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Full Length Research Paper

Tripterine induces apoptosis of human acute myelocytic leukemic cells via up-regulating Fas/FasL and down-regulating NF-κB

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This study investigated the mechanism underlying the effect of tripterine on apoptosis of human acute myelocytic leukemic cells (HL-60). The apoptosis of HL-60 cells after treatment with triperine of different dosages and durations were determined using flow cytometry and transmission electron microscopy. The expressions of Fas, FasL and NF-κB in the HL-60 cells treated with 1.5 μmol/L tripterine for different durations were measured by flow cytometry. Our results showed treatment with 0.5 ~ 2.5 μmol/L tripterine for 24 h could induce apoptosis of HL-60 cells to different extents (apoptotic rate: 6.13 ~ 36.71%) in which the maximal apoptotic rate was observed after 1.5 μmol/L tripterine treatment for 24 h. In addition, 1.5 μmol/L tripterine could cause gradually increased apoptotic rates in a time-dependent manner presenting typical apoptotic morphology in the HL-60 cells. In the 1.5 μmol/L tripterine-treated HL-60 cells, the number of Fas and FasL positive cells was significantly increased ($P < 0.05$) accompanied by a markedly decreased number of NF-κB positive cells ($P < 0.05$), which was in a time dependent manner. Tripterine could effectively induce apoptosis of HL-60 cells and its antitumor mechanism may be related to both the up-regulation of Fas and FasL and down-regulation of NF-κB.

Key words: Tripterine, HL-60 cells, apoptosis, Fas/FasL, NF-κB, regulation.

INTRODUCTION

Recently, numerous reports reveal that apoptosis is closely related to the generation and development of certain kinds of cancers and the curative effect of anti-neoplastic drugs (Nakahara et al., 2003; Birkenkamp et al., 2004). Chemotherapy with large-dose drugs may cause necrosis of cancer cells. However, some drugs at low doses play anti-neoplastic roles through inducing apoptosis of cancer cells (Luo et al., 2005). Since almost of all the currently used anti-neoplastic drugs in clinical practice have various side effects, apoptosis elicited by low dosage of the anti-neoplastic drugs has been a hot topic in the research of chemotherapy for cancers.

Currently, many active components from plants have been confirmed to have the capability to induce apoptosis of cancer cells *in vitro* and *in vivo* (Luo et al., 2005; Tsang et al., 2009; Zhang et al., 2009), which has attracted extensive attention in the field of Medicine. Tripterine is a monomeric chemical compound extracted from *Tripterygium wilfordii*, a Chinese traditional herb belonging to Euonymus family (Yang et al., 2006). It belongs to a member of triterpene pigments and its molecular formula is $C_{29}H_{38}O_4$ with a molecular weight of 450 kD (He et al., 2009; Zhang et al., 2006). It is reported that tripterine not only has anti-inflammatory and immunosuppressive effects (Li et al., 2008; Kiaei et al., 2005), but also possesses anti-neoplastic activity to a variety of cancers (Dai et al., 2009; Huang et al., 2008; Abbas et al., 2007). However, the specific anti-neoplastic mechanism of tripterine is still poorly understood.

The solid correlations of Fas and FasL (Friesen et al., 1996; Jin et al., 2009), and NF-κB associated signaling in

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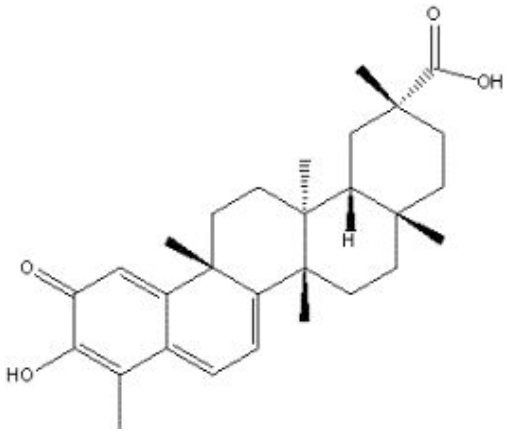

Figure 1. Basic chemical structure of tripterine.

apoptosis have been well investigated (Mankan et al., 2009; Sarkar et al., 2008). In this study, flow cytometry and transmission electron microscopy were performed to determine the effect of tripterine on the apoptosis of HL-60 cells, a cancer cell line derived from human acute myelocytic leukemia. Furthermore, the expression levels of Fas, FasL and NF-κB were also measured to investigate the anti-neoplastic mechanism of tripterine.

