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Triptolide induces apoptosis through extrinsic and intrinsic pathways in human osteosarcoma U2OS cells

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Triptolide, a diterpene derived from ***Tripterygium wilfordii*** Hook f., a Chinese medicinal herb, has been reported to inhibit cell proliferation and induce apoptosis in various human cancer cells, but its anticancer effects on human osteosarcoma cells have not yet been elucidated. In this study, we investigated whether triptolide induces apoptosis in human osteosarcoma cells and the underlying molecular mechanisms. We firstly demonstrated that triptolide inhibited cell growth and induced apoptosis in U2OS cells. Western blot analysis showed that the levels of procaspase-8, -9, Bcl-2, Bid and mitochondrial cytochrome ***c*** were downregulated in triptolide-treated U2OS cells, whereas the levels of Fas, FasL, Bax, cytosolic cytochrome ***c***, cleaved caspase-3 and cleaved PARP were upregulated. These results suggest that triptolide induces apoptosis in U2OS cells by activating both death receptor and mitochondrial apoptotic pathways.

Keywords: Triptolide, U2OS cell, Apoptosis

Osteosarcoma is the most current malignant bone tumor that occurs mainly in childhood and adolescence and the 5-yr survival rate of osteosarcoma patients is 61.6%[1]. Despite the advances of adjuvant chemotherapy and substantial improvement of survival rate, the prognosis for patients with osteosarcoma is still poor, because of recurrent metastasis and the induction of drug-resistant[2]. Thus, it is important to explore more effective chemotherapeutic agents for treating aggressive osteosarcoma. Moreover, chemotherapeutic agents currently used for cancer patients possess severe toxicity and significant side effects of chemotherapy[3]. As a new source of chemotherapy, natural compounds and their derivatives exerting their antitumor effect against cancer cells by inducing apoptosis have gradually gained considerable attention in order to reduce chemotherapy-associated side effects[4].

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Abbreviations: DAPI, 4', 6-diamidino-2-phenylindole; FADD, Fas-associated protein with death domain; FasL, Fas ligand; FCM, flow cytometry; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; PARP, poly (ADP-ribose) polymerase; PBS, phosphate-buffered saline; PI, propidium iodide;.

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It is well-known that apoptosis contributes to the anticancer activity of many chemotherapeutic drugs[5,6]. In cancer therapies especially, apoptosis is a crucial factor that affects sensitivity to chemotherapeutic agents. Most cytotoxic agents used in cancer chemotherapy induce apoptosis and inhibition of cell cycle progression[6].

Very recently, several natural products including columbamine[1], gambogic acid[7], bufalin[8], quercetin[9], oridonin[10], matrine[11], apigenin[12] and kaempferol[13] have been reported to inhibit cell proliferation and induce apoptotic cell death in human osteosarcoma cells. These studies have indicated that they inhibit proliferation and induce apoptosis through cell cycle arrest and the mitochondrial (intrinsic) and/or the death receptor-mediated (extrinsic) pathways.

Triptolide, a diterpene triepoxide and the major active component of the Chinese medicinal herb ***Tripterygium wilfordii*** Hook f., has been used for treatment of autoimmune and inflammatory diseases[14]. In recent years, numerous studies have demonstrated that triptolide could induce cell growth inhibition and apoptosis in various human tumor cell types, including colon cancer[15], glioblastoma[16], pancreatic cancer[17], myeloma[18], leukemia[19], thyroid carcinoma[20], adrenal cancer[21] and gastric cancer cells[22].

Although the anticancer effects of triptolide have been studied in various human cancer cells[15-22],

its effects on human osteosarcoma cells have not yet been reported. Therefore, in this study, we have investigated anticancer effects of triptolide on human osteosarcoma cells and have attempted to elucidate the mechanism of its action. We have firstly shown that triptolide-induced apoptosis in U2OS human osteosarcoma cells is mediated through G2/M phase arrest and activation of caspase-3, followed by PARP cleavage, which is dependent of both the death receptor-mediated (extrinsic) and the mitochondrial (intrinsic) pathways.

