Tanshinone IIA inhibits leukemia THP-1 cell growth by induction of apoptosis

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Abstract. Tanshinone IIA, a diterpene quinone extracted from the traditional herbal medicine, *Salvia miltiorrhiza* Bunge, has been reported to have anti-tumor effects on a large variety of cancer cells. The present study was undertaken to investigate the *in vitro* antiproliferation and apoptosis inducing effects of Tanshinone IIA on leukemia THP-1 cell lines and its mechanisms of action. MTT assay was used to detect the cell growth inhibitory rate; cell apoptotic rate and the mitochondrial membrane potential ($\Delta\psi m$) were investigated by flow cytometry (FCM), apoptotic morphology was observed by Hoechst 33258 staining and DNA fragmentation analysis. The expression of caspase-3 and different apoptosis modulators were analyzed by Western blotting. The results revealed that Tanshinone IIA inhibited the growth of THP-1 cells and caused significant apoptosis, the suppression was both in time- and dose-dependent manner. After treatment by Tanshinone IIA for 48 h, the percentage of disruption of $\Delta\psi m$ gradually increased in a dose-dependent manner along with marked changes of cell apoptosis. Western blotting showed cleavage of the caspase-3 zymogen protein (32-kDa) with the appearance of its 20-kDa subunit and a dose-dependent cleavage of PARP, with the appearance of 89-kDa fragment; The expression of Bcl-2 and survivin was down-regulated remarkably while Bax expression was up-regulated concurrently after the cells were treated with Tanshinone IIA for 48 h. We therefore conclude that Tanshinone IIA has significant growth inhibition effects on THP-1 cells by induction of apoptosis, and that Tanshinone IIA-induced apoptosis on THP-1 cells is mainly related to the disruption of $\Delta\psi m$ and activation of caspase-3 as well as down-regulation of anti-apoptotic protein Bcl-2, survivin and up-regulation of pro-apoptotic protein Bax. The results indicate that Tanshinone IIA may serve as a potential anti-leukemia reagent.

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Introduction

Recently, the use of herbal medicines to prevent the development as well as recurrence of a large variety of malignant diseases has become widely accepted as a realistic option for the treatment of malignant disease. There has been intense effort not only to identify new herbal medicines but also to understand how the existing constituents exhibit their activities (1).

Danshen (*Salvia miltiorrhiza* Bunge) is a widely used Chinese herbal medicine; its extracts contain diterpene quinine and phenolic acid derivatives, including tanshinone (I, IIA and IIB), cryptotanshinone, isocryptotanshinone, miltirone, tanshinol (I and II) and salviol (2). Tanshinone IIA is a derivative of phenanthrene-quinone isolated from Danshen (Fig. 1); it has anti-oxidant properties, inhibiting the association of lipid peroxidation products with DNA by breaking the chain reactions of peroxidation by scavenging lipid free radicals (3-5). Recent studies have shown that Tanshinone IIA has significant anti-proliferation effects by inducing apoptosis against multiple human cancer cell lines such as human breast cancer (6) and hepatocellular carcinoma (7).

Apoptosis (programmed cell death), a form of cell death defined by a characteristic set of morphological and biochemical changes which is a highly regulated process that involves the activation of a series of cellular events leading to cell death, plays an important role in the prevention of cancer development, and impaired apoptosis is now recognized to be a key step in tumorigenesis (8). Apoptosis is mediated through at least three major pathways, which are regulated by the death receptors, mitochondria and endoplasmic reticulum. Activation of apoptosis pathways is a key mechanism by which cytotoxic drugs kill tumor cells, and defects in apoptosis signalling contribute to tumor cell drug resistance (9,10). Thus, induction of apoptosis has now been considered as an important method of assessment for the clinical effectiveness of many anti-tumor drugs and a significant index for the selection of new anti-tumor drugs (10,11).

