferation in the GBM cell line U373. The parthenolide-induced effects in relation to survivin suppression and cell death were further investigated. Our results showed that parthenolide substantially inhibited cell viability with IC_{50} values of approximate 16 μM. Treatment with parthenolide at the dose of 16 μM led to considerable downregulation of survivin, G_2/M cell cycle arrest and Chk2 upregulation in cells. Parthenolide induced apoptosis in only a few cells and a slight increase in activated caspases 3 levels. By contrast, parthenolide induced a significant increase of intracellular autophagosomes and the expression of autophagy related proteins, including ULK1 and LC3 I/LC3 II, in the treated cells. These results suggested that parthenolide induced survivin inhibition, G_2/M cell cycle arrest, and triggered cell death through autophagic cell death in the GBM cell line.

Key Words: Autophagic cell death, Glioblastoma, Parthenolide, Survivin

Introduction

Malignant glioblastoma (GBM) accounts for more than 50% of all primary brain tumors and typically results in poor prognosis and survival in adults. The current strategy for the treatment of GBM is a combination of chemotherapy, surgical palliative resection and focal radiotherapy. Nevertheless, GBM often exhibits a high resistance to therapy. Although molecularly targeted agents hold promise for more effective therapy compared to conventional agents, such as approach still fails to offer long-term survival benefits for patients (39, 45). Therefore, it is crucial to develop alternative agents for GBM.

Prior studies have shown that therapeutic failure in GBM is closely associated with upregulation of survivin. Survivin is an inhibitor of the apoptotic protein (IAP) that plays an important role in apoptosis inhibition and promotion of the G_2/M phase cell cycle transition that is essential for cancer progression (6, 35, 49, 59). Survivin is highly expressed in GMB cells in comparison with normal brain cells and it is related to GBM patients with poor prognosis and apoptosis resistance (8, 19). Inhibition of survivin activity is considered as a crucial strategy in GBM treatment (21). Several therapeutic approaches

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Received: December 19, 2013; Revised: May 8, 2014; Accepted: June 5, 2014.
 ISSN : 0304-4920. http://www.cps.org.tw

for targeting survivin using immunotherapy or small-molecule antagonists, either as single agent or in combination with conventional chemotherapeutic agents, are currently being applied in clinical trial (14). Developing more alternative agents in modulating the action of survivin is required for GBM prevention or treatment.

Natural products, which are rich sources of chemotherapeutic compounds with diverse bioactivities, such as qauercetin (10), Huaier extract (24) and parthenolide (15, 18, 20, 44), have been extensively explored in both the fields of cancer treatment and prevention. In this study, we investigated parthenolide, a sesquiterpene lactone, which is identified to be the main bioactive component of feverfew (Tanacetum parthenium) leave. Parthenolide is known to cure migraines, arthritis, fever and stomach aches after ingested orally, or as an infusion extract. Increased evidence has shown antitumor activities of parthenolide in various cancer cell lines (15, 18, 20, 44). The most classical activity of parthenolide has been identified as a pharmacological NFκB inhibitor to normal astrocytes with low toxicity, but with selective cytotoxicity to GBM cells (57). Other important mechanisms of parthenolide action include DNA synthesis inhibition (58), mitochondrial disorder induction, G_2/M cell cycle arrest (44, 53) and sustained activation of c-Jun N-terminal kinase (JNK) (42). In addition, alternation in Ca^{2+} homeostasis is also involved in the bioactivity of parthenolide (47) to induce apoptosis through multiple pathways (12, 13, 22, 37, 50). Likewise, it has also been reported that parthenolide induced downregulation of a variety of antiapoptotic molecules, such as Bcl-xL, Mcl-1 and survivin in leukemia cells (31). Though parthenolide exhibits significant antitumor activities in many cancer cell lines, the effects of parthenolide on survivin inhibition and the cell death process on GBM are still controversial. In this study, we undertook to determine the effects of parthenolide in survivin inhibition, cell cycle arrest and cell death type in malignant GBM cell line.

Materials and Methods

Cell Culture

The human GBM cell line-U373 used has been characterized with a p53 mutation and high apoptosis resistance to chemotherapeutic agents (25). U373 cells were cultured in the Dulbecco Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing antibiotics and 10% fetal bovine serum (Invitrogen) at 37°C in a humidified atmosphere containing 5% CO_2.

