Triptolide induces apoptosis of PMA-treated THP-1 cells through activation of caspases, inhibition of NF-κB and activation of MAPKs

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Received April 17, 2013; Accepted June 7, 2013

DOI: 10.3892/ijo.2013.2033

Abstract. Triptolide is known to be involved in many cellular events, such as those related to immunosuppressive and anti-tumor activity. We investigated whether triptolide mediates these effects through multiple mechanisms, including activation of cell cycle arrest and caspase-dependent pathways, as well as by blocking nuclear factor-κB (NF-κB) activation and by potentiating the activities of the mitogen-activated protein kinase (MAPK) pathway, in phorbol myristate acetate (PMA)-differentiated THP-1 cells. Triptolide significantly inhibited cell proliferation in a dose- and time-dependent manner and it increased the apoptotic fraction in the cell cycle and the number of apoptotic THP-1 cells. Exposure of the cells to triptolide also increased caspase-3 activity in these cells. Furthermore, co-treatment of cells with triptolide and the pan-caspase inhibitor, Z-VAD-FMK, or the caspase-3 inhibitor, Z-DEVE-FMK, increased THP-1 cell growth. Triptolide treatment resulted in a significant decrease in mRNA expression levels in genes encoding Bcl-2, cyclin D1, p27 and survivin and an increase in those encoding Bax and p21 in THP-1 cells. Triptolide not only inhibited NF-κB activation, but also activated p38 MAPK and MEK/ERK phosphorylation. These results show that triptolide inhibits the growth of THP-1 cells by inducing apoptosis through caspase activation and the mechanism involves NF-κB inhibition and the MAPK pathway.

Introduction

Tripterygium wilfordii Hook F. has been used for centuries in traditional Chinese medicine to treat rheumatoid arthritis, an autoimmune disease associated with the increased production of the pro-inflammatory cytokine, tumor necrosis factor (TNF)-α. Triptolide, a compound originally purified from T. wilfordii Hook F., which has potent anti-inflammatory and immunosuppressant activities (1,2), has been reported to exert anti-neoplastic activity mainly by inducing apoptosis in various cancer cells (3-6). The exact mechanism responsible for the anti-neoplastic and anti-inflammatory effects of triptolide is not clearly understood.

Apoptosis is mediated through at least 3 major pathways, which are regulated by the death receptors, mitochondria and the endoplasmic reticulum. Activation of apoptosis pathways is a key mechanism by which cytotoxic drugs kill tumor cells and defects in apoptosis signaling contribute to tumor cell drug resistance (7,8). Among the important regulators of apoptosis are the members of the Bcl-2 family. This family of proteins includes both anti-apoptotic molecules, for instance Bcl-2 and Bcl-XL (12). Thus, suppression of the NF-κB pathway should be effective in inducing apoptosis of tumor cells.

Moreover, mitogen-activated protein kinases (MAPKs) play a critical role in the regulation of cell growth and differentiation in the control of cellular responses to stress and cytokines. MAPK signaling pathways have also been shown to play critical roles in tumorigenesis. The activities of MAPKs are negatively regulated via dephosphorylation of certain conserved tyrosine and threonine residues by a family of MAPK phosphatases (MKPs) (13). Triptolide has been reported to suppress the expression of MKP-1, which inactivates extracellular signal-regulated kinase (ERK), p38 MAPK and c-Jun N-terminal kinase (JNK), to exert its anti-proliferative and pro-apoptotic activities (14).

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Key words: apoptosis, caspase, nuclear factor-κB, mitogen-activated protein kinase, THP-1 cells, triptolide
In the present study, we used a human monocytic leukemia cell line, THP-1, which had been differentiated into macrophage-like cells by treatment with phorbol myristate acetate (PMA). We postulated that triptolide mediated its effects through multiple mechanisms, including activating cell cycle arrest and caspase-dependent pathways, as well as blocking NF-κB activation and potently activating the MAPK pathway.

Materials and methods

Cell culture. Human monocytic leukemia THP-1 cells were supplied by the Korean Cell Line Bank. Cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in 95% air. THP-1 cells were treated with 100 nM of phorbol myristate acetate (PMA, Sigma-Aldrich Co., St. Louis, MO, USA) for 24 h to induce differentiation of the cells into macrophages. After differentiation, non-attached cells were removed by aspiration and the adherent macrophages were washed with RPMI-1640 medium 3 times and then incubated in cell culture medium at 37°C.