Though Tanshinone IIA has been proved to be very effective in treating a variety of malignancies, many of its anti-tumor mechanisms have not been demonstrated. Currently there is no detailed laboratory evidence showing the

mechanisms of Tanshinone IIA on leukemic THP-1 cells. In order to clarify some of its anti-leukemia mechanisms, we investigated the apoptotic effects of various concentrations of Tanshinone IIA (10-50 μmol/l) on THP-1 cells *in vitro*, and detected the expressions of caspase-3 as well as some gene expression that related to apoptosis to provide laboratory evidence of Tanshinone IIA for its mechanisms in the treatment of leukemia.

Materials and methods

Reagent. Tanshinone IIA, isolated from *Salvia miltiorrhiza* Bunge, was provided by Professor Pei-Qing Liu, Institute of Pharmacy College, Sun Yat-sen University. Hoechst 33258 was purchased from Sigma Company. The antibodies used in this study, anti-Bcl-2, Bax, Bid, Bad and Bak as well as anti-survivin were purchased from Santa Cruz Company (Germany). Antibodies against caspase-3 and poly (ADP-ribose) polymerase (PARP) were from Upstate Inc.

Cell culture. Human leukemia cell line THP-1 T-cells (purchased from Shanghai Rui-jin hospital) were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated calf serum, and 100 U/ml penicillin in a humidified 5% incubator at 37˚C. Cells were passaged twice weekly and routinely examined for mycoplasma contamination.

Cell growth inhibitory rate. Cell growth inhibitory rate was assayed using the microculture tetrazolium method. Briefly, $2x10^5$ cells/well were dispensed within 96-well culture plates in 100 ml volumes. Then different concentrations of Tanshinone IIA (10, 20, 30, 40 and 50 μmol/l) were put in different wells. Every one of the concentrations above was regarded as one treated group while the control group contained no Tanshinone IIA. Each of the treated or control group contained 6 parallel wells. After culture plates were incubated for 0, 24, 48 and 72 h, 20 μl of MTT working solution was added and then incubated continuously for 4 h. All culture medium supernatant was removed from the wells after centrifugation and replaced with 100 μl of DMSO. Following thorough solublization, the absorbance (A value) of each well was measured using a microculture plate reader at 570 nm. Cell inhibitory rate was calculated according to the formula: inhibitory rate =100x (A value of control group-A value of treated group)/A value of control group. The 50% inhibitory concentration (IC_{50}) was determined from dose-response data from at least 3 indepen-dent experiments.

Flow cytometry analysis for cell apoptosis. For FCM analysis, $2x10^6$ cells treated with different concentrations of Tanshinone IIA were collected, pelleted, washed with PBS, and fixed in 75% ethanol at -20˚C overnight. Prior to analysis, cells were washed again with PBS and resuspended and treated with RNase 200 mg/l for 30 min at 37˚C, cells were then incubated with 20 mg/l PI in the darkness for 15 min. Then, the suspension was passed through a nylon mesh filter and analyzed using flow cytometry (Becton-Dickinson). All data were collected and analyzed by lysis II software (Becton-Dickinson). The experiments were repeated 3 times and the results were presented as mean ± SD.

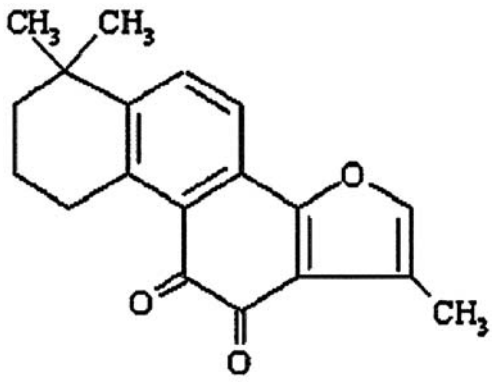

Figure 1. Molecular structure of Tanshinone IIA.