Cell Viability Assay

Cell viability was determined by the mitochondrial reduction assay (MTT, 3-(4, 5-dimenthylthiazol-2-yl)-2, 5-dimethyl-tetrazolium bromide), as described previously (33). Before the cell viability assay, U373 cells were grown to subconfluent levels in DMEM with 10% FBS and then plated onto 24-well culture plates (5×10^4 cells/well) in the a final volume of 1 ml of culture medium. After 24 h of incubation, culture medium was replaced by 1 ml serum-free DMEM and then various concentrations (0-20 μM) of parthenolide were added. The cell plates were incubated for 24 h, after which cells were incubated for 2 h at 37°C with MTT at 5 mg/ml in PBS. The quantity of the blue formazan product formed was measured by absorbance at wave length of 570 nm. All treated groups were performed and compared with the control group of untreated cells. Cell viability was calculated as follows:

Cell viability (%)

= (Absorbance value of treated group /

Absorbance value of control group) × 100%.

Growth Inhibition Measurement

In vitro growth effects of parthenolide on U373 cells were determined by counting viable cells with trypan blue staining (Sigma). The cells (5×10^3 cells/well) were seeded in a 96-well plate overnight and subsequently exposed to various concentrations of parthenolide (0, 8 or 16 μM) for 0~4 day. U373 cells treated with parthenolide were trypsinized, and trypan blue staining was used to count the viable cell numbers every day.

Cell Cycle Analysis

U373 cells (1×10^6 cells) were treated with culture medium containing parthenolide at 0 or 16 μM at 37°C for 24 h, and the cells were trypsinized and washed once with ice-cold phosphate-buffered saline solution (PBS). All collected cells were fixed with 70% ethanol at 4°C for 2 h. Subsequently, the fixed U373 cells were washed with PBS, suspended in 1 ml staining reagent containing 50 mg/ml propidium iodide, PI (Sigma) and 100 μg/ml RNase, and incubated for 20 min at room temperature in the dark. The percentage of cells in the G_1, S and G_2/M phase of the cell cycle was determined using flow cytometry (FACScan, Becton Dickinson, USA) and analyzed using Cell Quest software.

Determination of Apoptotic Cells

U373 cells (1×10^6 cells) were treated with culture

medium containing parthenolide (0, 8 and 16 μM) at 37°C for 0-24 h. After incubation, collected cells were trypsinized, washed with PBS and centrifuged at 1,500 rpm for 5 min. Resuspended cell pellets were stained with Annexin V-FITC, PI and binding buffer provided by the annexin V staining kit (Roche, Indianapolis, USA) at room temperature for 15 min in the dark. Cells stained with annexin V-FITC alone or with annexin V-FITC in the presence of PI were indicated as early and late apoptotic cells, respectively. Detection and analysis of apoptotic cells were conducted by using FACScan (Becton Dickinson) flow cytometry.

Western Blotting

Protein levels at 50 μg amounts were quantified and resolved on 10% or 12% polyacrylamide gels and subsequently transferred to PVDF membranes (Millipore, Bedford, MA, USA) by electroblotting. The membranes were blotted with anti-caspase 3 (Santa Cruz, CA, USA), anti-ULK1 (Santa Cruz), anti-p-Chk2 (Santa Cruz), anti-p-Cdc2 (Santa Cruz), anti-LC3 I/LC3 II (Abgent, CA, USA), and anti-β actin (Novus Biologicals, CO, USA) antibodies followed by horseradish peroxidase-conjugated secondary antibodies (Santa Cruz). The blots were visualized using an enhanced chemiluminescence kit (Amersham Biosciences, Norway). Densitometric analyses were used to quantify protein levels. The intensity of each protein band was normalized to the intensity of the β-actin band.

Statistical Analysis

Data were expressed as mean ± SEM and processed using analysis of variance (ANOVA) tests. Intergroup comparisons were made using the Duncan test. Differences were considered significant when $P < 0.05$. The statistical analyses were performed by SPSS (version 12.0).

Results

Parthenolide Inhibited U373 Cell Proliferation and Growth

U373 cells were treated with different concentrations of parthenolide (0-20 μM) for 24 h, and cell survival was evaluated by MTT assays. Parthenolide inhibited the proliferation of U373 cells in a dose-dependent manner after treatment for 24 h, and the IC_{50} values was below 16 μM for the parthenolide-treated groups (Fig. 1A). We subsequently assessed the effects of parthenolide (0, 8 and 16 μM) in growth inhibition for 4 days. The results showed that the group treated with 8 μM parthenolide did not cause