MTT assay. Cell proliferation was measured with CellTiter 96 Aqueous One Solution (Promega, Madison, WI, USA). Cells were seeded at 1x10⁴ cells per well in 96-well plates and incubated with different concentrations of triptolide (Sigma-Aldrich) at 37°C for 24, 48 and 72 h. Cell viability was determined using a colorimetric assay with PMS/MTS solution. The absorbance was determined at 490 nm, with background subtraction at 650 nm.

Cell cycle analysis. Cells (5x10⁴) were incubated with 5, 10 and 25 nM of triptolide for 48 h. After incubation, the cells were harvested and washed with PBS. Cells were fixed with 70% ethanol for 1 h, treated with RNase A (20 μg/ml) at 37°C for 1 h, before being stained with propidium iodide (50 μg/ml). DNA content at each cell cycle stage was analyzed using a FACSCalibur with CellQuest software (Becton-Dickinson, USA).

Apoptosis assay. For determining apoptosis in THP-1 cells, apoptotic cells were quantified using a cell death detection ELISA kit (Roche Molecular Biochemicals, Mannheim, Germany). Cells (1x10⁴) were incubated with 5, 10 and 25 nM of triptolide for 48 h. Cells were lysed with cell lysis buffer (200 μl). Cell lysates were assayed for DNA fragments using the cell death ELISA kit according to the manufacturer's protocol. DNA fragmentation was measured at 405 nm against the cell death ELISA kit according to the manufacturer's protocol. First-strand cDNA synthesis was then made using 1 μg of RNA using a reverse transcriptase system (Promega). Reverse transcription was primed using random hexamers. The primers and product sizes were as follows: cyclin D1 forward 5'-CCGTTCCATGCGGAAGATC-3', reverse 5'-ATGGCCACCGGGGAAGAC-3', 86 bp; p21 forward 5'-CAGACCACGATGACAGATTTC-3', reverse 5'-TTAGGGCTTCCCTCTTGAGA-3', 200 bp; survivin forward 5'-GGCCCAGTGTTTCTTCTGCTT-3', reverse 5'-CTCTTGAGGACAC-3', 120 bp; Bax forward 5'-GGATGCGTCCACCAAGAAG-3', reverse 5'-AGAAGAATCGTCGGTGTGCA-3', 216 bp; Bcl-2 forward 5'-GGTGTAGTGATGGTGTGCA-3', reverse 5'-CCTTGGCATGAGATGCAGGA-3', 200 bp; Bcl-2 forward 5'-GGATGCGTCCACCAAGAAG-3', reverse 5'-GCCCTTGGACACCATTGGC-3', 216 bp; and 25 nM of triptolide for 48 h. After treatment, cells were harvested and washed with cold PBS and lysed using lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM

These colorimetry substrates were solubilized in an assay buffer. After incubation with the substrates at 37°C for 1 h in the dark, color production in the lysates was measured with a microplate reader at 405 nm. Caspase-3, -8 and -9 activities were determined by direct comparison to the level of the uninduced control. To assess the effect of caspase inhibitor treatment, THP-1 cells (1x10⁶ cells) were pretreated with a pan-caspase inhibitor, Z-VA-FMK, or a caspase-3-specific inhibitor, Z-DEVD-FMK (R&D Systems), for 2 h, followed by addition of 50 nM triptolide. After 48 h, cell viability was determined by a colorimetric assay with PMS/MTS solution. The absorbance was determined at 492 nm with background subtraction at 650 nm.

Nuclear staining with Hoechst 33258. Cells (1x10⁴) were treated with 5, 10 and 25 nM of triptolide for 48 h and then washed with ice-cold PBS. The cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.05% Triton X-100 for 5 min and stained with 50 ng/ml of Hoechst 33258 (Sigma-Aldrich). The nuclear areas were observed and photographed with a fluorescent microscope and calculated with an Olympus 13x51 fluorescence microscope equipped with a Nuance 2.1 Multispectral Imaging System (Cambridge Research & Instrumentation, Inc., MA, USA).