DNA fragmentation assay. Apoptosis was confirmed by detection of fragmentation of chromosomal DNA with the classic DNA ladder method. Briefly, $2x10^6$ cells were immersed in cytolysis buffer and incubated for 3 h at 50˚C. DNA was extracted with phenol-chloroform, precipitated in 1/10 volume of NaAc 2 mol/l and 2 volumes of ethanol at -20˚C overnight, recovered by centrifugation at 1000 x g for 30 min at 4˚C, and then resuspended in TE buffer. RNase A was then added at a concentration of 200 mg/l, then the treated extract was incubated at 37˚C for 30 min and electrophoresed on a 1.2% agarose gel.

Hoechst 33258 staining. Hoechst 33258 (purchased from Sigma Company) staining was used to observe the apoptotic morphology of THP-1 cells especially after the cells treated with Tanshinone IIA for 48 h. Cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) for 10 min, stained by Hoechst 33258 (10 mg/l) for 1 h, and then subjected to fluorescence microscopy.

Analysis of the mitochondrial membrane potential ($\Delta\psi m$). The mitochondrial membrane potential ($\Delta\psi m$) was measured by FCM using the intramitochondrial dye JC-1 (Alexis Biochemical Co, Germany) after THP-1 cells treated with Tanshinone IIA for 48 h. The detection procedure was performed according to the manufacturer's instructions. Data were converted to dot plots using CellQuest software (Becton-Dickinson).

Western blot analysis. For Western blot analysis, $2x10^6$ cells were washed with ice-cold PBS twice and lysed for 30 min at 4˚C, then debris was removed by centrifugation for 15 min at 15,000 x g at 4˚C, and equivalent amounts of protein were separated by 10% SDS-PAGE and transferred onto nitrocellulose filter. The filters were first stained to confirm uniform transfer of all samples and then incubated in blocking solution for 2 h at room temperature. The filters were reacted firstly with monoclonal antibody (anti-caspase-3 and anti-PARP as well as anti-Bcl-2, Bid, Bad, Bak and Bax as well as survivin) at a dilution of 1:1000 for 2 h, followed by extensive washes with PBS twice and TBST twice. Filters were then incubated with 1:1000 diluted horseradish peroxidase-conjugated secondary antibody and washed with TBST. As an internal control, ß-actin was also detected. The

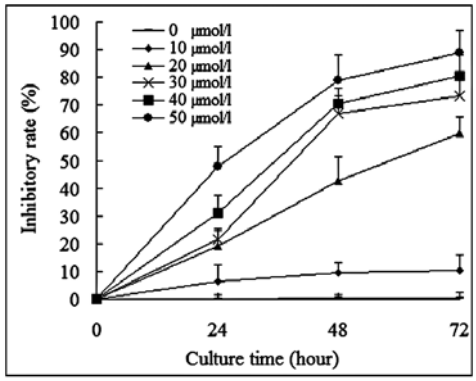

Figure 2. Cell growth inhibitory rate caused by Tanshinone IIA. After THP-1 cells were treated with different concentrations of Tanshinone IIA, MTT assay was used to detect cell growth inhibitory rate as described in Materials and methods. The inhibitory rate of Tanshinone IIA between 40-50 μmol/l is much higher than that of lower concentrations of Tanshinone IIA ($p<0.01$).

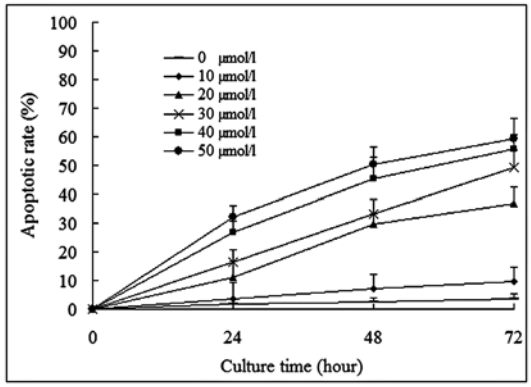

Figure 3. Cell apoptotic rate caused by Tanshinone IIA. After THP-1 cells were treated with different concentrations of Tanshinone IIA, cell apoptosis was analyzed by FCM. Tanshinone IIA (>20 μmol/l) induced apoptosis when cultured with THP-1 cells after 24-72 h. The percentage of apoptotic cells was gradually increased in a time- and dose-dependent manner. The percentage of apoptosis was high and over 50% when the cells were treated with 50 μmol/l Tanshinone IIA for 72 h.

immunoreactive proteins were detected using an ECL Western blotting detection system.