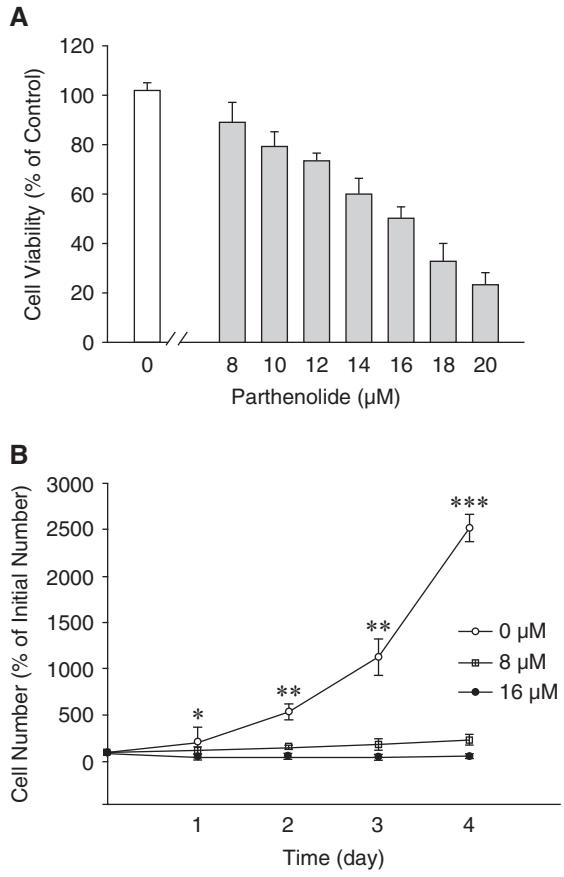

Fig. 1. Parthenolide could inhibit U373 cell viability and growth. (A) Cells were treated with parthenolide (0-20 μM) for 24 h, and cell viability was subsequently measured using MTT assay. (B) Cells were treated with 0, 8 and 16 μM parthenolide from 0 to 4 days, and the cell numbers were counted every day. Values are expressed as mean ± SEM (n = 3). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

cell death. Instead, 8 μM parthenolide inhibited cell growth. In the cells exposed to 8 μM parthenolide for 4 days, the cell numbers were less than that in the control group. In contrast, the number of viable cells of the 16 μM parthenolide-treated group decreased below initial level (Fig. 1B). These results suggested that parthenolide, at 16 μM concentration, had significant inhibitory effects on U373 cell viability.

Parthenolide Induced Survivin Inhibition in U373 Cells

Survivin is related to drug resistance in most cancers, including GBM (2, 3, 21). Since parthenolide had significant inhibitory effect in GBM U373 cells at 16 μM, we next examined the effect of parthenolide on survivin. Survivin activation can be enhanced by activating Cdc2, but is suppressed by activating Chk2. Thus, we measured the expression levels of survivin, activating Cdc2 and activating Chk2 of the

