Involvement of Mitochondrial Pathway in Triptolide-Induced Cytotoxicity in Human Normal Liver L-02 Cells

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Triptolide, a purified diterpenoid triepoxide compound derived from a traditional Chinese medicine, Tripterygium wilfordii Hook. f (TWHf), has been used in the treatment of autoimmune and inflammatory diseases. However, the toxicity of triptolide limits its application to a great extent. In the present study, we treated human normal liver L-02 cells (L-02 cells) with triptolide in vitro and investigated its toxic effects. The cytotoxicity was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for cellular viability and by flow cytometry and Hoechst 33258 staining for apoptosis. The mitochondrial membrane potential (ΔΨm) was evaluated by flow cytometry with JC-1 as probe. After treatment with triptolide, a decrease in the viability of L-02 cells and increase in apoptosis were observed. Triptolide-induced apoptosis was accompanied by loss of mitochondrial membrane potential and release of cytochrome c (cyt-c) from the mitochondria to the cytosol and down-regulation of anti-apoptotic protein Bcl-2 levels with concurrent up-regulation in pro-apoptotic protein Bax levels and tumor suppressor protein p53 levels. Triptolide-increased activity of caspase 9 and caspase 3 was also observed. These results indicate that triptolide induced cytotoxicity in L-02 cells by apoptosis, which is mediated through mitochondrial pathway.

Key words triptolide; cytotoxicity; human normal liver L-02 cell; mitochondria; apoptosis

Triptolide (Fig. 1), a highly oxygenated diterpene separated from Tripterygium wilfordii Hook. f (TWHf), is one of the main active components in TWHf.1) Many studies have shown that triptolide has various functions including immunosuppressive, anti-cancer, and contraceptive activities.2—4) The ethanol extract, ethyl acetate extract, and other extracts of TWHf containing triptolide have been used for the treatment of rheumatoid arthritis and autoimmune diseases clinically and triptolide was deemed to account for the immunosuppressive activity of the extracts.5—7) However, triptolide has severe toxicities on digestive, urogenital, and blood circulatory systems,8,9) which have largely limited its clinical application.

In recent years, many studies have shown that various extracts of TWHf containing triptolide could lead to hepatic injury in animals and humans.9—14) Our previous study has shown that oral administration of triptolide to rats leads to liver injury and death. Furthermore, Pharmacokinetics data in rats show that the concentrations of triptolide found in livers exceed those observed in other tissues such as spleen, lung, heart, and kidney (unpublished data). Based on our studies, we propose a hypothesis that liver is the major toxic target following triptolide treatment. Therefore elucidation of the possible mechanisms of hepatotoxicity is essential from a safety point of view.

In many types of tumor cells, triptolide has been shown to cause apoptotic cell death.15—19) The apoptotic effects of triptolide on tumor cells involves alterations of apoptotic signaling including decreased expression of anti-apoptotic proteins, and cytochrome c release as a result of mitochondrial damage.15) It is not known whether triptolide-induced hepatic injury is apoptotic or involves necrotic cell damage. It is important to understand the mechanisms of toxic effects in preventing liver damage of triptolide in clinical use.

The present study was conducted to investigate triptolide-induced toxicity in human hepatocytes and the possible role of mitochondrial pathway in the toxic effects. Under experimental conditions, triptolide inhibited proliferation and induced apoptosis of L-02 cells in a concentration-dependent manner. The results suggest that triptolide-induced apoptosis in L-02 cells is associated with increased expression of p53 and Bax protein and decreased Bcl-2 protein. Furthermore, the data show that triptolide treatment of L-02 cells causes loss of mitochondrial membrane potential (ΔΨm), releases of cytochrome c from the mitochondrial intermembrane space toward the cytosol, and proteolytic activation of caspases 9 and 3.