Materials and Methods

Materials

Triptolide, 4', 6-diamidino-2-phenylindole (DAPI) and propodium iodide (PI) were obtained from Sigma Chemical Co. (St, Louis, MO, USA). Antibodies to Fas, FasL, FADD, caspase-8, -9, Bid, Bcl-2, Bax, cytochrome ***c*** and poly (ADP-ribose) polymerase (PARP) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against caspase-3, -8 and -9 and Bid were purchased from Cell Signaling (Beverly, MA, USA). GAPDH was purchased from Millipore Corporation (Temecula, CA, USA). Secondary antibodies, horseradish peroxidase-conjugated goat anti-mouse and rabbit IgG were purchased from Stressgene Biotechnologies (Victoria, BC, Canada). Annexin V-FITC apoptosis detection kit and Bradford protein assay kit were obtained from BD Biosciences (San Jose, CA) and Bio-Rad Laboratories (Hercules, CA), respectively.

Cell cultures

Human osteosarcoma cancer cell line U2OS was obtained from American Type Culture Collection (Rockville, MD, USA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM; WelGENE Co., Daegu, Korea), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C under 5% CO_2.

Cell viability

Cell viability assay was performed using a similar procedure as described previously[23]. Briefly, cells were plated in 24-well culture plate (2×10^4 cells /well). After 24 h, the cells were treated with various concentrations of triptolide. After triptolide treatment for 24 h, cells were washed with PBS and MTT (0.5 mg/ml) was added to each. After incubation for 3 h, DMSO was added to dissolve the formazan crystal from MTT reduction and the amount of forrmazan salt was determined by measuring the optical density (OD) at 590 nm using an ELISA plate reader (Bio-rad, Hercules, CA, USA). Cell viability was quantified as a percentage compared to the control.

Flow cytometric analysis

Flow cytometric analysis was performed by the same method as described previously[23]. After treatment with 200 nM of triptolide for various times, the cells were collected and fixed with 70% ethanol. Cells were suspended in PBS containing 0.5 mg/ml propidium iodide, 0.5 mg/ml RNase and 0.03% NP-40, incubated in the dark for 30 min at room temperature and analyzed using CXP and WinCycle softwares (Beckman-coulter, Miami, FL). To determine apoptosis, triptolide-treated cells were washed in PBS, stained using the Annexin V-FITC apoptosis detection kit according to the instructions of the manufacturer and analyzed by flow cytometry. Each experiment was repeated at least twice to ensure reproducibility.

Western blot analysis

Western blot analysis was also performed as described previously[23]. For total protein preparation, cells were lysed in M-PER buffer (Pierce, Rockford, IL, USA) containing protease and phosphatase inhibitor cocktail. Lysate was centrifuged at 14,000 × ***g*** for 15 min at 4°C. The protein concentration was measured using the Bradford protein assay Kit. Total proteins were separated by electrophoresis, transferred on to a PVDF membrane by electroblotting and then probed using primary Abs. The detection of specific proteins was carried out with an enhanced chemiluminescence kit (Amersham Biosciences, UK), according to the recommended procedure. Mitochondrial and cytosolic fractions were prepared by mitochondria isolation kit (Pierce, Rockford, IL, USA) according to the instructions of manufacturer.

DAPI staining

Cells treated with 200 nM of triptolide for various times were washed with PBS and fixed with 4% paraformaldehyde (Sigma Chemical Co.) for 10 min at room temperature. Fixed cells were washed and stained with DAPI solution for 10 min at room temperature. Nuclear morphology of the cells was analyzed with a fluorescent microscope (Carl Zeiss, Germany).

Results

Effect of triptolide on growth of human osteosarcoma cells

To investigate the effects of triptolide on cell growth, U2OS cells were treated with triptolide at different dose levels for 24 h or with 200 nM triptolide for different times and then subjected to MTT assays. As shown in Fig. 1, triptolide treatment inhibited cell growth in dose- and time-dependent manner. After treatment with 100 nM triptolide for 24 h, cell viability was more than 80%, but triptolide at the concentrations of more than 200 nM significantly inhibited the cell proliferation. The concentration giving 50% growth inhibition (IC_{50} values) was approximately 200 nM in U2OS cells. Therefore, we used triptolide at the concentration of 200 nM to examine its effect on apoptosis in U2OS cells.

