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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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Abstract. Triptolide is known to be involved in many cellular events, such as those related to immunosuppressive and antitumor 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, such as Bcl-2 and pro-apoptotic molecules, such as Bax (9). Triptolide induces apoptosis in T-cell hybridomas and peripheral T cells (10) and can sensitize TNF-α-resistant tumor cell lines to TNF-α-induced apoptosis (5).

Nuclear factor-κB (NF-κB) is active in most tumor types and regulates a series of pivotal events in the tumor progress, including cell survival, cell proliferation, angiogenesis and invasion (11). For cell survival, NF-κB mainly suppresses apoptosis by increasing the transcription of genes encoding anti-apoptotic proteins, for instance Bcl-2 and Bcl-X_L (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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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% CO_2 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 1x10^4 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 (5x10^5) 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^{plus} kit (Roche Molecular Biochemicals, Mannheim, Germany). Cells (1x10^4) 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^{plus} kit according to the manufacturer's protocol. DNA fragmentation was measured at 405 nm against an untreated control. To measure the enzymatic activity of caspase proteases, a caspase colorimetric assay kit (R&D Systems, Inc. Minneapolis, MN, USA) was used. THP-1 cells (2x10^6) were treated with 5, 10 and 25 nM of triptolide for 48 h. Cells were harvested and cell pellets were lysed in 50 μl of lysis buffer on ice for 10 min. The proteins concentration in the supernatant (cytosolic extract) was measured by BCA assay. The activities of caspase-3-, -8 and -9-like proteases were measured by proteolytic cleavage of substrates, including DEVD-*p*NA (caspase-3 substrate), IETD-pNA (caspase-8 substrate) and LEHD-*p*NA (caspase-9 substrate) respectively. 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 (1x10^4 cells) were pretreated with a pan-caspase inhibitor, Z-VAD-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 (1x10^5) 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-Bee 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 primer sequences and product sizes were as follows: cyclin D1 forward 5'-CCGTCCATGCGGAAGATC-3', reverse 5'-ATGGCCAGCGGGAAGAC-3', 86 bp; p21 forward 5'-CAGACCAGCATGACAGATTTC-3', reverse 5'-TTAGGGC TTCCTCTTGGAGA-3', 66 bp; p27 forward 5'-CCGGCTAA CTCTGAGGACAC-3', reverse 5'-AGAAGAATCGTCGGT TGCAG-3', 120 bp; Bcl-2 forward 5'-GATTGATGGGATCGT TGCCTTA, reverse 5'-CCTTGGCATGAGATGCAGGA-3', 200 bp; Bax forward 5'-GGATGCGTCCACCAAGAAG-3', reverse 5'-GCCTTGAGCACCAGTTTGC-3', 216 bp; survivin forward 5'-GGCCCAGTGTTTCTTCTGCTT-3', reverse 5'-GCAACCGGACGAATGCTTT-3', 91 bp; β-actin forward 5'-GCGAGAAGATGACCCAGATC-3', reverse 5'-GGATAGC ACAGCCTGGATAG-3', 77 bp. Real-time PCR was performed on a StepOneplus real-time PCR system (Applied Biosystems, Foster, CA, USA) with Power SYBR Green PCR Master Mix. PCRs were performed with 1 μl cDNA in 20-μl reaction mixtures that consisted of 10 μl Power SYBR Green PCR Master Mix, 2 μl primers and 7 μl PCR-grade water. The reactions were performed with a denaturation step at 95˚C for 10 min, followed by 40 cycles each consisting of 95˚C for 15 sec and 60˚C for 1 min. The crossing point of target genes with β-actin was calculated using the formula $2^{-(\text{target gene - }\beta\text{-actin})}$ and the relative amounts were quantified.