MATERIALS AND METHODS

Materials

HL-60 cells were provided by the Institute of Basic Medicine, Chinese Academy of Medical Sciences (Beijing, China). Fas-FITC, FasL-FITC monoclonal antibody and Annexin V apoptosis kit were purchased from Sino-American (Luoyang, China). Rabbit anti-human NF-κBP65 monoclonal antibody was from Bio-Lab (Beijing, China). RPMI-1640, acridine orange (AO), ethidium bromide (EB), Hoec-hst 33258, Propidium iodide (PI) were all purchased from Sigma (USA). Tripterine was kindly provided by the Pharmaceutical Research Division, Affiliated Zhongshan Hospital of Fudan University, China. The basic chemical structure of tripterine is shown in Figure 1.

Cell lines and culture

The HL-60 cells were obtained from Cell Bank of the Shanghai Institute of Cell Biology, Chinese Academy of Science. The cells were maintained in RPMI-1640 medium (GIBCO Invitrogen, NY, USA) supplemented with 10% fetal calf serum (FCS, GIBCO), 100 U/ml penicillin and 100 μg/ml streptomycin (Thermo Scientific HyClone, USA) at 37°C in a humidified atmosphere of 5% CO_2.

Tripterine treatment

HL-60 cells were seeded into 24-well plates (Corning, USA) at a density of 1×10^6/ml. The cells were pre-incubated in 5% CO_2 at 37°C for 24 h. The medium was then replaced with fresh RPMI1640 medium containing tripterine of different concentrations (0.5, 1.0, 1.5 and 2.0 μmol/L) followed by incubation for an additional 24 h. In another experiment, the HL-60 cells were treated with 1.5 μmol/L tripterine at 37°C for 0, 4, 8, 16 or 24 h. The cells were harvested by centrifugation at 2000 rpm for 10 min and washed with PBS (0.01 mol/L, pH7.4) three times. At the same time, cells without tripterine treatment were used as negative controls.

Detection of apoptosis by flow cytometry

Cell apoptosis was detected using Annexin V-FITC Apoptosis Detection Kit (BioVision, CA, USA) according to the instructions of the manufacturer. Briefly, the tripterine-treated HL-60 cells were collected and re-suspended in the FITC-conjugated annexin-V buffer followed by incubation for 15 min in dark. After PI was added, the apoptosis was immediately detected by FACSCalibur flow cytometry (Beckman Coulter XL, USA) and the data were analyzed with CellQuest Pro software (Beckman Coulter, Fullerton, CA). Apoptotic cells can bind to annexin-V but not PI (annexin V^+/PI^-), whereas the necrotic cells can bind annexin-V and PI (annexin V^+/PI^+).

Detection of apoptotic morphology by transmission electron microscopy

After treatment with 1.5 μmol/L tripterine for 24 h, the HL-60 cells were fixed in 2.5% formaldehyde for 2 h at 4°C. The fixed cells were then washed with PBS three times followed by post-fixation with osmium tetroxide. These fixed cells were rinsed, dehydrated and embedded in epoxy resin (Sigma, USA), and ultrathin sections were collected on 100 to 150 mesh nickel grids (Plano, Wetzlar, Germany). Lead citrate and uranyl acetate were used for staining and the stained sections were examined by a transmission electron microscope (Philips, TECNAI-10, Holland).

Detection of Fas and FasL expressions

After treatment with 1.5 μmol/L tripterine for 0, 12 and 24 h, the HL-60 cells were re-suspended in PBS. Then, 100 μl of each suspension were mixed with 10 μl of mouse anti-human Fas or FasL monoclonal antibody conjugated to FITC (eBioscience, USA) followed by washing three times with PBS. In addition, IgG1-FITC was used as negative control. The mixtures were incubated at 37°C for 30 min. The percentages of Fas and FasL positive cells were measured by FACSCalibur flow cytometry and analyzed with CellQuest Pro software (Beckman Coulter, Fullerton, CA).