MATERIALS AND METHODS

Materials RPMI-1640 medium and fetal bovine serum were obtained from Gibco BRL (Grand Island, NY, U.S.A.). Dimethylsulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Hoechst 33258

Fig. 1. Structure of Triptolide

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were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). Triptolide was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (purity >98% (HPLC), Beijing, China). Triptolide was dissolved in DMSO and stock solutions (0.5 mg/ml) were stored at −20°C. Triptolide was freshly diluted to the indicated concentrations with culture medium before use. DMSO concentration in experimental conditions never exceeded 0.01% (v/v). 5,5′,6,6′-Tetra-chloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) was purchased from Molecular Probes (Leiden, Netherlands). Annexin V-FITC apoptosis detection kit was bought from Becton Dickinson (CA, U.S.A.). Anti-rabbit p53, Bcl-2, Bax, cytochrome c, and β-actin antibodies and HRP-conjugated goat anti-rabbit Ig were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). An enhanced chemiluminescence (ECL) detection kit was from Pierce Biotechnology Inc. (Rockford, IL, U.S.A.). Bradford protein assay kit and caspase 3, caspase 8, and caspase 9 activity assay kit were from Beyotime Institute of Biotechnology (Haimen, China). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

**Cell Culture** Human normal liver L-02 cells (Cell Bank, Type Culture Collection of Chinese Academy of Sciences) were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. Cells were incubated at 37°C in a humidified atmosphere (5% CO2).

**Cell Viability Assay** Cell viability was determined by MTT assay.20 Briefly, L-02 cells were seeded at an initial density of 5×10^4 cells/ml in a 96-well plate for 24 h. Cells were then incubated with fresh medium containing various concentrations of triptolide (0, 60, 120, 180 μM) for 24 h, or 120 μM of triptolide for 12, 24, and 48 h. After incubation, MTT was added into each well at a final concentration of 0.5 mg/ml. The insoluble formazan was collected and dissolved in dimethylsulfoxide (DMSO) and measured with an ELISA reader (Bio-Rad, U.S.A.) at a wavelength of 540 nm.

**Assessment of Cell Morphological Changes** L-02 cells were cultured and treated without or with triptolide (60, 120, 180 μM) for 24 h. After exposure, the culture plates were examined and photographed by phase contrast microscope (Nikon, Japan).

**Hoechst 33258 Staining** Cells cultured on slides were washed twice with PBS and fixed with 4% paraformaldehyde for 10 min then incubated for 10 min with Hoechst 33258 (5 μg/ml). After being washed and dried, the slides were examined by fluorescence microscopy (DIAPHOT300, Nikon, Japan).

**Annexin V/PI Staining Assay** Annexin V/PI staining assay was employed further to classify L-02 cells in early apoptosis and late apoptosis/necrosis stages. L-02 cells (1×10^5 cells/ml) in 10-cm culture dishes were treated without or with triptolide (60, 120, 180 μM) for 24 h. Cells were labeled with annexin V-FITC and PI using an apoptosis detection kit according to the manufacturer’s protocol. The cells were pelleted and analyzed in BD FACSCanto flow cytometer analyzer. Excitation wave was set at 488 nm and the emitted green fluorescence of Annexin V (FL1) and red fluorescence of PI (FL2) were collected using 525 and 575 nm band pass filters, respectively. A total of at least 10000 cells were analyzed per sample. Light scatter was measured on a linear scale of 1024 channels and fluorescence intensity on a logarithmic scale. The amount of early apoptosis and late apoptosis/necrosis was determined as the percentage of Annexin V+/PI− and Annexin V+/PI+ cells, respectively.

**Measurement of Mitochondrial Membrane Potential (Δψm)** The loss of Δψm was monitored with the dye 5,5′,6,6′-tetra-chloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1). JC-1 is capable of selectively entering mitochondria, where it forms monomers and emits green fluorescence when Δψm is relatively low. At a high Δψm, JC-1 aggregates and gives red fluorescence.21 The ratio between green and red fluorescence provides an estimate of Δψm that is independent of the mitochondrial mass. Briefly, L-02 cells (1×10^5 cells/ml) in 10-cm culture dishes were treated with or without triptolide (60, 120, 180 μM) for 24 h. Cells were trypsinized, washed in ice-cold PBS, and incubated with 10 μM JC-1 at 37°C for 20 min in darkness. Subsequently, cells were washed twice with PBS and analyzed by flow cytometry. Excitation wave was set at 488 nm and the emitted green fluorescence of Annexin V-FITC (FL1) and red fluorescence of PI (FL2) were collected using 525 and 575 nm band pass filters, respectively.