Statistical analysis. All experiments were performed in triplicate and the results were expressed as mean ± SD. Statistical analysis were performed with a Student's t-test using SAS 6.12 software. Statistical significance was accepted at the level of $p<0.05$.

Results

Cell growth inhibition caused by Tanshinone IIA. Tanshinone IIA (10 μmol/l) had little inhibitory effect on THP-1 cells, but it inhibited the proliferation of THP-1 cells significantly at a higher concentration, especially when the concentration was over 30 μmol/l. The inhibitory rate of Tanshinone IIA between 40-50 μmol/l is much higher than that of lower concentrations of Tanshinone IIA ($p<0.01$) (Fig. 2). The IC_{50} values after 24, 48 and 72 h of treatment were 44.9, 35.2 and 21.3 μmol/l.

Cell apoptosis caused by Tanshinone IIA. As shown in Fig. 3, Tanshinone IIA (>20 μmol/l) induced apoptosis when cultured with THP-1 cells after 24-72 h. The percentage of apoptotic cells was gradually increased in a time- and dose-dependent manner. Tanshinone IIA at 50 μmol/l caused an apoptotic rate much higher (>50%) than that of lower concentrations of Tanshinone IIA ($p<0.05$).

DNA fragmentation analysis. The intergrity of DNA was assessed by agarose gel electrophoresis. Incubation of THP-1 cells with Tanshinone IIA at 20-50 μmol/l for 72 h elicited a characteristic 'ladder' of DNA fragments representing integer multiples of the internucleosomal DNA length (~180-200 bp) (Fig. 4).

Morphology of cell apoptosis. Cells were treated with different concentrations of Tanshinone IIA for 72 h, then harvested and observed by Hoechst staining. No remarkable apoptotic

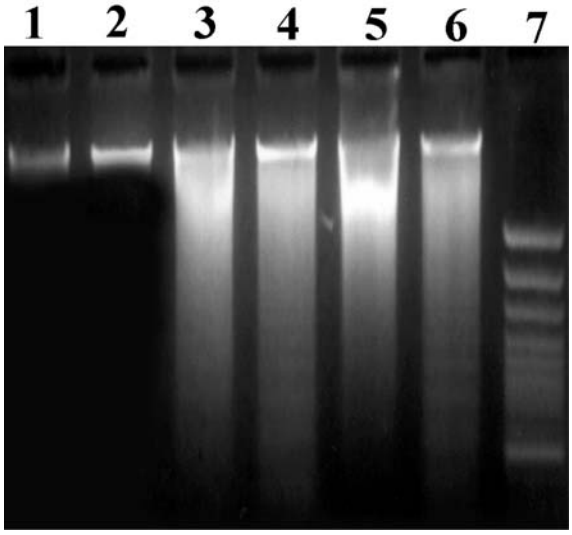

Figure 4. DNA fragmentation analysis. After cells were incubated with different concentrations of Tanshinone IIA for 72 h, apoptosis was confirmed by detection of fragmentation of chromosomal DNA with the classic DNA ladder method. As shown in Fig. 4, DNA ladder was observed clearly when the Tanshinone IIA concentrations were >20 μmol/l. Lanes 1, 2, 3, 4, 5 and 6 and were 0, 10, 20, 30, 40 and 50 μmol/l Tanshinone IIA. Lane 7, DNA Marker.