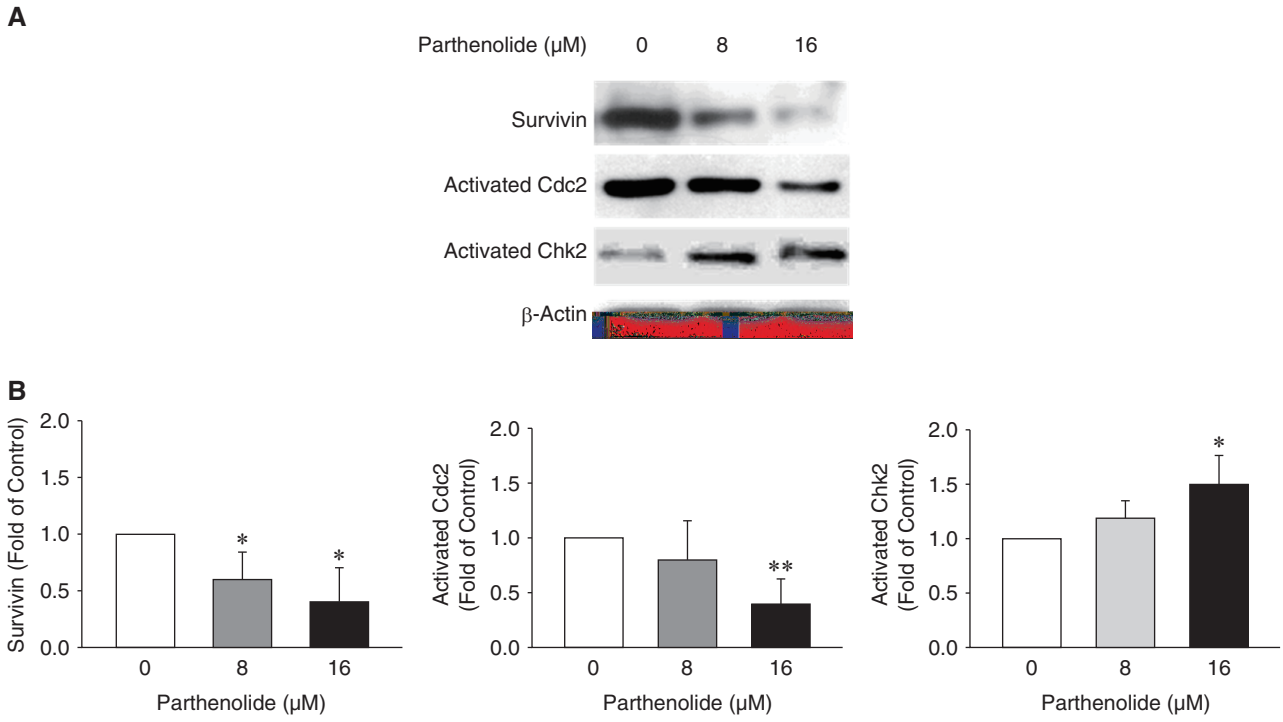

Fig. 2. Parthenolide induced survivin inhibition in U373 cells. (A) After U373 cells were treated with parthenolide (0, 8, 16 μM) for 24 h, the expression levels of survivin, activated Cdc2 (phosphorylated at Thr^{161}), and Chk2 (phosphorylated at Thr^{68}) of the cells were determined using western blotting using β-actin as an internal control. (B) Quantity of survivin activated Cdc2 and activated Chk2 expression as compared with the control group. Values are expressed as mean ± SEM (n = 3). $*P < 0.05$, $**P < 0.01$, as compared with the control group.

treated U373 cells after various doses of parthenolide (0, 8, and 16 μM) for 24 h by western blotting. The survivin level and the activating Cdc2 level of treated-cells were substantially downregulated (Figs. 2A and 2B). By contrast, parthenolide increased the activating Chk2 level of the cells. These results suggested that parthenolide exerted considerable effects in survivin inhibition.

Parthenolide Induced Cell Cycle Arrest of the G_2/M Phase in U373 Cells

Survivin and activating Cdc2 enhances G_2/M cell cycle progression, but activated Chk2 causes G_2/M phase of the cell cycle arrest (23). To further explore whether the effect of parthenolide on cell cycle was consistent with survivin and Cdc2 inhibition, cell cycle analysis was performed on the tested cells. As shown in Fig. 3A, numerous cells exhibited a shrinking/floating morphology and few cells adhered to the culture plate after parthenolide treatment at the dose of 16 μM for 24 h. According to cell cycle analysis, data showed that the percentage of G_2/M cells was 48 ± 5.7%, and was significantly increased in the shrinking/floating population. But the fraction of G_2/M cells of the adhered cells was 21 ± 5.4%, which was similar to the G_2/M cell percentage of the control group at 23 ± 3.2% (Fig. 3B).

Parthenolide Induced Cell Death in U373 Cells Regardless of Apoptosis

Besides the effect of parthenolide on the inhibition of cell viability and on cell cycle arrest, we further investigated whether parthenolide was capable of inhibiting U373 cell growth by inducing apoptotic death. U373 cells were treated with or without 16 μM parthenolide for 0-24 h and the apoptosis indices, including Annexin V detection and caspase 3 levels, were determined. The apoptotic cell percentage quantified using flow cytometry analysis showed that the parthenolide-treated group for 8 h displayed less than 5% apoptotic cells (Fig. 4A). The apoptotic cell percentages at 16 h and 24 h of the treated group also increased slightly and were counted as 9.7 ± 2.5% and 10.6 ± 3.8%, respectively. Another apoptosis index, the activating caspase 3 level, examined by Western blotting, was also increased insignificantly after parthenolide treatment (Fig. 4, B and C). These results indicated that apoptosis might not play an important role in parthenolide-induced cell death of U373 cells.

