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Induction of apoptosis and inhibition of cell adhesive and invasive effects by tanshinone IIA in acute promyelocytic leukemia cells *in vitro*

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Abstract

Tanshinone IIA, a diterpene quinone extracted from the traditional herbal medicine, *Salvia miltiorrhiza Bunge*, is used widely and successfully in clinics in China for treating inflammatory diseases. Recently tanshinone IIA has been reported to have apoptosis inducing effects on a large variety of cancer cells. In this study, the anti-proliferation and apoptosis inducing effects of tanshinone IIA as well as its influence on cell adhesion to and invasion through the extracellular matrix (ECM) on acute promyelocytic leukemia (APL) NB4 cells *in vitro* were studied. Cell proliferation was assessed by MTT assay, cell apoptosis was observed by Hoechst 33258 staining and flow cytometry (FCM); The variation of caspase-3 and apoptotic related genes were assayed by Western blotting, cell mitochondrial membrane potential as well as cell adhesive and invasive effects were also investigated by using standard methods. The results showed that tanshinone IIA exhibited induction of apoptosis by activation of caspase-3, downregulation of anti-apoptotic protein bcl-2 and bcl-xl and upregulation of pro-apoptotic protein bax, as well as disruption of the mitochondrial membrane potential. Furthermore, treatment by tanshinone IIA could reduce cell adhesion to and invasion through ECM in leukemia NB4 cells. These data provide a potential mechanism for tanshinone IIA-induced apoptosis and cell growth inhibition in leukemia NB4 cells, suggesting that tanshinone IIA may serve as an effective adjunctive reagent for the treatment of APL.

Introduction

Acute myeloid leukemia (AML) is a group of several different diseases, the treatment and outcome of which depend on several factors, including leukemia karyotype, patient age, and comorbid conditions. Despite advances in understanding the molecular biology of AML, its treatment remains challenging. Standard regimens using cytarabine and anthracyclines for induction followed by some form of postremission therapy produce response rates of 60–70%, with less than 20% of all patients achieving long-term disease-free survival [1, 2]. Acute promyelocytic leukemia (APL) is a rare disease accounting for approximately 10% of AML. This kind of leukemia is associated with a high risk of early mortality before the onset of therapy or in the early treatment phase resulting from severe coagulopathy, frequently inducing fatal cerebral hemorrhage, and the early mortality

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rate is approximately 10% [3]. Therefore, it is a permanent subject to find new anti-leukemia drugs and effective therapies for the clinical treatment of myeloid leukemia.

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 have been intense activities not only to identify new herbal medicines but also to understand how the existing constituents exhibit their activities [4]. Danshen (*Salvia miltiorrhiza Bunge*) is a widely used Chinese herbal medicine; its extracts contain diterpene quinone and phenolic acid derivatives, including tanshinone (I, IIA and IIB), cryptotanshinone, isocryptotanshinone, miltirone, tanshinol (I and II) and salviol [5]. Tanshinone IIA is a derivative of phenanthrene-quinone isolated from Danshen (Figure 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 [6–8]. 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 [9] and hepatocellular carcinoma [10].

Though tanshinone IIA has been proved to have anti-tumor effects in many different human cancer cells, many of its anti-proliferation and apoptotic mechanisms remain to be demonstrated. To date, no detailed data are available about the role and mechanisms of tanshinone IIA in leukemia NB4 cells. In order to understand the roles of tanshinone IIA in NB4 cells and possible clinical application of tanshinone IIA in leukemia therapy, we examined the anti-proliferation effects of tanshinone IIA as well as its influence on cell adhesion to and invasion through extracellular matrix (ECM) on NB4 cells *in vitro*.

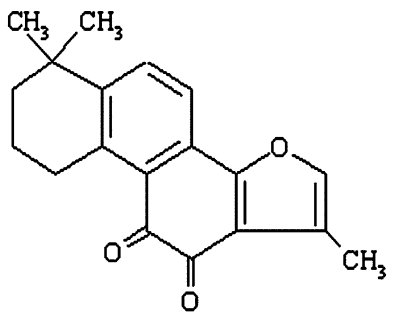

Figure 1. Molecular structure of tanshinone IIA.