**Detection of Cytochrome c Release from the Mitochondria to the Cytosol** Cytochrome c determination in cytosolic and mitochondrial fractions was done by western blotting. The cells were harvested after the respective treatments and washed once with ice-cold PBS. For isolation of mitochondria and cytosol, the cells were sonicated in buffer containing 10 mM Tris–HCl pH 7.5, 10 mM NaCl, 175 mM sucrose, and 12.5 mM EDTA and the cell extract centrifuged at 10000 g for 10 min to pellet nuclei. The supernatant thus obtained was centrifuged at 180000 g for 30 min to pellet the mitochondria and purified as previously described.22 The resulting supernatant was termed the cytosolic fraction. The pellet was lysed and protein content estimated in both fractions by Bradford’s method. Equal amounts of protein were separated on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) then were electrotransferred to PVDF membrane (PolyScreen, NEN life Science Products, Inc. Boston, MA, U.S.A.). The membrane was then incubated in 5% non-fat milk in TBST (TBS: Tris-buffered saline, 10 mM Tris, 0.15 M NaCl, pH 7.6) and incubated with primary antibody (anti-β-actin antibodies and HRP-conjugated goat anti-rabbit Ig). After extensive washing with TBST, the immune complexes were detected by enhanced chemiluminescence detection kit.

**Western Blot Analysis** L-02 cells grown at a density of 1×10^5 cells/ml in 10 cm culture dishes were treated with or without triptolide (60, 120, 180 μM) for 24 h, then the cells were harvested and lysed with 50 mM HEPES buffer containing 150 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EGTA, 20 mM NaF, 50 mM β-glycerophosphate, 2 mM phenylmethylsulfonyl fluoride, 1 mM NaN3, 10 mg/m of leupeptin, and 10 mg/m of aprtinin and incubated on ice for 25—30 min. The lysates were centrifuged at 10000 g at 4°C for 12 min and the protein concentration in the supernatant was determined by Bradford protein assay kit. Equal amounts of
protein were separated on 10—15% SDS–PAGE then were electrotransferred to PVDF membrane (PolyScreen, NEN Life Science Products, Inc., Boston, MA, U.S.A.). The membrane was then incubated in 5% non-fat milk in TBST for 2 h followed by overnight incubation with the primary antibody separately. The incubated membranes were extensively washed with TBST before incubation for 2 h with the secondary antibody. After extensive washing with TBST, the immune complexes were detected by enhanced chemiluminescence detection kit.

**Assay for Caspase 8, Caspase 9, and Caspase 3 Activity**
The measurement of caspase 8, caspase 9, and caspase 3 activity was performed according to the manufacturer’s experimental instructions. In brief, cell lysates were incubated with substrates, Ac-IETD-pNA (for caspase 8), LEHD-pNA (for caspase 9), and Ac-DEVD-pNA (for caspase 3) in assay buffer. After further incubation at 37 °C for 3 h, the absorbance of samples was measured by ELISA reader (Bio-Rad, U.S.A.) at 405 nm.

**Statistical Analysis** Values are expressed as the mean±standard error (S.E.) of at least three experiments. Data were analyzed by one-way analysis of variance (ANOVA) with further post hoc tests using the statistical software SPSS 10.0. A value of $p<0.05$ was considered statistically significant.

**RESULTS**

**Effect of Triptolide on L-02 Cell Growth** MTT assay was used to quantify the effects of triptolide on L-02 cells growth. As shown in Fig. 2, triptolide decreased cell viability in L-02 cells in a concentration- and time-dependent manner when compared with the control. Treatment with triptolide for 24 h resulted in a significant decrease in cell viability by approximately 39.1% and 52.3% at 120 and 180 nM, respectively (Fig. 2A). To investigate the time-course of cytotoxic effects of triptolide, significant cell death induced by triptolide in L-02 cells was observed at as early as 12 h treatment (Fig. 2B).