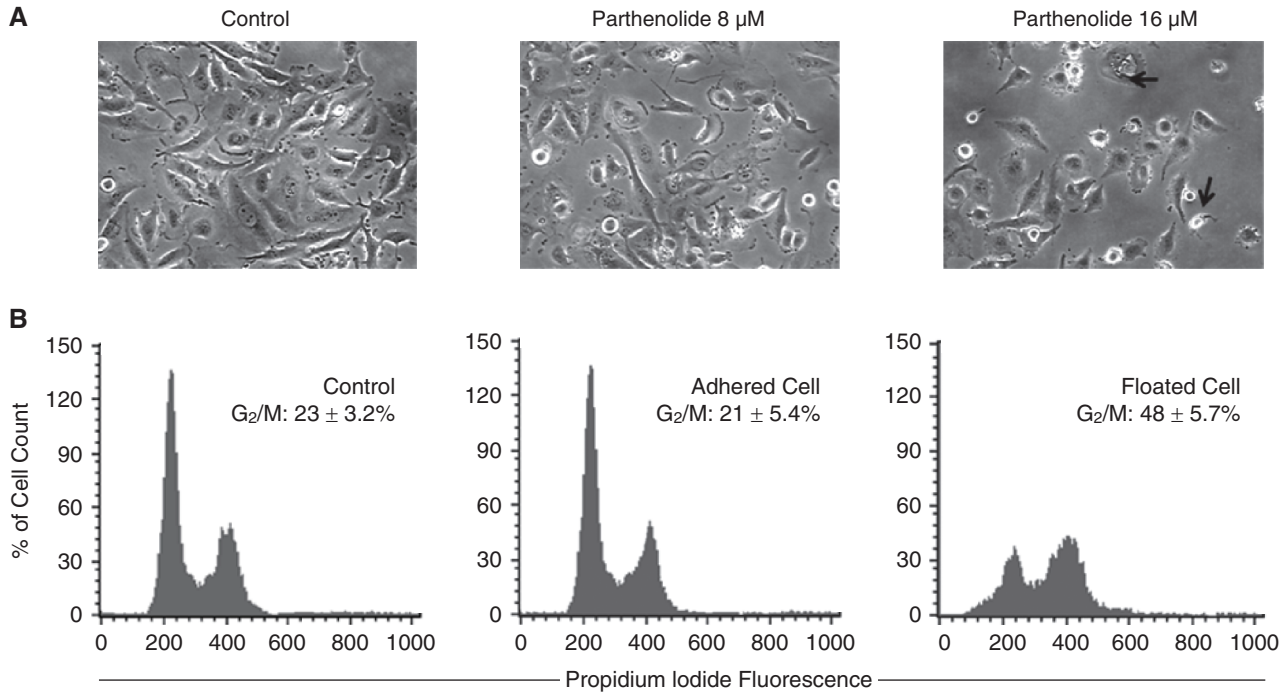

Fig. 3. Parthenolide induced U373 cells to arrest at G_2/M phase of the cell cycle. (A) Cell morphology obtained by microscopy (100×) with or without parthenolide treatment. Shrinking/floating cells observed in the 16 μM parthenolide treated-groups are indicated by arrows (→). (B) After parthenolide treatment, the shrinking/floating cells and adhered cells were collected respectively to determine cell cycle distribution by flow cytometry.

Parthenolide Induced Autophagic Cell Death in U373 Cells

Autophagy is usually considered as a cell survival and protection mechanism to limit the therapeutic efficacy of chemotherapeutic agents in apoptosis-resistant cells (26, 27). However, studies have reported that excessive and sustained autophagy by various anticancer therapies can eventually induce cell death in many cancer types (4, 17), which suggests that autophagy may not only act as a guardian but also an executor. Since caspase-dependent cell death (apoptotic death, type I cell death) was not a distinct feature of parthenolide-treated cells, we further investigated whether the cytotoxic effect of parthenolide was related to autophagic cell death (type II cell death). Extensive vacuolation in cytoplasm is a typical feature of autophagic cell death (29). Thus we first examined the amount of cytoplasm vacuole by microscopic images and found that cytoplasmic vacuoles were frequently observed in parthenolide treated-groups (Fig. 5 A).

UNC51-like kinase 1 (ULK1), a mammalian serine/threonine protein kinase, plays an essential role in autophagy initiation. ULK1 activation recruits a series of signals to trigger the autophagosome formation process (9). The microtubule-associated protein light chain 3 (LC3) is a downstream signal of ULK1, which is commonly used as a biochemical marker of autophagosomal formation (5). LC3 can be detected as two distinct bands by Western blotting analysis, recognized as LC3 I/LC3 II. LC3 II is cleaved from LC3 I, which represented an index of autophagosomal formation (40). To further identify the involvement of autophagy in parthenolide-mediated cell death, ULK1 and LC3 I/LC3 II expression levels derived from U373 cells were analyzed. The expression levels of ULK1 and LC3 I/LC3 II increased significantly in 16 μM parthenolide-treated group (Fig. 5). These results suggested that parthenolide was an inducer of autophagic cell death in U373 cells.