Triptolide induces G2/M phase arrest in U2OS cells

Previous reports have shown that triptolide inhibits the proliferation of various human tumor cells through cell cycle arrest and induce apoptotic cell death[14-22]. To determine the role of triptolide in the control of cell cycle, in the present study, cell cycle distribution analyses were conducted in U2OS cells. Triptolide-treated cells were collected at 12 h and subjected to FCM analysis. As shown Fig. 2A, cells in the control group without triptolide exposure showed 32.7% population in G2/M phase, whereas triptolide treatment increased the accumulation of G2/M phase in time-dependent manner, indicating G2/M phase arrest in triptolide-treated U2OS cells. To further confirm G2/M phase arrest in U2OS cells, the levels of cell cycle-related proteins were analyzed by

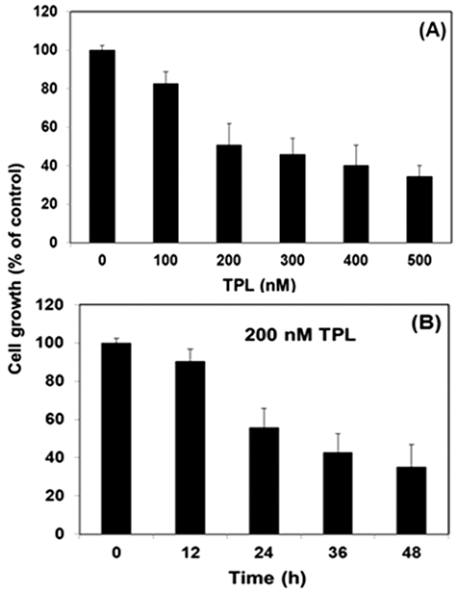
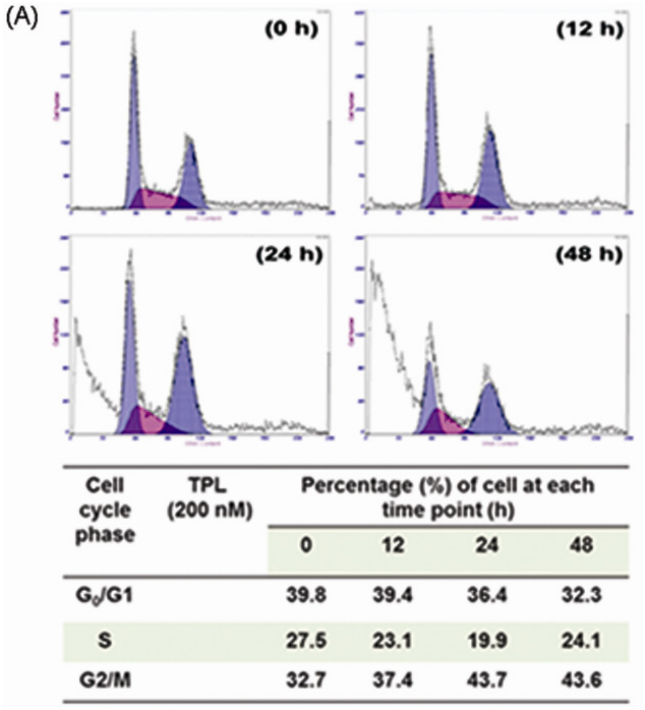


Fig. 1—Effect of triptolide on cell growth of human osteosarcoma U2OS cells [U2OS cells were treated with various concentrations of triptolide for 24 h (A) or with 200 nM triptolide for different times. Cell growth was measured by MTT assay. Data were presented as percentage of control and were mean ± SEM (n = 3-4)]