Immunoblot analysis. Cells (2x10^6) 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 Na_2EDTA, 1 mM

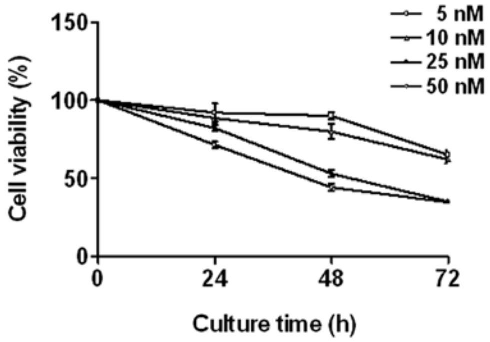

Figure 1. Effects of triptolide on cell proliferation in THP-1 cells. Cells were treated with triptolide (0-100 nM) for 24, 48 and 72 h. Cell proliferation was determined using an MTT assay. Values represent the mean ± SD of 3 independent experiments.

EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 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-phospho-MEK1/2, anti-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

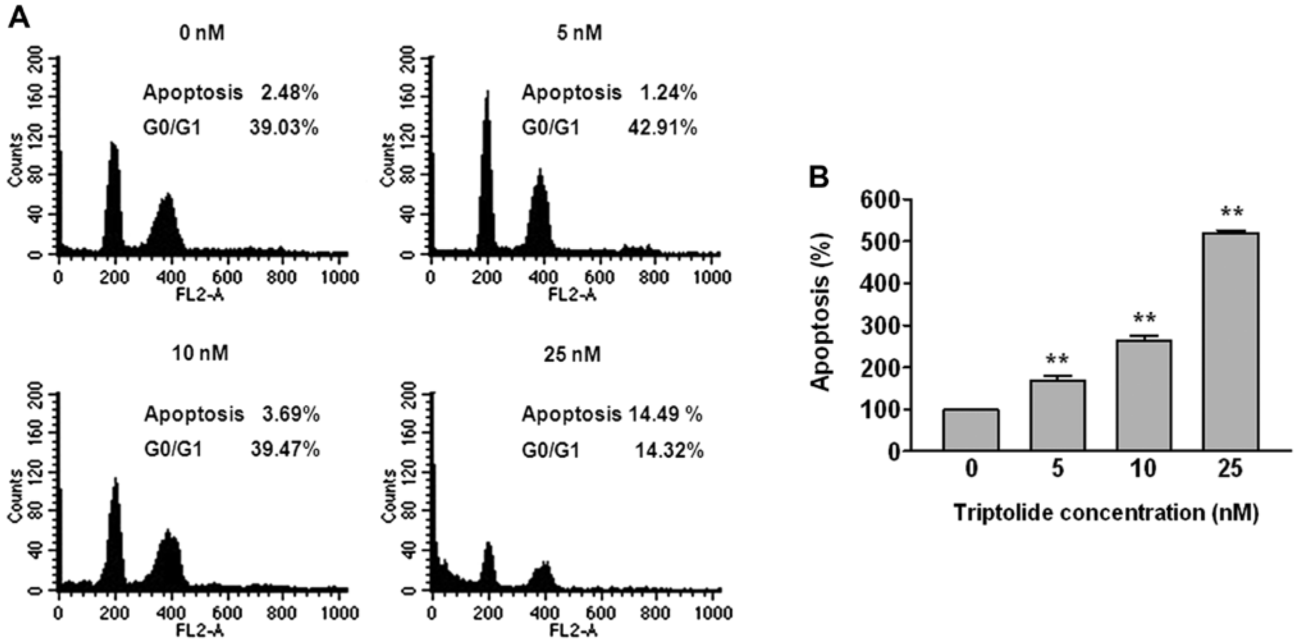

Figure 2. Effects of triptolide on apoptosis in THP-1 cells. Cells were treated with 5, 10 and 25 nM of triptolide for 48 h. (A) Cells stained with propidium iodide. The DNA content was analyzed by flow cytometry. The percentage of cells in apoptosis and in G0/G1 phases was calculated. (B) Apoptotic cells were measured using a cell death detection ELISA. Values represent the mean ± SD of 3 independent experiments. $^{**}P<0.01$ compared to control.