Discussion

Ongoing studies have developed new therapeutic agents such as temozolomide for GBM treatment. However, clinical benefits of these therapeutic agents are still insufficient because aberrant signaling networks tend to cause GBM cells to be resistant to apoptosis (7, 32). Survivin is expressed in low levels and is tightly regulated by serial signalling cascades in normal cells (11, 19, 46). However, survivin is highly expressed in cancers, including GBM, and is a pivot molecule of therapeutic resistance. Mounting evidences are reporting that inhibition of survivin expression can sensitize GBM cells to cell death induced

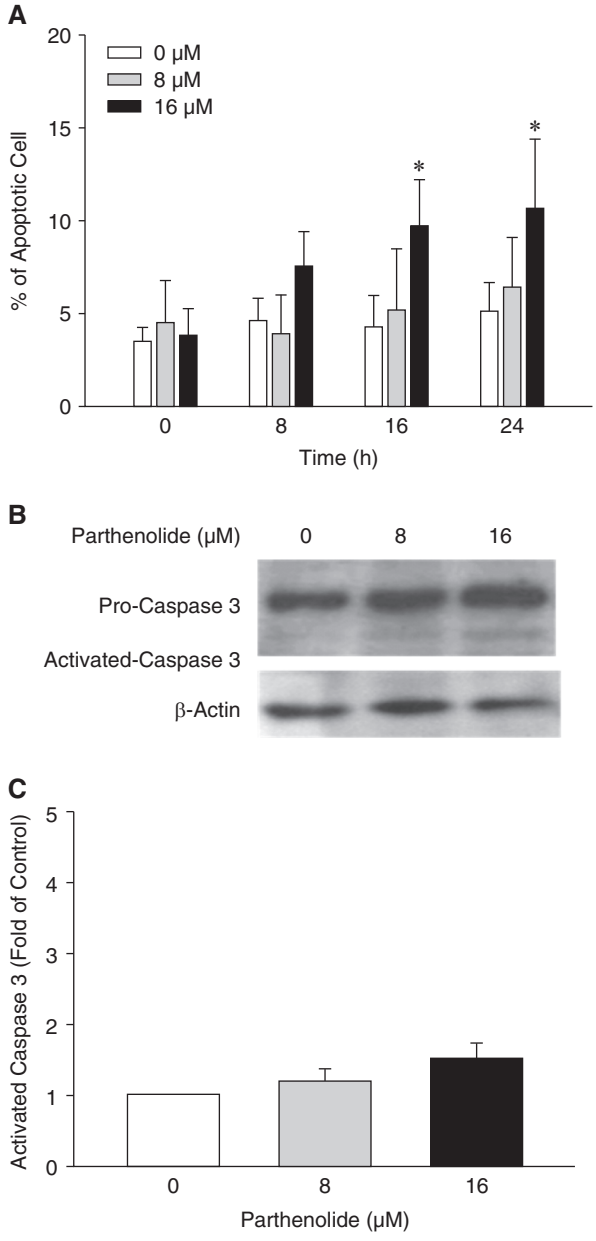

Fig. 4. Parthenolide induced indistinct apoptosis in U373 cells. (A) Annexin V staining kit was used to detect apoptosis in U373 cells induced by parthenolide treatment. Values are expressed as mean ± SEM (n = 3). *$P < 0.05$ indicates the apoptosis percentage of the control group compared to the 16 μM parthenolide treated-group. (B) Western blot quantification of expression levels of activating caspase 3 determined in 24 h parthenolide-treated groups. (C) Insignificant quantity of activating capase 3 levels in parthenolide-treated groups as compared with the control group. Values are expressed as mean ± SEM (n = 3).

by radiotherapy (46). The GBM cell line U373, characterized with mutations in several genes, are highly resistant to apoptosis (51). In the present study, we speculated that parthenolide decreased survivin expression significantly in apoptosis resistant-U373 cells (Fig. 2; $P < 0.05$). Thus parthenolide might be an responsible agent for survivin inhibition in GBM cells.