RNA extraction and real-time PCR procedures. Total RNA was purified from cultured cells using the RNA-Beet solution kit (Tel-test, Friendswood, TX, USA), following the manufacturer's protocol. First-strand cDNA synthesis was then made using 1 μg of RNA using a reverse transcriptase system (Promega). Reverse transcription was primed using random hexamers. The primers and product sizes were as follows: cyclin D1 forward 5'-CCGTTCCATGCGGAAGATC-3', reverse 5'-ATGGCCACCGGGGAAGAC-3', 86 bp; p21 forward 5'-CAGACCACGATGACAGATTTC-3', reverse 5'-TTAGGGCTTCCCTCTTGAGA-3', 200 bp; survivin forward 5'-GGCCCAGTGTTTCTTCTGCTT-3', reverse 5'-CTCTTGAGGACAC-3', 120 bp; Bax forward 5'-GGATGCGTCCACCAAGAAG-3', reverse 5'-GCCCTTGGACACCATTGGC-3', 216 bp; Bcl-2 forward 5'-GGTGTAGTGATGGTGTGCA-3', reverse 5'-CCTTGGCATGAGATGCAGGA-3', 200 bp; Bcl-2 forward 5'-GGATGCGTCCACCAAGAAG-3', reverse 5'-GCCCTTGGACACCATTGGC-3', 216 bp; survivin forward 5'-GGCCCAGTGTTTCTTCTGCTT-3', reverse 5'-GCAACCCGGAGATGCTTGT-3', 91 bp; β-actin forward 5'-GGGAGAAGATGACCCAGAC-3', reverse 5'-GGGAGAAGATGACCCAGAC-3', 216 bp; and 25 nM of triptolide for 48 h. After treatment, cells were harvested and washed with cold PBS and lysed using lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM

Immunoblot analysis. Cells (2x10⁶) were treated with 5, 10 and 25 nM of triptolide for 48 h. After treatment, cells were washed with cold PBS and lysed using lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM

β-actin was calculated using the formula 2⁻ΔΔCt and the relative amounts were quantified.
EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 µg/ml leupeptin containing 1 mM PMSF. The protein concentration was determined by means of a BCA protein assay according to the manufacturer's protocol. Thirty micrograms of protein was fractionated on 12% SDS-PAGE and transferred by electrophoresis onto a nitrocellulose membrane. The membranes were blocked with 5% non-fat dry milk for 1 h at room temperature and incubated with anti-NF-κB p65, anti-p38, anti-phospho-p38, anti-MEK1/2, anti-ERK1, anti-phospho-ERK1, anti-phospho-ERK1/2 (Cell Signaling Technology, Danvers, MA, USA) and β-actin antibodies (Sigma-Aldrich) at a 1:1,000 dilution with Tris-buffered saline containing 0.05% Tween-20 (TBS-T) at 4˚C for 18 h. After washing with TBS-T for 1 h, the membranes were treated with horseradish peroxidase-conjugated secondary antibody, diluted 1:2,500 with TBS-T, for 1 h at room temperature. After washing the membranes with TBS-T for 1 h, proteins were detected using an Enhanced Chemiluminescence kit (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Protein expression levels were analyzed using a Chemiluminescence Imaging system (Davinch-Chemi™, Seoul, Korea).

Statistical analysis. Values are expressed as the mean ± SD. Student’s t-test was used to evaluate differences between the control samples and triptolide-treated samples. Inhibition of apoptosis was estimated by the differences between the triptolide-treated sample and samples treated with a combination of caspase inhibitor and triptolide. *P<0.05 and **P<0.01 were considered statistically significant.

Results

Triptolide inhibits cell proliferation in THP-1 cells. THP-1 cells were treated with various concentrations of triptolide (0-50 nM) for 24, 48 and 72 h. The effect of triptolide on cell proliferation was measured using an MTT assay. Triptolide induced a significant decrease in THP-1 cell proliferation in a dose- and time-dependent manner (Fig. 1).

Triptolide induces cell populations. THP-1 cells were treated with 5, 10 and 25 nM of triptolide for 48 h, after which flow cytometric analyses were performed (Fig. 2A). The apoptosis fraction increased by 2.48, 1.24, 3.69 and 14.49%, whereas the G0/G1 phase decreased by 39.03, 42.91, 39.47 and 14.32%, respectively, in THP-1 cells.

Triptolide induces apoptosis. THP-1 cells were treated with 5, 10 and 25 nM of triptolide for 48 h and apoptotic cells were quantified using a cell death detection ELISA (Fig. 2B). The number of apoptotic cells increased in a dose-dependent manner.

Figure 1. Effects of triptolide on cell proliferation in THP-1 cells. Cells were treated with triptolide (0-100 nM) for 24, 48 and 72 h. Cell proliferation was determined using an MTT assay. Values represent the mean ± SD of 3 independent experiments.