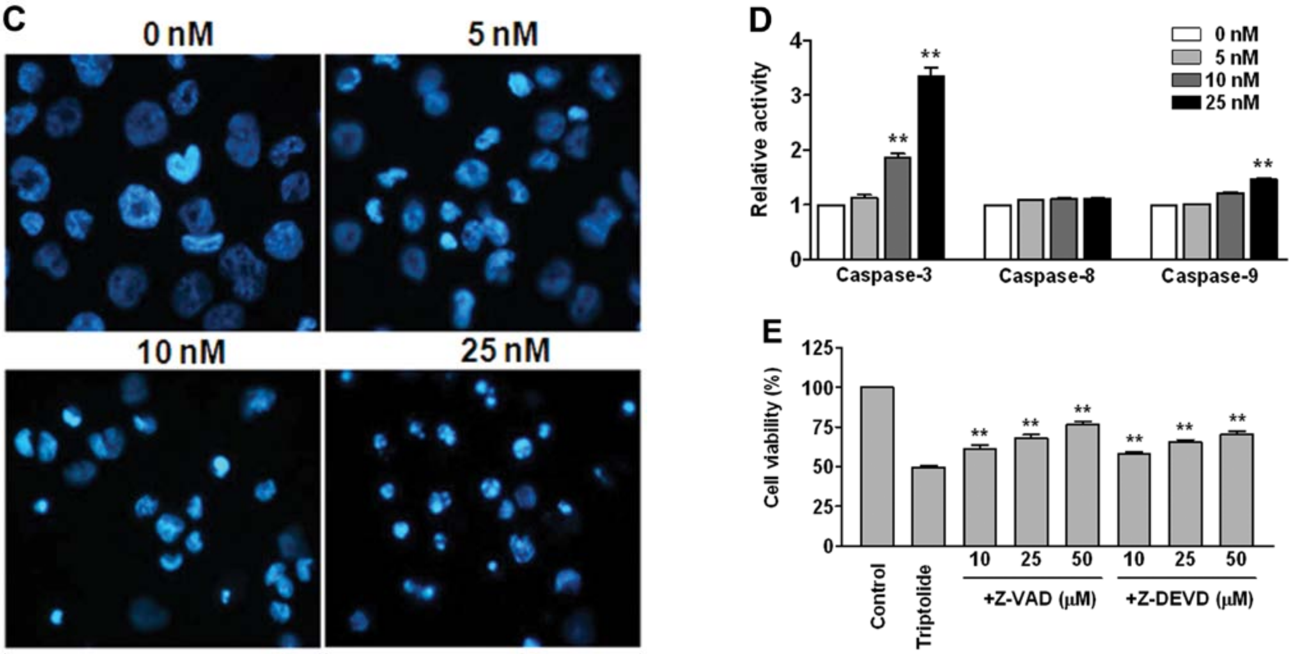

Figure 2. Continued. (C) Hoechst 33258 staining of THP-1 cells was observed in the cell cultured with triptolide for 48 h (x400). (D) The enzymatic activity of caspase proteases was measured by caspase colorimetric assay. Control cells were assigned a value of 1 and other values were expressed relative 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.