Cell cycle phase	TPL (200 nM)	Percentage (%) of cell at each time point (h)			
		0	12	24	48
$G_0/G1$		39.8	39.4	36.4	32.3
S		27.5	23.1	19.9	24.1
G2/M		32.7	37.4	43.7	43.6

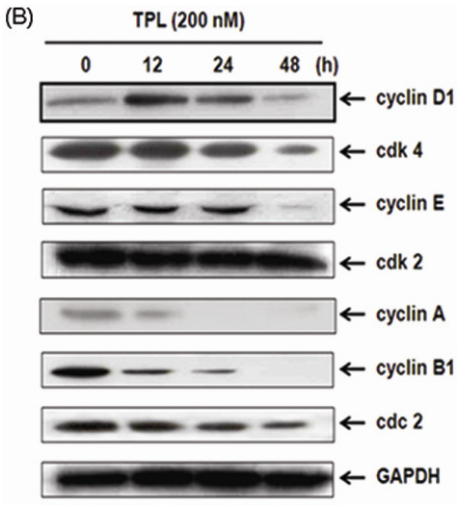


Fig. 2—Effect of triptolide on cell cycle of human osteosarcoma U2OS cells [U2OS cells were treated with 200 nM triptolide for 0, 12, 24, and 48 h. (**A**): Cells were washed, fixed, stained with PI and analyzed for DNA content by FCM; and (**B**): Cells were collected and cell lysates were analyzed by Western blotting. Cell cycle-related proteins were detected using their antibodies. GAPDH was used as an internal control]

Western blot analysis. As shown in Fig. 2B, in U2OS cells, the levels of cyclins A and B1 were remarkably decreased after 12 h of triptolide treatment compared to control level, while the level of their partner cdc2 showed a slight decrease. These results clearly indicated that triptolide induced G2/M phase arrest in U2OS cells.

Triptolide induces apoptosis in U2OS cells

To investigate whether G2/M phase arrest leads to apoptosis in triptolide-treated U2OS cells, the effects of triptolide on cell cycle progression and apoptosis were analyzed by flow cytometry (FCM). The result showed that the percentage of U2OS cells at the apoptotic sub-G1 phase significantly increased in a time-dependent manner, whereas those at G1 phase were decreased (Fig. 3A). To further identify apoptosis of U2OS cells by triptolide treatment, the percentage of apoptotic cells was determined by Annexin V-PI double staining after treatment with 200 nM triptolide for the indicated times. Annexin V-FITC binds to exposed phosphatidylserine on apoptotic and necrotic cells, and PI staining exhibits entry into the late apoptotic cells and necrotic cells. The result showed the time-dependent increase in U2OS cells positive for Annexin V and Annexin V-PI (Fig. 3B). These results indicated that the cytotoxic effect of triptolide on U2OS cells was associated with its apoptosis-inducing activity.