Figure 2. Effects of triptolide on apoptosis in THP-1 cells. Cells were treated with 5, 10 and 25 nM of triptolide for 48 h. (A) Cells stained with propidium iodide. The DNA content was analyzed by flow cytometry. The percentage of cells in apoptosis and in G0/G1 phases was calculated. (B) Apoptotic cells were measured using a cell death detection ELISA. Values represent the mean ± SD of 3 independent experiments. *P<0.01 compared to control.
Figure 2. Continued. (C) Hoechst 33258 staining of THP-1 cells was observed in the cell cultured with triptolide for 48 h (x400). (D) The enzymatic activity of caspase proteases was measured by caspase colorimetric assay. Control cells were assigned a value of 1 and other values were expressed relative to these and were plotted against the time after triptolide treatment. Values represent the mean ± SD of 3 independent experiments. **P<0.01 compared to control. (E) THP-1 cells were pre-treated with Z-VAD-FMK (10-50 µM) and Z-DEVD-FMK (10-50 µM) for 2 h and were then incubated with 25 nM triptolide for 48 h. Then, the cells were assessed using an MTT assay. Values represent the mean ± SD of 3 independent experiments. **P<0.01, comparison of triptolide-treated cells vs cells pretreated with caspase inhibitor before treatment with triptolide.

Figure 3. Effects of triptolide on the mRNA expression in THP-1 cells. Cells were treated with 5, 10 and 25 nM of triptolide for 48 h. mRNA levels were measured by real-time PCR. Values represent the mean ± SD of 3 independent experiments and were expressed as the relative mRNA accumulation corrected using β-actin mRNA as an internal standard. **P<0.01 compared to control.
Hoechst 33258 staining was used to observe the morphology of cell apoptosis. When THP-1 cells were exposed to triptolide for 48 h, apoptotic cells characterized by morphological alteration such as condensed nuclei and cell shrinkage were observed and the apoptotic cell number increased with the dose (Fig. 2C). Caspases are cysteine-aspartate proteases that play critical roles during the initiation and execution of apoptosis. To further elucidate the mechanism involved in the observed apoptosis, intracellular caspase-3, -8 and -9 activities were measured in THP-1 cells treated with various concentrations of triptolide (Fig. 2D), using a colorimetric ELISA. Caspase-3 and -9 activities were increased in response to triptolide treatment in a dose-dependent manner at 48 h. To confirm whether the activation of caspases is involved in triptolide-induced apoptosis, cell growth of THP-1 cells by triptolide was determined by MTT assay in the presence of the pan-caspase inhibitor Z-VAD-FMK and the caspase-3-specific inhibitor Z-DEVD-FMK. As shown in Fig. 2E, treatment with 25 nM triptolide resulted in an increase in proliferation of THP-1 cells after pretreatment with 10-50 µM Z-VAD-FMK as well as by 10-50 µM Z-DEVD-FMK.

Triptolide mediates gene expression. The level of mRNAs transcribed from cell cycle-related genes (cyclin D1, p21, p27) and apoptosis-related genes (Bcl-2, Bax, survivin) were examined by real-time PCR (Fig. 3). THP-1 cells were treated with 5, 10 and 25 nM of triptolide for 48 h. The levels of Bcl-2, cycline D1, p27 and survivin mRNA were decreased, whereas those of Bax and p21 mRNA were increased in a dose-dependent manner.

Triptolide inhibits NF-κB activation and enhances p38 MAPK and MEK/ERK phosphorylation. THP-1 cells were incubated with triptolide at 5, 10 and 25 nM for 48 h. After triptolide treatment, protein expression levels were measured by western blot analysis (Fig. 4). The levels of NF-κB p65 significantly declined after cells were exposed to triptolide. Triptolide induced a marked increase in the levels of phosphorylated p38 MAPK, MEK and ERK1/2.

Discussion

Triptolide is known to affect many cellular events, to have anti-neoplastic activity and induce apoptosis. Triptolide inhibits cell growth in variety of tumor cells (15,16). In this study, human monocytic leukemia THP-1 cells that had been PMA-differentiated showed inhibition of cell proliferation after triptolide treatment, in a dose-and time-dependent manner. To elucidate whether triptolide decreased cell viability by inducing apoptosis, we investigated its effect on the expression of apoptosis-related factors and found that triptolide increased the apoptosis fraction at cell cycle and the number of apoptotic THP-1 cells in a dose-dependent manner. These results suggested that triptolide not only inhibits THP-1 cell growth and blocks cell cycle progression at the G1 phase, but also induces apoptosis. Triptolide has also been reported to induce significant apoptosis and minor accumulation in the S phase in THP-1 cells (17). However, treatment with triptolide was shown to reduce the viability of HeLa and Caski cells in a concentration-dependent manner and to increase accumulation of sub-G1 phase cells and apoptotic bodies (16).