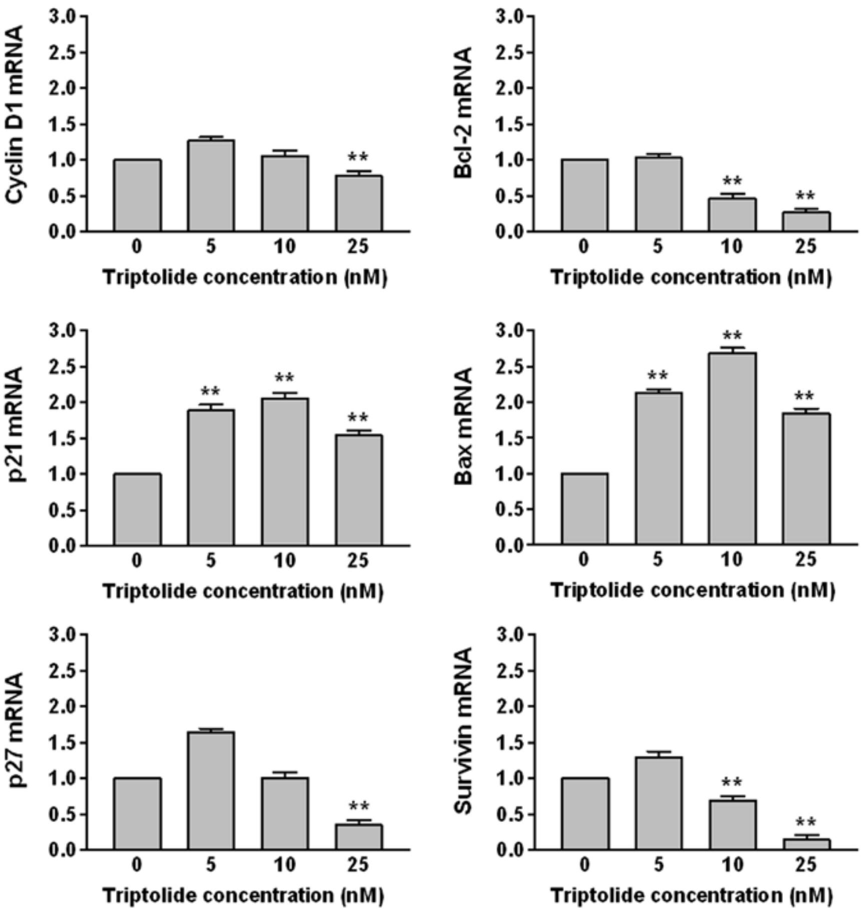

Figure 3. Effects of triptolide on the mRNA expression in THP-1 cells. Cells were treated with 5, 10 and 25 nM of triptolide for 48 h. mRNA levels were measured by real-time PCR. Values represent the mean ± SD of 3 independent experiments and were expressed as the relative mRNA accumulation corrected using β-actin mRNA as an internal standard. **P<0.01 compared to control.