Materials and methods

Main reagents

Tanshinone IIA, isolated from *Salvia miltiorrhiza Bunge*, was provided by Professor Gu Lian-Quan, Institute of Pharmacy Synthesis, Sun Yat-sen University. Annexin V/FITC and propidium iodide (PI) apoptosis detection kit was obtained from R&D, USA. Hoechst 33258 was purchased from Sigma Company. The antibodies used in this study, anti-bcl-2, bcl-xl, bax, bid, bak and bad were purchased from Santa Cruz Company (Germany). Antibodies against caspase-3 was bought from Upstate Inc. Caspase inhibitor (z-DEVD-FMK) was purchased from R&D systems Inc. (Minneapolis, MN, US). TRIZOL was from GIBCO (USA), and caspase colorimetric assay kit was from MBI (USA).

Cell culture

Leukemia NB4 cells were provided by central laboratory of Sun Yat-sen university cancer center. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin, in a humidified incubator with 5% CO_2 at 37 °C. All the cells were passaged twice weekly and routinely examined for mycoplasma contamination. Cells in logarithmic growth phase were used for further experiments.

Cell growth inhibition assay

The cell growth inhibition effects caused by tanshinone IIA was assessed by MTT assay, which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product. Briefly, leukemia NB4 cells in logarithmic growth-phase were collected, and 2×10^5 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 there was no tanshinone IIA in the control group. Each of the treated or

control group contained 6 parallel wells. Before MTT assay, the cells were first incubated in serum free RPMI for 24 h. After tanshinone IIA was absorbed completely, culture plates were then maintained in RPMI containing 10% fetal calf serum for 0, 12, 24 and 48 h prior to the addition of tetrazolium reagent. MTT working solution was prepared as follows: 5 mg MTT/ml PBS was sterile by being filtered with 0.45 μm filter units. Each of the above cultured wells was added 20 μl of MTT working solution and then incubated continuously for 4 h. All culture medium supernatant was removed from each wells after centrifugation and replaced with 100 μl of DMSO. Following thorough solubilization, the absorbance (OD) of each well was measured using a microculture plate reader at 570 nm. Each condition was tested in triplicate. The growth inhibitory rate was calculated by the following formula:

$$\text{Growth inhibitory rate} = (\text{average OD value in the control group} - \text{average OD value in the treatment group}) / \text{average OD value in the control group} \times 100\%.$$

The 50% inhibitory concentration (IC50) was determined from dose–response curve from at least three independent experiments. The percentages of cell growth were used to obtain the full dose–response curves and to determine the IC50 values (concentration inhibiting of 50% the cell growth compared with control).

Apoptosis assays

After the cells treated with 40 μmol/l tanshinone IIA for 0, 12, 24 and 48 h, Apoptosis was assayed by annexin V and propidium iodide (PI) staining and analyzed by flow cytometry (FACScan, Becton Dickinson; Mountain View, CA) according to the manufacturer's protocol.

Hoechst 33258 staining

After the cells treated with 40 μmol/l tanshinone IIA for 0, 12, 24 and 48 h, the morphology of NB4 cells exposed to tanshinone IIA for different time was observed firstly under inverted microscope. Then hoechst 33258 staining was used to observe the apoptotic morphology. 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. After treatment with tanshinone IIA, the morphologic changes including reduction in the volume and nuclear chromatin condensation were observed.

Adhesion assay

In vitro adhesion assays were performed to evaluate the effects of tanshinone IIA on the adhesive properties of NB4 cells. The plates for the adhesion assays were precoated with the ECM proteins laminin, fibronectin, vitronectin, or type IV collagen (each at a final concentration of 1 mg/ml in PBS) overnight at 4 °C and dried. To study the effects of tanshinone IIA on cell adhesion, exponentially growing cells were incubated with tanshinone IIA (40 μmol/l) for 24 h in a humidified 5% CO_2 atmosphere. The cells were centrifuged, washed twice with serum-free medium, counted, and resuspended in serum-free medium to a final concentration of 5×10^5 cells/ml. 1×10^5 cells were added to each well, and cells were allowed to adhere for 1 h at 37 °C in a humidified 5% CO_2 atmosphere. The nonadherent cells were removed by gently washing the cells with PBS, and then the adherent fraction was quantitated using MTT assays as described above.