**Effects of Triptolide on Morphological Changes in L-02 Cells** L-02 cells were used for investigating the potential cytotoxic effects of triptolide on human normal liver cells in vitro. Morphological changes induced by triptolide were observed concentration-dependently at 60, 120, and 180 nM under a phase contrast microscope. Under control conditions, cells appeared spindle-shaped. After exposure to triptolide for 24 h, L-02 cells shrank and retracted from their neighbors, accompanied with floating apoptotic cells in the culture medium (Fig. 3).

**Morphological Observation of Nuclear Change** Hoechst 33258 staining showed that there were significant morphological changes in the nuclear chromatin (Fig. 4). In the control group, the nuclei were stained a less bright blue and the color was homogeneous, however, when cells were treated with 120 nM of triptolide for 24 h, the blue emission light in apoptotic cells was much brighter than the control cells. Condensed chromatin and fragmented nuclei could also be found in many treated cells, which are classic characteristics of apoptotic cells.

**Differentiation of Apoptosis and Necrosis Induced by Triptolide** We characterized triptolide-induced apoptosis by Annexin V/PI dual staining. Apoptotic cells lose asymmetry of membrane phospholipid, which leaves phosphatidylser-
ine (PS) on the outer leaflet of the plasma membrane. One of the tools for studying apoptosis is to quantify the PS externalization by binding of Annexin V. PI is a non-specific DNA intercalating agent, which is excluded by the plasma membrane of living cells, and thus can be used to distinguish necrotic cells from apoptotic and living cells by supravital staining without prior permeabilization. This assay divides apoptotic cells into two stages: early (Annexin V+/PI−) and late apoptotic/necrotic (Annexin Annexin V+/PI+). As shown in Fig. 5A, treatment with various concentrations of triptolide (60, 120, 180 nM) for 24 h resulted in a concentration-dependent increase in both early and late apoptotic/necrotic cells. Furthermore, the percentage of early apoptotic cells was greater than that of late apoptotic/necrotic cells (Fig. 5B).

Mitochondria Membrane Potential (Δψm) Determination Disruption of mitochondrial integrity is one of the early events leading to apoptosis. To assess whether triptolide affects the function of mitochondria, potential changes in mitochondrial membrane were analyzed by employing a mitochondria fluorescent dye, JC-1. As shown in Fig. 6, exposure to triptolide for 24 h resulted in a significant decrease in the ratio between red and green fluorescence by approximately 85.8%, 67.5%, and 63.7% at 60, 120, and 180 nM, respectively. This suggests that treatment with various concentrations of triptolide (60, 120, 180 nM) for 24 h resulted in significant decreases of Δψm. The results imply that triptolide induces Δψm dissipation in a concentration-dependent manner.

Cytochrome c Release from Mitochondria to Cytosol Cytochrome c release from mitochondria is a critical step in the apoptotic cascade since this activates downstream caspases. To investigate the release of cytochrome c in triptolide-treated L-02 cells, we conducted western blotting in both the cytosolic and mitochondrial fractions. The results demonstrate a concentration-dependent increase in the cytosolic cytochrome c after treatment with triptolide. Simultaneously, there was a decrease in cytochrome c in the mitochondrial fraction. The mean relative densities (versus control) were reduced to 80.1%, 49.6%, and 33.7% in the mitochondrial cytochrome c level at 60, 120, and 180 nM, respectively. This was accompanied by simultaneous increases in cytochrome c level in the cytosol to 121.3%, 154.5%, and 181.6%, respectively (Fig. 7).

Expression of p53, Bax, and Bcl-2 Protein Death-promoting members of the Bcl-2 family such as Bax and Bid play key roles in the chemical-induced release of cytochrome c. Bid and Bax in the cytosol receive death signals from upstream events and induce the release of cytochrome c, thereby activating the mitochondrial apoptotic pathway. Fig. 7 shows details of the levels of Bax, Bcl-2, and p53 in whole-
cell lysates of L-02 cells treated with triptolide. Triptolide could up-regulate the expression of p53 and Bax, and down-regulated the expression of Bcl-2 in a concentration-dependent manner (Fig. 7) compared with control.