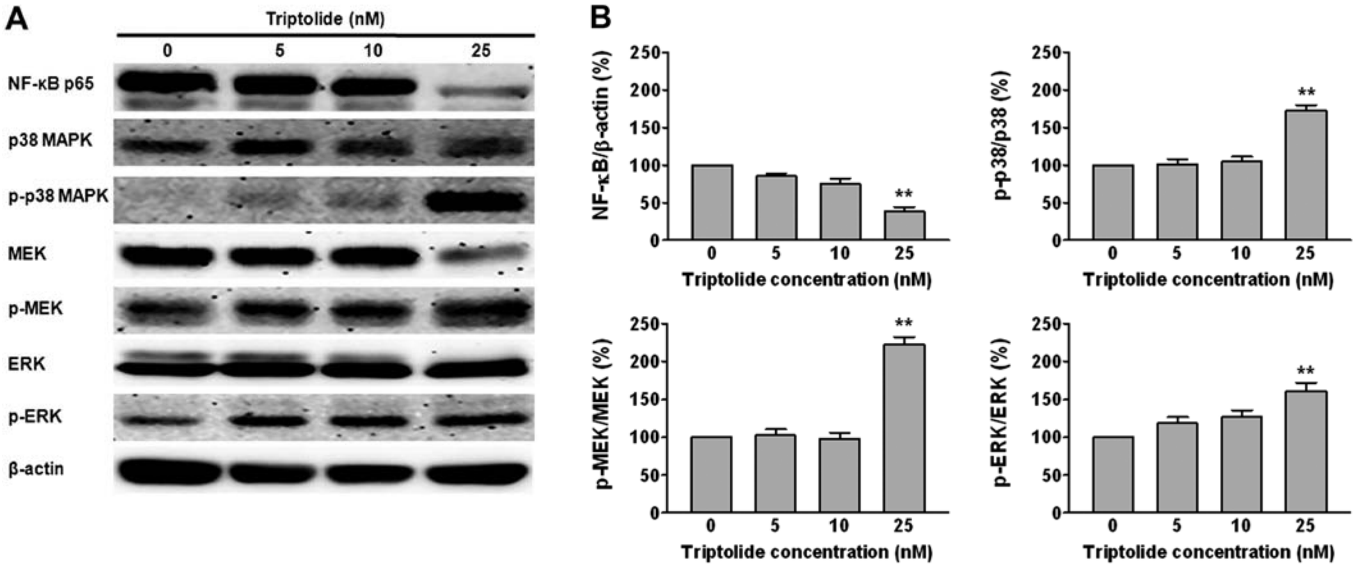

Figure 4. Effects of triptolide on NF-κB p65, p38 MAPK, MEK and ERK signaling pathways in THP-1 cells. Cells were treated with 5, 10 and 25 nM of triptolide for 48 h. Cells were lysed and 30 μg of soluble protein was separated by electrophoresis on an SDS-PAGE gel. (A) Protein expression was detected by western blot analysis using the antibodies. (B) Data are presented as the relative protein accumulation corrected using β-actin protein as an internal standard. Values represent the mean ± SD of 3 independent experiments. **P<0.01 compared to control.

manner. 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-X_L 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-X_L (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). Danshensu 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.

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References

1. Chen BJ: Triptolide, a novel immunosuppressive and anti-inflammatory agent purified from a Chinese herb *Tripterygium wilfordii* Hook F. Leuk Lymphoma 42: 253-265, 2001.
2. Qiu D and Kao PN: Immunosuppressive and anti-inflammatory mechanisms of triptolide, the principal active diterpenoid from the Chinese medicinal herb *Tripterygium wilfordii* Hook. f. Drugs R D 4: 1-18, 2003.
3. Shamon LA, Pezzuto JM, Graves JM, *et al*: Evaluation of the mutagenic, cytotoxic and antitumor potential of triptolide, a highly oxygenated diterpene isolated from *Tripterygium wilfordii*. Cancer Lett 112: 113-117, 1997.
4. Tengchaisri T, Chawengkirttikul R, Rachaphaew N, Reutrakul V, Sangsuwan R and Sirisinha S: Antitumor activity of triptolide against cholangiocarcinoma growth in vitro and in hamsters. Cancer Lett 133: 169-175, 1998.
5. Lee KY, Chang W, Qiu D, Kao PN and Rosen GD: PG490 (triptolide) cooperates with tumor necrosis factor-α to induce apoptosis in tumor cells. J Biol Chem 274: 13451-13455, 1999.
6. Chan EW, Cheng SC, Sin FW and Xie Y: Triptolide induced cytotoxic effects on human promyelocytic leukemia, T cell lymphoma and human hepatocellular carcinoma cell lines. Toxicol Lett 122: 81-87, 2001.
7. Tsuruo T, Naito M, Tomida A, *et al*: Molecular targeting therapy of cancer: drug resistance, apoptosis and survival signal. Cancer Sci 94: 15-21, 2003.
8. Debatin KM: Apoptosis pathways in cancer and cancer therapy. Cancer Immunol Immunother 53: 153-159, 2004.
9. Levine B, Sinha S and Kroemer G: Bcl-2 family members: dual regulators of apoptosis and autophagy. Autophagy 4: 600-606, 2008.
10. Yang Y, Liu Z, Tolosa E, Yang J and Li L: Triptolide induces apoptotic death of T lymphocyte. Immunopharmacology 40: 139-149, 1998.
11. Aggarwal BB: Nuclear factor-κB: the enemy within. Cancer Cell 6: 203-208, 2004.
12. Karin M: Nuclear factor-κB in cancer development and progression. Nature 441: 431-436, 2006.
13. Keyse SM: Protein phosphatases and the regulation of mitogen-activated protein kinase signaling. Curr Opin Cell Biol 12: 186-192, 2000.
14. Liu Q: Triptolide and its expanding multiple pharmacological functions. Int Immunopharmacol 11: 377-383, 2011.
15. Zhu W, Hu H, Qiu P and Yan G: Triptolide induces apoptosis in human anaplastic thyroid carcinoma cells by a p53-independent but NF-κB-related mechanism. Oncol Rep 22: 1397-1401, 2009.
16. Kim MJ, Lee TH, Kim SH, Choi YJ, Heo J and Kim YH: Triptolide inactivates Akt and induces caspase-dependent death in cervical cancer cells via the mitochondrial pathway. Int J Oncol 37: 1177-1185, 2010.
17. Pigneux A, Mahon FX, Uhalde M, *et al*: Triptolide cooperates with chemotherapy to induce apoptosis in acute myeloid leukemia cells. Exp Hematol 36: 1648-1659, 2008.
18. Yinjun L, Jie J and Yungui W: Triptolide inhibits transcription factor NF-kappaB and induces apoptosis of multiple myeloma cells. Leuk Res 29: 99-105, 2005.
19. Carter BZ, Mak DH, Schober WD, *et al*: Triptolide induces caspase-dependent cell death mediated via the mitochondrial pathway in leukemic cells. Blood 108: 630-637, 2006.
20. Kim R, Emi M and Tanabe K: Caspase-dependent and -independent cell death pathways after DNA damage (Review). Oncol Rep 14: 595-599, 2005.
21. Denault JB and Salvesen GS: Caspases: keys in the ignition of cell death. Chem Rev 102: 4489-4500, 2002.