Survivin is degraded rapidly in cytoplasm, but it could be stabilized by the Cdc2/cyclin B1 complex (34, 43). Cdc25 is necessary for Cdc2 to associate with cyclin B1, which can be impeded by phosphorylated Chk2 (52). Phosphorylated Chk2 has effects in the phosphorylation process of p53 and Cdc25 to arrest cell cycle. Phosphorylated p53 causes cell cycle arrest at the G_1/S phase, and phosphorylated Cdc25 results in cell cycle arrest at both the S and G_2/M phase (30). In this regard, we found that the effect of parthenolide in downregulating survivin coincided with Cdc2 downregulation and the upregulation of phosphorylation Chk2. Survivin also acts as an upstream signal on G_2/M transition and cellular proliferation (6). In this work, we also observed the effects of parthenolide involving G_2/M cell cycle arrest in the tested cells. The effects of survivin alteration and cell cycle arrest of parthenolide-induced effects may, thus, provide potentials to overcome drug resistance in GBM cells.

Several reports have suggested that parthenolide is a promising agent that can be used alone or in combination with therapeutic agents by inducing apoptosis (44), and the drug is tolerated without considerable side effects in patients with cancer (15, 53). When the apoptosis induction of parthenolide was examined, the results showed that only a minor fraction of apoptotic cells could be detected in the treated groups. Moreover, the expression level of activating caspase 3 also increased insignificantly. Therefore, the results indicated that other mechanism(s) might be involved in parthenolide-induced cell death.

GBM is known to be resistant to therapies that induce apoptosis (32, 59). However, recent evidences indicate that GBM cells seem to be less resistant to therapies by autophagy induction (35). Autophagy is classically related to tumorigenesis and tumor progression, but increasing evidences show that autophagy is also involved in tumor suppression and sensitized apoptosis resistant-cell to death under certain conditions (48, 54). Autophagy regulation is complex, because it enhances cancer cell survival in response to stress, and it also suppresses the initiation of cell growth (48). In this regard, there are increasing interests in agents with pharmacological effects by autophagy induction (26, 27, 38). In this study, most cells exposed to parthenolide could be observed with a lot of cytoplasmic vacuoles by microscopy (Fig. 5). The ULK1 level, which is closely related to autophagic capacity and initiation (9), was significantly increased in U373 cells after parthenolide treatment. Subsequently, autophagosome formation index indicated by LC3I/LC3II (40) could be upregulated