morphology was observed after the cells were treated with 0 μmol/l (control) and 10 μmol/l (Fig. 5A and B). Apoptotic cells gradually increased when cells were treated by 20 μmol/l and 30 μmol/l Tanshinone IIA (Fig. 5C and D). Tanshinone IIA (40 and 50 μmol/l) caused typical cell apoptotis (Fig. 5E and F). Marked morphological changes of cell apoptosis such as condensation of chromatin and nuclear fragmentations were found clearly using Hoechst 33258 staining. Apoptotic cells gradually increased in a dose-dependent manner in Tanshinone IIA treated cells.

Variation of caspase-3 activity. After treatment for 72 h, the cleavage of procaspase-3 was detected by Western blotting. The results revealed that caspase-3 was activated, as measured

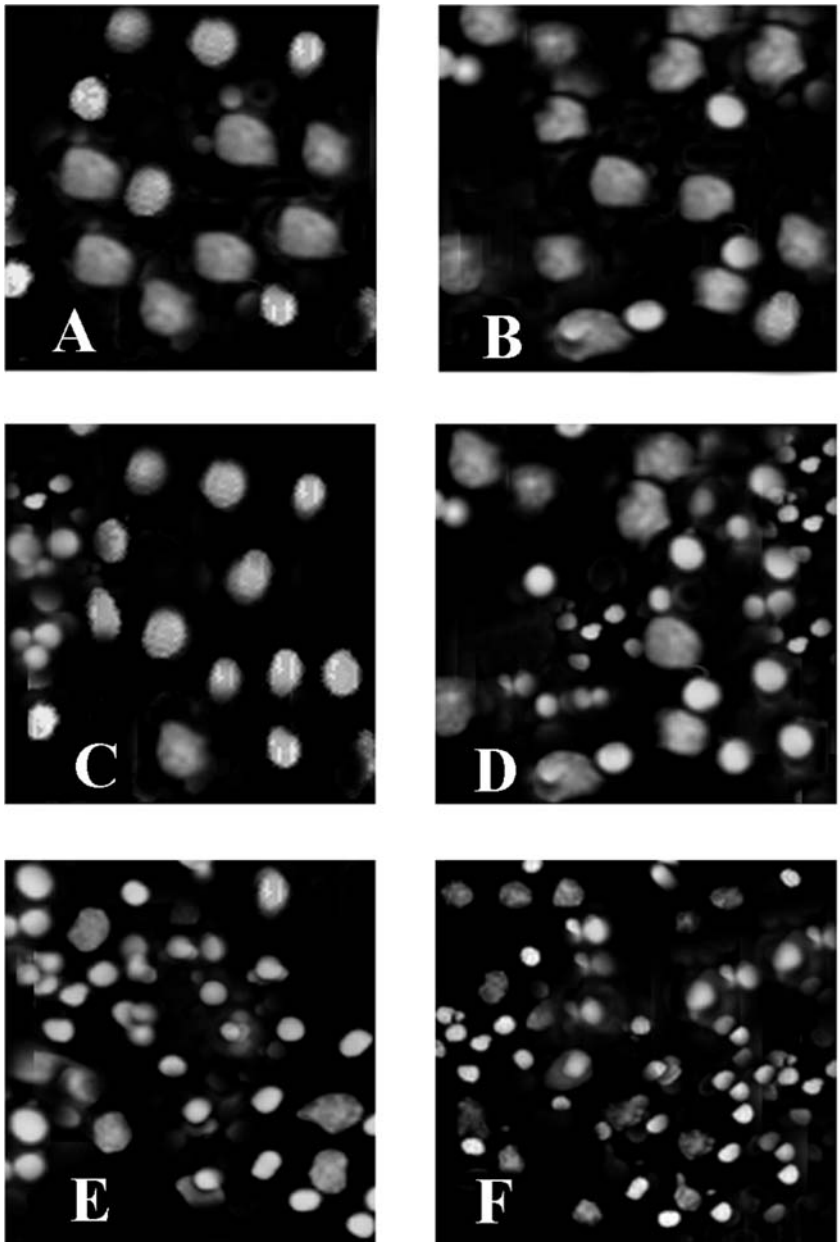

Figure 5. Cell apoptosis observed by Hoechst 33258 staining (x200). After the cells had been exposed to different concentrations of Tanshinone IIA for 72 h, Hoechst 33258 staining was used to observe the morphology of cell apoptosis. Apoptotic cells gradually increased in a dose-dependent manner when the concentrations of Tanshinone IIA was over 20 μmol/l. Marked morphological changes of cell apoptosis including condensation of chromatin and nuclear fragmentation were clearly observed. (A) Control (0 μmol/l); (B) 10 μmol/l; (C) 20 μmol/l; (D) 30 μmol/l; (E) 40 μmol/l and (F) 50 μmol/l.

by the loss of caspase-3 proenzyme (32 kDa) and the appearance of its 17 kDa subunit, after the cells had been exposed to Tanshinone IIA (>20 μmol/l) (Fig. 6A). Caspase-3 activation was found to increase concomitantly with increased concentration of Tanshinone IIA treatment. To confirm Tanshinone IIA induced activation of caspase-3, the cleavage of poly (ADP-ribose) polymerase (PARP), a known substrate of caspase-3, was also examined by Western blotting. As shown in Fig. 6B, Tanshinone IIA treatment caused a dose-dependent cleavage of PARP, with the appearance of 89-kDa fragment and disappearance of the intact 116-kDa PARP (Fig. 6B).

Disruption of the $\Delta\psi m$. The changes in the membrane potential of the mitochondria in Tanshinone IIA treated cells were examined after the cells were treated for 48 h. The results showed that the cells lost their mitochondrial membrane potential following Tanshinone IIA treatment. After Tanshinone IIA treatment for 48 h, the cells exhibited a significant alterations in $\Delta\psi m$, and the percentage of disruption of $\Delta\psi m$ gradually increased in a dose-dependent manner (Fig. 7). This suggests that Tanshinone IIA induced apoptosis involves the mitochondrial signaling pathway.

Western blot analysis of Bcl-2 family members and survivin expression. After Tanshinone IIA (>20 μmol/l) treatment for 72 h, cell apoptosis was found clearly by using Hoechst staining and DNA Fragmentation Analysis. To determine whether apoptosis related gene expression might play roles in this event, we detected the expression of Bcl-2 family members such as Bcl-2, Bax, Bid, Bad and Bak as well as the

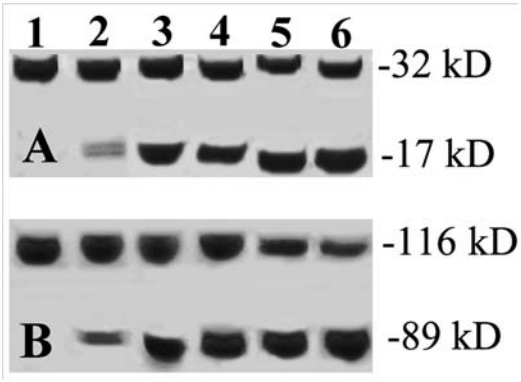

Figure 6. Western blot analysis of caspase-3 PARP. Caspase-3 was activated by the loss of caspase-3 proenzyme (32-kDa) and the appearance of its 17-kDa subunit (A) after the cells exposed to Tanshinone IIA for 72 h. Tanshinone IIA treatment also caused a dose-dependent cleavage of PARP, with the appearance of 89-kDa fragment (B). Lane 1, Control. Lanes 2, 3, 4, 5 and 6 were 10, 20, 30, 40 and 50 μmol/l Tanshinone IIA.