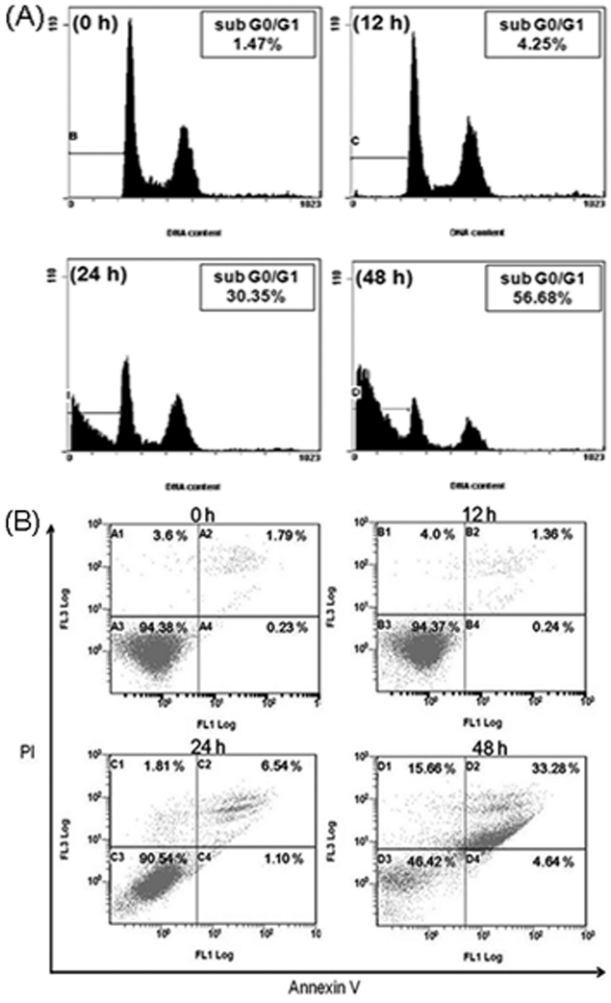


Fig. 3—Effect of triptolide on apoptosis of human osteosarcoma U2OS cells [U2OS cells were treated with 200 nM triptolide for 0, 12, 24, and 48 h. (**A**): Cells were washed, fixed, stained with PI and analyzed for DNA content by FCM; and (**B**): Apoptotic cells were measured by Annexin V assay and followed by analysis with flow cytometry]

To further confirm whether triptolide inhibited the proliferation of human osteosarcoma cells by inducing apoptosis, the effects of triptolide on the induction of apoptosis in U2OS cells were examined by direct observation using an inverted microscope and DAPI staining. After treatment with 200 nM triptolide for the indicated times, the morphologies of U2OS cells changed to irregular and shrunk shape in a time-dependent manner (Fig. 4A). In addition, nuclear DAPI staining using a fluorescent microscope revealed the typical morphological features of apoptosis with nuclear fragmentation in triptolide-treated cells (Fig. 4B). These results clearly indicated that U2OS cells were undergoing apoptosis by triptolide.

Caspase-3, a key protease in the apoptotic machinery, cleaves a number of proteins that are essential for cell survival[24,25]. Caspase-3 synthesized

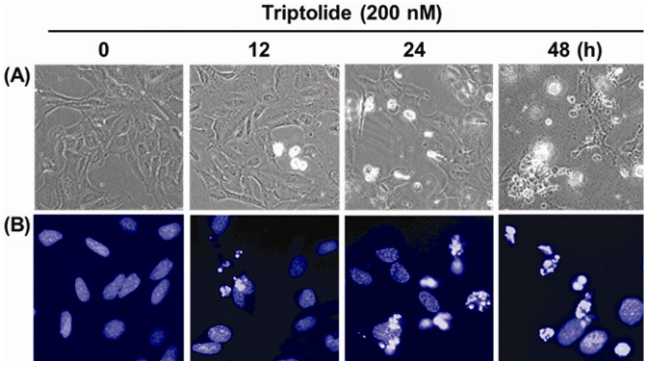


Fig. 4—Time-dependent changes of cell morphology in response to triptolide [U2OS cells were cultured in DMEM containing 200 nM triptolide for 0, 12, 24 and 48 h. At each time point, cells were observed using an inverted microscope (A, 200X) or were stained with DAPI solution for 10 min and then photographed with a fluorescence microscope using a blue filter (B, 200X). The fragmented and condensed nuclei of apoptotic cells showed a bright staining pattern]