22. Tao Y, Zhang ML, Ma PC, *et al*: Triptolide inhibits proliferation and induces apoptosis of human melanoma A375 cells. Asian Pac J Cancer Prev 13: 1611-1615, 2012.
23. Chao DT and Korsmeyer SJ: BCL-2 family: regulators of cell death. Annu Rev Immunol 16: 395-419, 1998.
24. Li J, Zhu W, Leng T, *et al*: Triptolide-induced cell cycle arrest and apoptosis in human renal cell carcinoma cells. Oncol Rep 25: 979-987, 2011.
25. Zhou GX, Ding XL, Huang JF, *et al*: Apoptosis of human pancreatic cancer cells induced by Triptolide. World J Gastroenterol 14: 1504-1509, 2008.
26. Lee KY, Park JS, Jee YK and Rosen GD: Triptolide sensitizes lung cancer cells to TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by inhibition of NF-κB activation. Exp Mol Med 34: 462-468, 2002.
27. Frese S, Pirnia F, Miescher D, *et al*: PG490-mediated sensitization of lung cancer cells to Apo2L/TRAIL-induced apoptosis requires activation of ERK2. Oncogene 22: 5427-5435, 2003.
28. Rubinfeld H and Seger R: The ERK cascade: a prototype of MAPK signaling. Mol Biotechnol 31: 151-174, 2005.
29. Fan M and Chambers TC: Role of mitogen-activated protein kinases in the response of tumor cells to chemotherapy. Drug Resist Updat 4: 253-267, 2001.
30. Davis RJ: The mitogen-activated protein kinase signal transduction pathway. J Biol Chem 268: 14553-14556, 1993.
31. Lee SJ, Park K, Ha SD, Kim WJ and Moon SK: Gleditsia sinensis thorn extract inhibits human colon cancer cells: the role of ERK1/2, G2/M-phase cell cycle arrest and p53 expression. Phytother Res 24: 1870-1876, 2010.
32. Yin Y, Guan Y, Duan J, *et al*: Cardioprotective effect of Danshensu against myocardial ischemia/reperfusion injury and inhibits apoptosis of H9c2 cardiomyocytes via Akt and ERK1/2 phosphorylation. Eur J Pharmacol 699: 219-226, 2013.
33. Stanciu M, Wang Y, Kentor R, *et al*: Persistent activation of ERK contributes to glutamate-induced oxidative toxicity in a neuronal cell line and primary cortical neuron cultures. J Biol Chem 275: 12200-12206, 2000.
34. Wang X, Martindale JL and Holbrook NJ: Requirement for ERK activation in cisplatin-induced apoptosis. J Biol Chem 275: 39435-39443, 2000.