Detection of NF-κB expression

After treatment with 1.5 μmol/L tripterine for 0, 12 and 24 h, the HL-60 cells were re-suspended in PBS. Then, 100 μl of each suspension were mixed with 100 μl of rabbit anti-human NF-κBP65 antibody (Jackson ImmunoResearch Laboratories Inc., USA). The mixture was incubated at 37°C for 30 min followed by washing three times with PBS. Thereafter, FITC conjugated mouse anti-rabbit antibody (Jackson ImmunoResearch Laboratories Inc., USA) was added followed by incubation at 37°C for 30 min in dark. These cells were then washed three times with PBS and fixed in 1% paraldehyde. The percentages of NF-κB positive cells in specimens were measured by FACSCalibur flow cytometry and analyzed with CellQuest Pro software (Beckman Coulter, Fullerton, CA).

Statistical analysis

Data of apoptotic rate and the expression levels of Fas, FasL and NF-κB were expressed as the mean ± standard deviation ($\bar{x} \pm s$). One-way analysis of variances (ANOVA) followed by Dunnett's

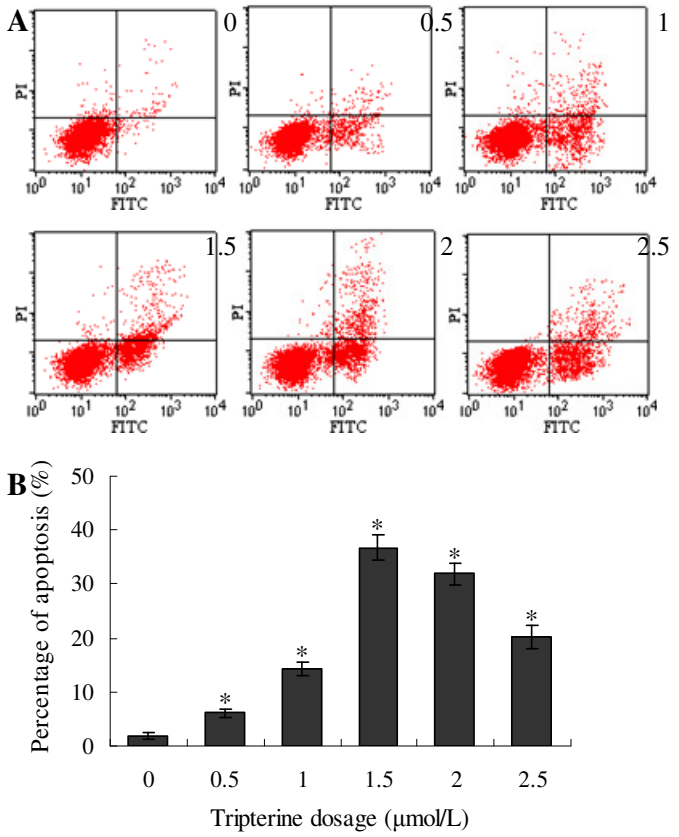

Figure 2. Apoptosis of HL-60 cells treated with tripterine of different concentrations for 24 h (n = 3). (A) Apoptosis of HL-60 cells treated with tripterine of different concentrations for 24 h was detected by FITC-annexin V/PI flow cytometry: X axis stands for FITC which indicated an increase of the apoptosis percentage, while Y axis stands for PI. Apoptosis was found in HL-60 cells treated with tripterine of different concentrations for 24 h, and the percentages of them were significantly higher than the blank control. *: P < 0.05 vs. blank control. (B) Comparing the apoptotic percentages in HL-60 cells treated with different concentrations for 24 h, it peaked at 1.5 μmol/L.

multiple comparisons were used to determine the significant differences. A value of P < 0.05 was considered statistically significant.

RESULTS

Apoptotic rates of tripterine-treated cells

As shown in Figure 2, treatment with 0.5 ~ 2.5 μmol/L tripterine for 24 h could induce evident apoptosis of HL-60 cells (Figure 2, P < 0.05), in which the highest apoptotic rate was observed after treatment with 1.5 μmol/L tripterine. Unexpectedly, the higher dosages of tripterine (2.0 and 2.5 μmol/L) resulted in relatively lower apoptotic rates. Furthermore, at 1.5 μmol/L tripterine doasge, the apoptotic rates of HL-60 cells were increased in a time dependent manner (Figure 3).