**Triptolide Activation of Caspase 9 and Caspase 3**

It has been previously demonstrated that triptolide induces apoptosis in different cell types by a pathway dependent on caspase activation.\(^{15,26}\) In the current study, we demonstrated that triptolide increased caspase 9 and caspase 3 activities in a concentration-dependent manner (Fig. 8).

**DISCUSSION**

The Chinese medicinal herb *Tripterygium wilfordii* Hook. f and one of its main active components, triptolide, a diterpenoid triepoxide, have been shown to have anti-inflammatory,\(^{27,28}\) contraceptive,\(^{29,30}\) and anti-neoplastic\(^{8,31}\) activities. However, the clinical uses of triptolide were limited because of its severe toxicities. While previous studies have shown that triptolide induces apoptotic cell death through a mitochondrial pathway in several types of tumor cells,\(^{15,32,33}\) this is the first study that demonstrates involvement of mitochondrial pathway in triptolide-induced cytotoxicity and apoptosis in L-02 cells, a normal human liver cell line. This could explain the triptolide-induced liver damage in human cells in vitro. The current study indicates that this agent causes toxic effects in normal liver cells by similar mechanisms as it does in tumor cells. Moreover, the apoptotic effects of triptolide in normal liver cells occur at concentrations close to those of its antitumor activities.\(^{15}\) The apoptotic effects of triptolide on tumor cells suggest its usefulness in treatment for leukemias and other malignancies.\(^{15,17}\) The current study revealed that liver damage of triptolide occurs by mechanisms of apoptosis, although the exact mechanism of how triptolide causes liver damage needs further investigation. It is possible that blocking apoptotic signaling during clinical use of this agent could protect the liver from triptolide injury. However, the antitumor activities of this agent would be reduced or blocked as a result of blocking its effects in tumor cells. The findings in the current study suggest that it is necessary closely to monitor liver functions and therefore prevent severe liver damage when triptolide is clinically used.

Apoptosis can be initiated via two alternative signaling pathways: the death receptor-mediated extrinsic apoptotic pathway and the mitochondrion-mediated intrinsic apoptotic pathway.\(^ {34,35}\) Mitochondria play critical roles in the regulation of various apoptotic processes including drug-induced apoptosis.\(^{36}\) The mitochondrial death pathway is controlled by members of the Bcl-2 family, which play a central regulatory role to decide the fate of the cells via the interaction between pro- and anti-apoptotic members.\(^ {37,38}\) The Bcl-2 family consists of pro-apoptotic and anti-apoptotic members.\(^ {39}\) During apoptosis, Bcl-2 family pro-apoptotic proteins including Bim, Bax and Bid can translocate to the outer membrane of mitochondria, promote the release of pro-apoptotic factors, and induce apoptosis. On the other hand, Bcl-2 family anti-apoptotic proteins including Bcl-2 and Bcl-XL, sequestered in mitochondria, inhibit the release of pro-apoptotic factors and prevent apoptosis. When interacting with activated pro-apoptotic proteins, the anti-apoptotic proteins lose inhibiting ability of pro-apoptotic factors’ release, and again promote apoptosis. Alteration in the levels of anti- and pro-apoptotic Bcl-2 family proteins influences apoptosis.\(^ {40}\) In the present study, cells treated with triptolide increased the level of the pro-apoptotic protein Bax, and decreased the level of the anti-apoptotic protein Bcl-2, suggesting that triptolide induced apoptosis in L-02 cells by modulating Bcl-2 family proteins.
Early cellular apoptosis is accompanied by disruption of the mitochondrial membrane, resulting in a rapid collapse in the electrochemical gradient. The significance of cytochrome c from the mitochondria into the cytosol. The significance of cytochrome c to the apoptotic process was revealed by the finding that mitochondrially released cytochrome c combines with apoptosis protease activating factor-1, procaspase-9, and dATP in the cytosol, producing active caspase-9. The activation of the mitochondrial pathway by toxicants frequently results in the release of cytochrome c from the mitochondria into the cytosol.

In conclusion, our data indicate that triptolide induced apoptosis in L-02 cells via mitochondrial pathway, and these findings may provide new insights for understanding the toxic effects of triptolide.

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