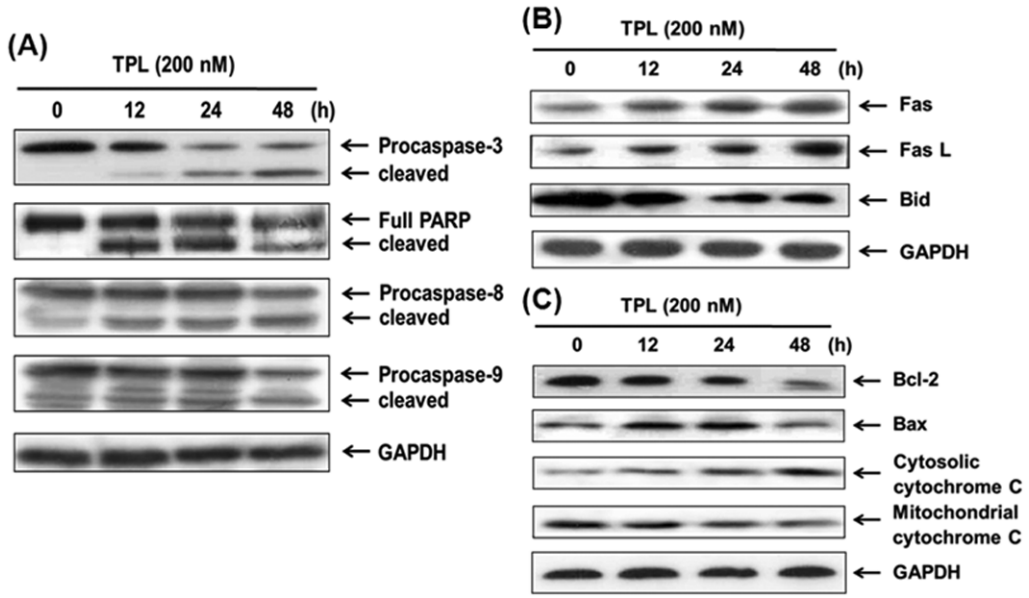


Fig. 5—Effect of triptolide on activations of capases, PARP cleavage and expression levels of proteins related to apoptosis pathways in U2OS cells [U2OS cells were treated with 200 nM triptolide for 0, 12, 24 and 48 h. Activations of capase-3, -8, -9, PARP cleavage and protein expression levels were determined by Western blot analysis. GAPDH was used as an internal control]

as procaspase-3 is activated by cleavage into two smaller subunits when the cells undergo apoptosis[24,25]. Activation of caspase-3 subsequently causes apoptotic cell death through cleavage of PARP which plays a crucial role in maintaining genomic integrity. Therefore, we investigated whether triptolide induced apoptosis through the activation of caspase-3 and subsequent cleavage of PARP protein in U2OS cells. As shown in Fig. 5A, treatment of cells with 200 nM triptolide for different times resulted in a time-dependent increase in the cleavage of procaspase-3 and PARP. This result indicated that triptolide induced apoptosis through the cleavage of procaspase-3 and PARP in U2OS cells.

Triptolide induces apoptosis through both extrinsic and intrinsic pathways in U2OS cells

It is well-known that caspase-3 is activated through cleavage into two smaller subunits by caspase-8 and/or caspase-9, which plays important roles in induction of apoptosis through the mitochondrial (intrinsic) and/or the death receptor-mediated (extrinsic) pathways, respectively[24,25]. To clarify the pathway by which triptolide induced apoptosis in U2OS cells, protein levels of caspase-8 and -9 were analyzed by Western blot analysis after the cells were treated with 200 nM triptolide during indicated times. As shown in Fig. 5A, protein levels of both procaspase-8 and -9 were decreased in time-dependent manner, whereas those of their cleaved forms were increased, indicating the activation of both caspases by triptolide in U2OS cells.

Thus, we checked downstream of extrinsic and intrinsic pathways in triptolide-treated U2OS cells. In the extrinsic pathway, the binding of FasL to Fas receptor leads to the activation of procaspase-8[24,25]. To verify triptolide-mediated apoptosis through extrinsic pathway in U2OS cells, levels of Fas and FasL were investigated. As shown in Fig. 5B, the expression levels of Fas and FasL were increased in time-dependent manner in triptolide-treated cells, indicating triptolide-induced apoptosis through extrinsic pathway. Moreover, the level of Bid was decreased, which presumably resulted in production of truncated Bid form (tBid). Bid is cleaved by active caspase-8, resulting in generating tBid and translocates to the mitochondria and then enhances cytochrome ***c*** release by its interaction with Bax or Bak24[24,25]. Thus, this result suggested that triptolide-induced apoptosis in U2OS cells might be mediated through intrinsic pathway via extrinsic pathway mediated by tBid.