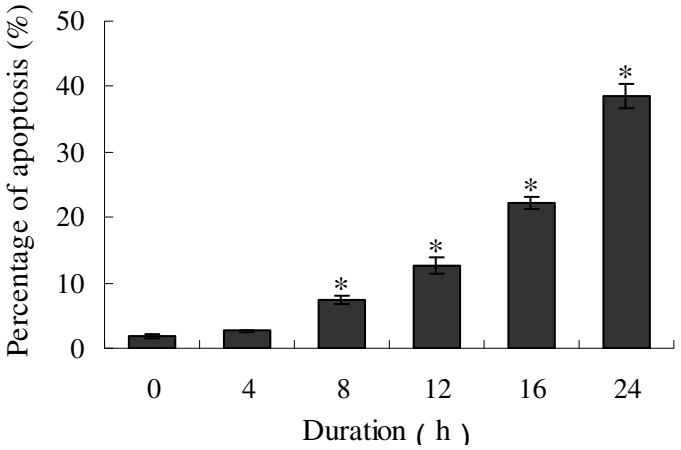

Figure 3. Apoptosis of HL-60 cells treated with 1.5 μmol/L tripterine for different durations. Apoptosis rate of HL-60 cells treated with 1.5 μmol/L tripterine increased with the duration, which was significantly higher than the blank control. *: P<0.05 vs. blank control.

Apoptotic morphology of tripterine-treated cells

After the HL-60 cells were treated with 1.5 μmol/L tripterine for 24 h, typical apoptotic features such as crescent-shaped nuclei and apoptotic body could be found (Figure 4). Furthermore, the tripterine-treated cells displayed circular and shrunken shapes, with plenty of vacuoles in the cytoplasm.

Expressions of Fas, FasL and NF-κB in tripterine-treated cells

Treatment of the HL-60 cells with 1.5 μmol/L tripterine for 12 and 24 h brought about elevated expressions of both Fas and FasL (P < 0.05). On the contrary, percentages of NF-κB positive cells were remarkably decreased when treated with 1.5 μmol/L tripterine for 24 h (P < 0.05) (Table 1). Nevertheless, the expressions of Fas, FasL and NF-κB exhibited a time dependent manner following the tripterine treatment.

DISCUSSION

Apoptosis, also called programmed cell death (PCD), is one of important mechanisms controlling cell proliferation, differentiation and death (Dirks et al., 1997). Particularly, induction of cancer cell apoptosis by drugs derived from natural components of plants is considered to be a practical and promising strategy for anti-neoplastic therapy (Tsang et al., 2009; Zhang et al., 2009; Chu et al., 2009). So far, several anti-neoplastic drugs derived from plants have been used in clinical practice, such as vinblastine, vinflunine and paclitaxel (Kruczynski et al., 2002; Zunino and Storms, 2009; Ohguri et al., 2009).

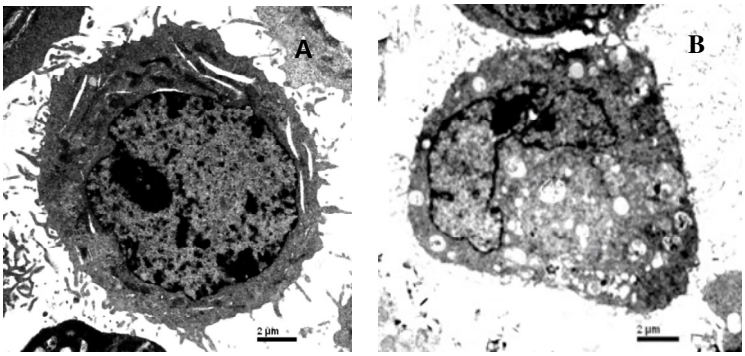

Figure 4. Morphology of HL-60 cells treated with 1.5 µmol/L tripterine. A: normal HL-60 cells; B: HL-60 cells after treatment with 1.5 µmol/L tripterine for 24 h.