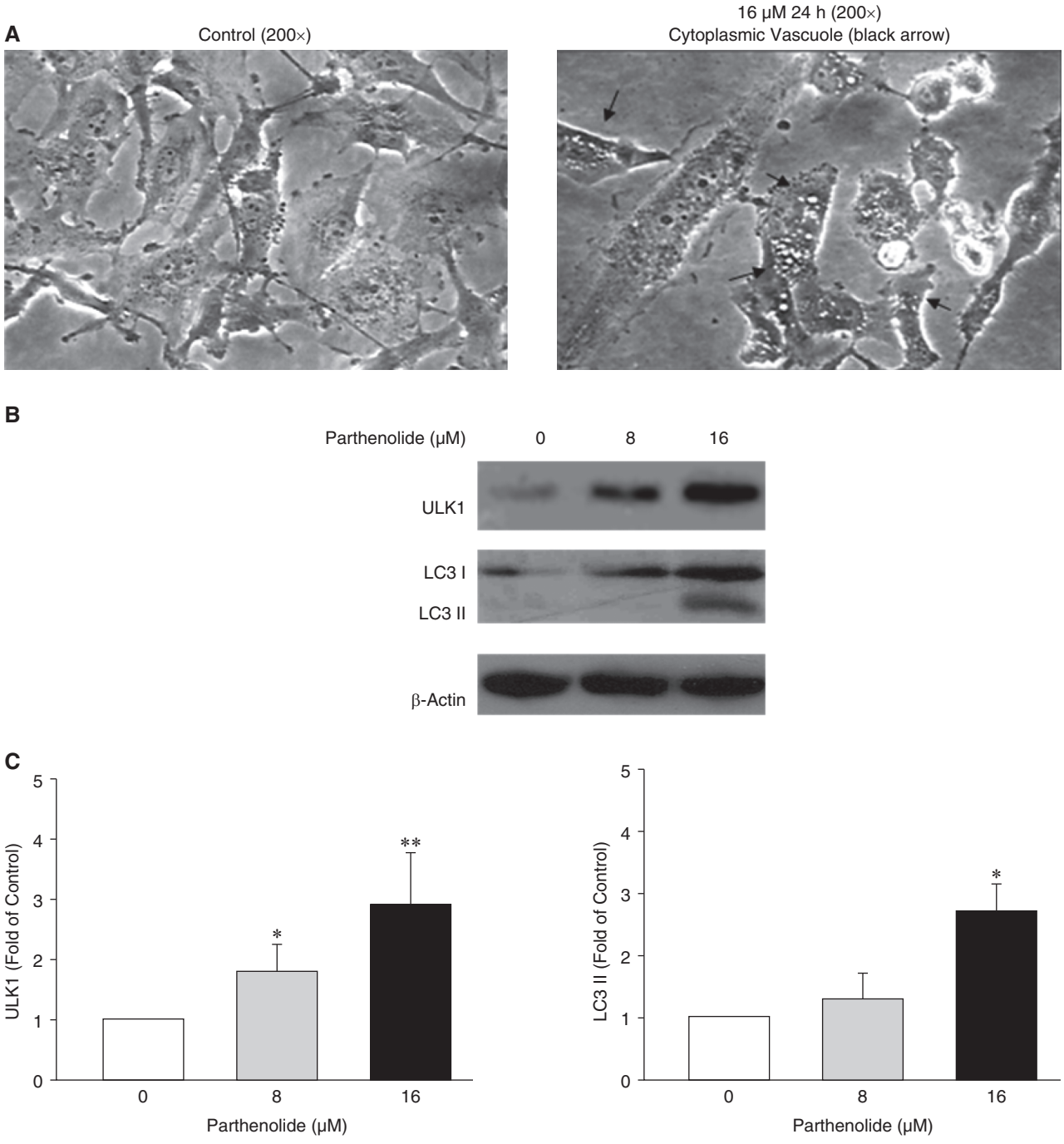

Fig. 5. Autophagic cell death was involved in parthenolide-induced U373 cell death. (A) Morphology (200×) of U373 cells with or without 16 μM parthenolide treatment for 24 h. Cytoplasmic vacuoles in U373 cells was labeled by arrows (→). (B) After treatment with parthenolide (0, 8 and 16 μM) for 24 h, expression of the autophagy index ULK1, and LC3 I/LC3 II was measured using Western blotting. (C) Quantity of ULK1 and LC3 II expression as compared with the control group. Values are expressed as mean ± SEM (n = 3). *$P < 0.05$, **$P < 0.01$, as compared with the control group.

after exposure to parthenolide. Thus, the data suggest that parthenolide stimulates the progression of autophagic cell death in U373 cells.

Autophagy initiation is closely linked to mTOR, the mammalian target of rapamycin. mTOR has a pleiotropic function in cell growth, and it can be activated by the protein kinase B, Akt (4). Inhibition of mTOR may trigger autophagy process in apoptosis-resistant cancer cells through blockage of the Akt/mTOR pathway (4). Akt is similar to survivin in being highly expressed in GBM cells, and in its correlation with invasive activities of GBM (8). Several studies have

discussed the possibility that interactions between autophagy and simultaneous inhibition of Akt and survivin can enhance GBM autophagic cell death (1). The effects of parthenolide in U373 cells included both survivin inhibition and autophagic cell death, which provided a connection between parthenolide and Akt signalling. The role of interaction of parthenolide-induced survivin inhibition with autophagic cell death and the Akt/mTOR pathway will be clarified in our future work.

NFκB, an inflammatory-associated transcription factor, is similar to survivin, which is also constitutively activated or aberrantly expressed in GBM cell lines or solid tumors compared to normal astrocytes. (57). A number of strategies have been employed to augment therapeutic sensitivity in cancers through inhibiting NFκB and survivin simultaneously (55). Likewise, targeting at Akt and NFκB is considered a promising strategy on GBM prevention or therapy (16, 56). Thus, it may be proposed that agents with inhibitory effects on survivin, Akt and NFκB are more beneficial to improve GBM treatment or prevention. Parthenolide, a sesquiterpene lactone compound, is considered as an NFκB inhibitor by targeting a component of the IκB kinase complex in immune response (20). Ongoing investigations conducted by other groups have observed that sesquiterpene lactone-helenalin triggers autophagic cell death in A2780, RKO and MCF-7 cell lines through NFκB inhibition (36). In addition, it has been demonstrated that parthenolide has antitumor activity by dislodging the Akt/NFκB pathway in GBM cells (41). Thus, by taking together our results and current evidences in the literature, it may be deduced that parthenolide-induced effects in GBM cells may have cooperative relationship in survivin inhibition, NFκB inhibition, Akt inactivation and autophagic cell death.

These results suggested that parthenolide exhibited significant *in vitro* antitumor efficacy of GBM.

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