To confirm triptolide-mediated apoptosis through intrinsic pathway in U2OS cells, levels of Bcl-2, Bax and cytochrome ***c*** were investigated. As shown in Fig. 5C, the level of Bcl-2, an anti-apoptotic protein was decreased, whereas the level of Bax, a pro-apoptotic protein was increased after triptolide exposure. Furthermore, cytochrome ***c*** was released from

mitochondria to cytosol in a dose-dependent manner by triptolide treatment. This result indicated that triptolide-induced apoptosis in U2OS cells was mediated through intrinsic (mitochondrial-mediated) pathway.

Discussion

In this study, we demonstrated for the first time that triptolide induced apoptosis in human osteosarcoma cells by activating both mitochondrial (intrinsic) and the death receptor-mediated (extrinsic) pathways. Our result clearly revealed the growth inhibitory and apoptotic effects of triptolide on U2OS cells, as evidenced by FCM analysis and Annexin V-PI double staining. One mechanism for causing apoptosis in cancer cells is growth arrest by inhibition of positive cell cycle regulators[26]. In this study, we found that triptolide induced G2/M cell cycle arrest in U2OS cells, as evidenced by FCM analysis and Western blotting which showed that the levels of cyclins A and B1 were remarkably decreased after 12 h of triptolide treatment compared to control level and cdc2 level also reduced slightly. This result was in agreement with the previous finding that triptolide induces G2/M cell cycle arrest in multiple myeloma U266 cells[18].

We also found here that triptolide induced activations of caspase-3, -8, and -9 and subsequent cleavage of PARP, which are regarded as hallmark of apoptosis. These results provided a reasonable explanation for induction of apoptosis by triptolide in U2OS cells. Triptolide-induced apoptosis is known to be mediated through the mitochondrial (intrinsic) and/or the death receptor-mediated (extrinsic) pathways[14]. Activations of caspase-8 and -9 are characteristic of extrinsic and intrinsic pathways, respectively. The extrinsic pathway is initiated by the interaction of the death receptor (Fas/CD95) with its ligand (FasL). The binding of FasL to Fas results in the activation of procaspase-8 and subsequently promotes the activation of downstream procaspase-3[24,25]. Our result showed that triptolide significantly increased the levels of Fas and FasL, which resulted in the subsequent activation of procaspase-8 and -3 in U2OS cells, demonstrating triptolide-induced apoptosis through the death receptor-mediated (extrinsic) pathway.

We also demonstrated that triptolide activated intrinsic pathway that was initiated with cytochrome ***c*** release from mitochondria and the subsequent activation of procaspase-9 cascade. Bcl-2 and its family proteins, including tBid and Bax are important modulators of cytochrome ***c*** release from mitochondria[24,25]. Bcl-2 is an anti-apoptotic protein that regulates the mitochondrial release of cytochrome ***c***, the interaction of Apaf-1 with procaspase-9 and binding to Bax, whereas Bax is a pro-apoptotic Bcl-2 homologue that exists in the cytoplasm or in the cell membrane and can antagonize the protective role of Bcl-2[24]. We found in this study that triptolide decreased Bcl-2 level and increased Bax level in U2OS cells. Moreover, the increased cytochrome ***c*** level in the cytosol compared to mitochondria was observed in triptolide-treated cells. These results clearly indicated that the increased Bax/Bcl-2 ratio contributed to the disruption of mitochondrial potential and the release of cytochrome ***c*** into cytosol, which resulted in activation of procaspase-9, as described in the previous reviews[24,25]. Bid is a pro-apoptotic protein that connects extrinsic pathway with intrinsic pathway and tBid produced by active caspase-8 translocates to the mitochondria and then enhances cytochrome ***c*** release by its interaction with Bax[24]. We also found that Bid mediated the communication between extrinsic pathway and intrinsic pathway in triptolide-treated U2OS cells.

In conclusion, our study has demonstrated for the first time that triptolide treatment in human osteosarcoma U2OS cells leads to accumulation of G2/M phase and induction of apoptosis that is mediated by death receptor-mediated (extrinsic) pathway and/or the mitochondrial (intrinsic) pathways.

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