Table 1. Expressions of Fas, FasL and NF-κB in tripterine-treated HL-60 cells (n = 3).

Duration (h)	Percentage (%)					
	Fas positive cells	Fas negative control	FasL positive cells	FasL negative control	NF-κB positive cells	NF-κB negative control
0	14.23 ± 1.42	14.82 ± 1.16	15.61 ± 1.10	14.95 ± 1.94	8.34 ± 1.52	8.69 ± 1.38
12	47.91 ± 1.21	17.79 ± 0.92	37.92 ± 1.73	18.64 ± 2.47	7.11 ± 1.29	8.14 ± 1.91
24	65.74 ± 1.20	21.95 ± 3.61	43.50 ± 2.11	18.13 ± 3.82	4.78 ± 0.30	6.91 ± 1.20

After HL-60 cells were treated with 1.5 µmol/L tripterine for 12 and 24 h, the percentages of Fas and FasL positive cells were significantly higher that of their negative control($P < 0.05$), and after treatment for 24 h, the percentage of NF- κB positive cells was significantly lower than its negative control ($P < 0.05$).

Recently, increasing evidence has been reported for components extracted from plants including tripterine to possess the potential as novel antitumor drugs (Hur et al., 2009; MacCarrone et al., 2000).

HL-60 is a cell line from human acute myelocytic leukemia (Nagase et al., 2003) which is a malignant tumor (Jemal et al., 2008). In the present study, our results demonstrated that 0.5 ~ 1.5 µmol/L tripterine could induce apoptosis of HL-60 cells in a dose dependent manner, in which 1.5 µmol/L tripterine caused the highest apoptotic rate. Unexpectedly, tripterine of higher doses (2.0 and 2.5 µmol/L) produced relatively lower apoptotic rates in the HL-60 cells when compared to 1.5 µmol/L tripterine, implying the presence of effective concentration threshold of tripterine. It was surprising that the apoptotic rate of HL-60 cells decreased rather than increased when treated with over 2.0 µmol/L tripterine, which indicated the presence of effective concentration threshold of tripterine but the mechanism still needs to be further studied. Under a transmission electron microscope, the HL-60 cells treated with 1.5 µmol/L tripterine also presented typical apoptotic morphology such as crescent-shaped nuclei and apoptotic body. All these results above confirmed that tripterine had anti-neoplastic effects via inducing cancer cell apoptosis.

Anti-neoplastic drugs can not only directly kill tumor cells, but also induce cancer cell apoptosis by activating expression of apoptotic genes. It is reported that the apoptosis of leukemic cells caused by chemotherapeutics is related to the interaction between Fas/FasL (Zhang et al., 2006; Friesen et al., 1996). Fas receptor is a type I transmembrane protein and its ligand (FasL) is a type II membrane protein, both of which belong to the receptor family of tumor necrosis factors (Friesen et al., 1996; Jin et al., 2009). The interaction between Fas and FasL can switch on death signal transduction resulting in apoptosis of target cells (Friesen et al., 1996; Jin et al., 2009; Fathi and Karp, 2009). In the present study, our results demonstrated the tripterine-treated HL-60 cells exhibited significantly higher expressions of both Fas and FasL than the tripterine-untreated cells ($P < 0.05$). This indicates that up-regulation of Fas and FasL required to activate and amplify intracellular apoptotic signals may be one of the important anti-neoplastic mechanisms of tripterine.

Apoptosis is a complicated cell death process which is controlled by many related genes. NF-κB is a nuclear transcription factor regulating numerous target genes and plays a critical role in NF-κB signal transduction pathway involved in inflammation, immune response, cell proliferation and apoptosis. Usually, NF-κB can regulate a series of genes related to cell survival presenting an inhibitive effect on apoptosis (Sarkar et al., 2008; Sethi et al., 2007). Furthermore, the elevation of NF-κB expression and activity is closely associated with the

resistance of cancer cells to chemotherapy (Nakahara et al., 2003; Lee et al., 2006). The significantly low NF-κB levels after 1.5 μmol/L tripterine treatment in the present study suggested that the inhibition of NF-κB expression might be another anti-neoplastic mechanism of tripterine.

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