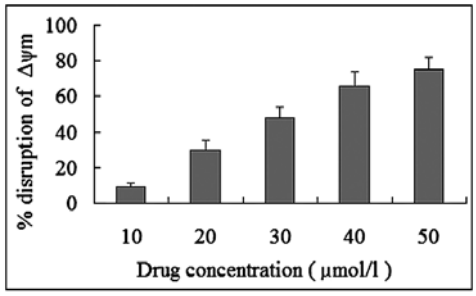

Figure 7. Disruption of the $\Delta\psi$m. After Tanshinone IIA treatment for 48 h, the cells exhibited a significant alteration in $\Delta\psi$m, and the percentage of disruption of $\Delta\psi$m gradually increased in a dose-dependent manner.

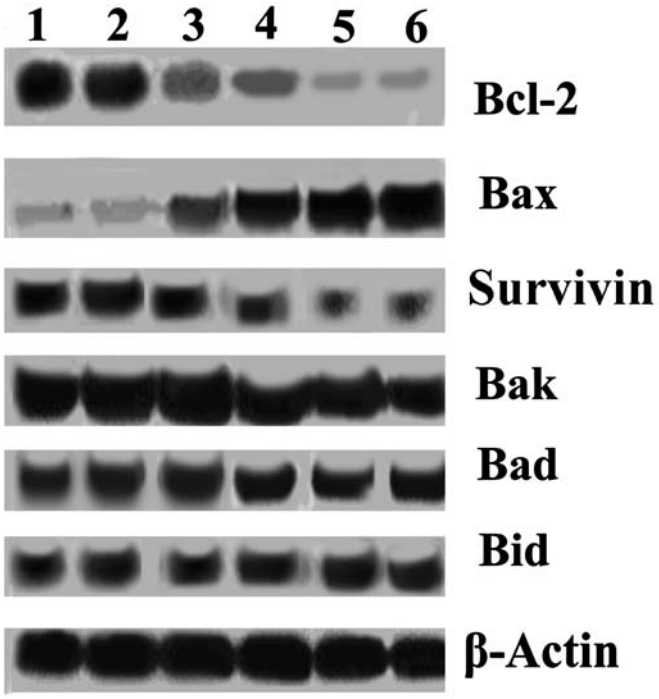

Figure 8. Western blot analysis of apoptosis related modulators. After the cells had been treated with Tanshinone IIA for 48 h, the expressions of Bcl-2 family and survivin was detected by Western blotting. The expression of Bcl-2 and survivin was down-regulated while Bax expression was up-regulated, and the expression of other Bcl-2 family members including Bak, Bid and Bad remained constant before and after apoptosis occurred. Lane 1, Control. Lane 2, 3, 4, 5 and 6 were 10, 20, 30, 40 and 50 μmol/l Tanshinone IIA.

expression of survivin after the cells were treated by Tanshinone IIA for 48 h. Western blot analysis revealed that Bcl-2 and survivin expression was down-regulated while Bax expression was up-regulated concomitantly, and there was no variation of other Bcl-2 family members (Fig. 8).

Discussion

In recent years, the use of herbal medicines to prevent the development or recurrence of cancers has become widely accepted as a realistic option for the treatment of malignant disease and herbal medicines have been proved to play an important role in integrative cancer treatment (12). Tanshinone IIA (as shown in Fig. 1), a derivative of phenanthrene-quinone isolated from Danshen, is now widely used in the treatment of inflammatory and cardiological disease. Previous studies have shown that tanshinone IIA has a large variety of pharmacological activities such as inhibition of clotting (13), inhibition of NO synthase (14) and dose-dependent inhibition on the basic fibroblast growth factor (bFGF)-induced human Smooth muscle cell (SMC) proliferation (15). Recent data have demonstrated that tanshinone IIA has anti-cancer activities on a large variety of cancer cells including solid tumor (6,7) as well as certain type of leukemia cells (16).