Triptolide not only regulates cell growth, but also induces programmed cell death in several types of cells. It appears that triptolide induces apoptosis by activating caspases, the proteases responsible for cell death in multiple myeloma cells and leukemic cells (18,19). Caspases play important roles in the apoptotic process (20,21). When investigating the molecular mechanism underlying apoptosis of THP-1 cells in response to triptolide, we found that treatment of THP-1 cells with triptolide produced increases in intracellular caspase-3 activity.
This finding was confirmed by experiments using the pan-caspase inhibitor Z-VAD-FMK and the caspase-3-specific inhibitor Z-DEVD-FMK, which enhanced cell growth in triptolide-treated cells. These results suggest that the apoptotic effect of triptolide in THP-1 cells may result from the regulation of the caspase pathways. Caspase-3 and -9 activity play important roles in apoptosis through a mitochondria-dependent pathway. It has been shown that the induction of apoptosis in cervical cancer cells by triptolide is associated with activation of caspases (16). Moreover, it was reported that triptolide may induce apoptosis through a mitochondria-mediated apoptotic pathway in a caspase-dependent way in human melanoma A375 cells (22).

Bcl-2 gene-family members have been widely considered to be regulators of cell death (23). In this study, our results revealed that triptolide treatment of THP-1 cells upregulated the mRNA expression of genes encoding Bax and p21 and downregulated those encoding Bcl-2, cycline D1, p27 and survivin. These results suggested that triptolide regulated transcription factors of cell cycle-related genes and apoptosis-related genes. It has previously been shown that triptolide treatment leads to increased Bax expression and decreased Bcl-2 expression (24). In contrast, Bax expression was significantly up-regulated in SW1990 cells treated with triptolide, while Bcl-2 mRNA was not (25).

Triptolide has also been shown to decrease anti-apoptotic mechanisms through NF-κB inhibition (26-27). We investigated whether this regulatory mechanism is also involved in the process of apoptosis in THP-1 cells; our results indicated that triptolide suppressed NF-κB activation, suggesting that triptolide induced apoptosis through inhibition of NF-κB activation. Recent reports demonstrated that the NF-κB signal is clearly downregulated during apoptosis induced by triptolide (15). Bcl-2 and Bcl-XL are members of the Bcl-2 family whose expression is regulated by NF-κB. Triptolide induces apoptosis by means of inhibiting NF-κB through downregulating the expression of the genes encoding Bcl-2 and Bcl-XL (22). Our study supports that the NF-κB pathways are involved in the process of THP-1 cell apoptosis induced by triptolide.

The MAPK pathway is a key signaling mechanism that regulates many cellular functions, such as cell growth, transformation and apoptosis (28). MAPKs can mediate apoptotic signaling induced by antineoplastic agents (29). ERK, JNK and p38 MAPK constitute 3 major subfamilies of MAPKs that appear to mediate cellular responses, including proliferation, differentiation and apoptosis (30). Gleditsia sinensis thorns (WEGS) induced phosphorylation of ERK1/2, p38 MAPK and JNK in human colon cancer cells (31). DanShenSuan increased phosphorylation of Akt and ERK1/2 in H9c2 cardiomyocytes (32). Induction of cell death can be mediated by activating ERK1/2 (33.34). We found that triptolide enhanced the level of p38 MAPK and MEK/ERK phosphorylation in THP-1 cells. These results suggest that triptolide mediates cell growth and apoptosis through the activation of the MEK/ERK pathway.

In conclusion, we demonstrated that triptolide inhibits the growth of THP-1 cells by inducing apoptosis through the activation of caspases, as well as inhibiting NF-κB and activating MAPK pathways. However, studies are needed to explore further details of the mechanisms underlying the effect of triptolide on human monocytic leukemia cells.

Acknowledgements
This study was supported by Bio-industry Technology Development Program, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea (grant no. 311059-4) and partially supported by the Rural Development Administration, Republic of Korea (grant no. PJ008475022012). We thank Waterborne Virus Bank for the technical support of real-time PCR work.

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