Acute lymphoid leukemia (ALL) is the main type of leukemia in children, though ~80% of ALL children can reach complete remission and will be long-term survivor with current treatment protocols (17), the prognosis of many individuals such as Philadelphia chromosome positive (Ph+) ALL and HTLV-I-associated adult T-cell leukemia is still very poor (18,19). Thus, there is a permanent need to find new anti-leukemia drugs and effective therapies for the clinical treatment of myeloid leukemia.

In this study, we found that Tanshinone IIA inhibited the growth of THP-1 cells and caused apoptosis significantly, the suppression was both in time- and dose-dependent manner. Marked changes of cell apoptosis were observed very clearly after the cells had been exposed to Tanshinone IIA for 72 h. Western blotting showed cleavage of the caspase-3 zymogen protein (32-kDa) with the appearance of its 20-kDa subunit when apoptosis occurred. The expression of Bcl-2 and survivin was down-regulated remarkably while Bax expression was up-regulated concurrently after the cells were treated with Tanshinone IIA for 48 h. We therefore conclude that Tanshinone IIA has significant antiproliferation effects on THP-1 cells by induction of apoptosis, and that Tanshinone IIA induced apoptosis on THP-1 cells is mainly related to the disruption of $\Delta\psi$m and activation of caspase-3 as well as down-regulation of anti-apoptotic protein Bcl-2 and survivin as well as up-regulation of pro-apoptotic protein Bax. Our data indicated that the Tanshinone IIA-induced apoptosis on THP-1 cells mainly involved the mitochondrial signaling pathway. The results revealed that Tanshinone IIA may serve as a potential anti-leukemia reagent.

The Bcl-2 family consists of about 20 homologues of important apoptotic regulators of programmed cell death. This family of proteins now includes both anti-apoptotic molecules such as Bcl-2, and pro-apoptotic molecules such as Bax (20). Cancers with high levels of Bcl-2 and Bcl-XL proteins are resistant to drug-induced apoptosis in a wide spectrum of chemotherapeutic agents, so Bcl-2 as well as Bcl-XL have become attractive targets for designing new anticancer drugs (21). Pro-apoptotic members of the Bcl-2 family, especially Bax and Bid, play important roles in drug-induced apoptosis by control of mitochondrial permeability due to their ability to form channels in membranes and to regulate pre-existing channels (22). In this study, our results revealed that Tanshinone IIA treatment of THP-1 cells up-regulated the expression of Bax as well as down-regulation of Bcl-2.

The caspases are a family of intracellular cysteine proteases with specificity for aspartic acid residues (23,24). Two of these groups, named 'initiator' and 'effector' caspases, play important roles in the apoptotic process (25,26). Caspase-3 is one of the most important executioners which is capable of cleaving many important cellular substrates (27), and caspase-3 mediated cell death plays an important role in pathogenesis and therapy of a variety of hematological malignancies (26-28).

Survivin, a member of apoptosis inhibitor family, is expressed in most human malignancies and implicated in mitosis regulation and preservation of cell viability (29). Previous data have revealed that survivin is one of the genes most consistently overexpressed in tumor cells which plays important roles in both cell proliferation and cell death (30), down-regulation of survivin expression may lead to programmed cell death (31), indicating that survivin may be an appealing new target for novel therapy in cancer (32). Our results showed that Tanshinone IIA may trigger apoptosis on THP-1 cells via activation of caspase-3 and down-regulation of anti-apoptosis protein Bcl-2 and survivin levels.

In summary, our results demonstrated that Tanshinone IIA has significant antiproliferation effects on THP-1 cells by induction of apoptosis, and that Tanshinone IIA induced apoptosis on THP-1 cells is mainly related to the disruption of $\Delta\psi m$ and activation of caspase-3 as well as down-regulation of anti-apoptotic protein Bcl-2 and survivin as well as up-regulation of pro-apoptotic protein Bax. The results indicate that Tanshinone IIA may serve as a potential anti-leukemia reagent.

Acknowledgements

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