In vitro invasion assay

The *in vitro* invasiveness of cells was assayed using a previously published method that uses Matrigel-coated Costar 24-well transwell cell culture chambers (''Boyden chambers'') with 8.0-mm pore polycarbonate filter inserts. The chamber filters were coated with 50 mg/ml of Matrigel matrix, incubated overnight at room temperature under a

laminar flow hood, and stored at 4 °C. To study the effects of tanshinone IIA on the invasiveness of NB4 cells, exponentially growing cells were incubated with different concentrations of tanshinone IIA in 0.1% DMSO overnight. The cells were washed twice with serum-free RPMI 1640 containing 0.1% BSA, counted, and resuspended at 1×10^5 cells/ml. An 0.5-ml cell suspension containing 1×10^5 cells in a serum-free RPMI 1640 containing tanshinone IIA or vehicle was added to the Matrigel-coated and rehydrated filter inserts. Next, 750 ml of NIH fibroblast-conditioned medium was placed as a chemoattractant in 24-well plates, and the inserts were placed in wells and incubated at 37 °C for 24 h. After the incubation period, the invasive cells that migrated into the lower chamber were counted under a light microscope. The invasive fractions of cells treated with tanshinone IIA were compared with those of DMSO (0.1%)-treated control cells, and the percentage inhibition of invasiveness was determined.

Western blot analysis

Cells were treated with 40 μmol/l tanshinone IIA for 0, 12, 24 and 48 h, then Western blotting analysis was used to detect the expression of Bcl-2 family members. Briefly, 2×10^5 cells were harvested and 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 × *g* at 4 °C, and equivalent amounts of protein were separated by 10% SDS-PAGE and transferred onto nitrocellulose filters. 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 the primary antibodies at 4 °C overnight, followed by extensive washes with PBS twice and TBST twice. Filters were then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h, washed with TBST and developed using the Super Signal West Pico Kit.

Analysis of the mitochondrial membrane potential ($\Delta\psi m$)

The mitochondrial membrane potential ($\Delta\psi$m) were measured by FCM using the intramitochondrial dye JC-1(Alexis Biochemical Co., Germany) after NB4 cells treated with different concentrations of tanshinone IIA for 24 h. The detection procedure was performed according to the manufacture's instructions. Data were converted to dot plots using Cell Quest software (Becton Dickison, Germany).

Caspase activity assay

After NB4 cells were treated 40 μmol/l tanshinone IIA for 0, 12, 24 and 48 h, the activity of caspases was determined by Caspase colorimetric assay kit, according to the manufacturer's protocol. Briefly, tanshinone IIA treated cells were washed with ice-cold PBS and lysed in a lysis buffer. The cell lysates were tested for protease activity using a caspase-specific peptide, conjugated to the color reporter molecule *p*-nitroanaline. The chromophore *p*-nitroanaline, cleaved by caspases, was quantitated with a spectrophotometer at a wavelength of 405 nm. The caspase enzymatic activities in cell lysate are directly proportional to the color reaction.

Western blot analysis was also used to detect the variation of caspase-3 after the cells exposed to 40 μmol/l tanshinone IIA for 24 h. To confirm the contribution of caspase-3 to tanshinone IIA induced cell apoptosis, the cells were pretreated with a caspase-3-specific inhibitor, z-DEVD-FMK (20 μmol/l), prior to tanshinone IIA treatment. The cells were firstly treated with z-DEVD-FMK for 1 h, then the cells were incubated with 40 μmol/l tanshinone IIA for 24 h and Western blot was used to detect the expression of caspase-3.

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 effects of tanshinone IIA on NB4 cells

To investigate the cytotoxicity of tanshinone IIA on leukemia cells, NB4 cells were treated with

various concentrations of tanshinone IIA for 12, 24 and 48 h. As shown in Figure 2, tanshinone IIA had significant growth inhibition effects on NB4 cells in a dose- and time-dependent manner. The inhibitory rate of tanshinone IIA between 30 and 50 μmol/l is much higher than that of lower concentrations of tanshinone IIA ($p < 0.01$). The IC50 values after 12, 24 and 48 h of treatment were 45.8, 24.7 and 19.3 μmol/l.

Apoptosis assay

To observe tanshinone IIA induced apoptosis, NB4 cells were treated with 40 μmol/l tanshinone IIA for 0, 12, 24 and 48 h, cells were collected and stained with annexin V and PI, and then subjected to flow cytometry assay to obtain an apoptosis scatter plot. As shown in Figure 3, by using double staining with annexin V and PI, apoptotic cells in the early-stage of apoptosis (positive for annexin V and negative for PI) is very higher, and the necrotic cells (both positive for annexin V and PI) is very lower, and the percentage of apoptotic cells in NB4 cells was increased in a time-dependent manner. Along with the enhancement of culture time, apoptotic cells gradually increased and the percentage of apoptotic cells reached up to over 60% when the cells were treated with 40 μmol/l tanshinone IIA for 48 h (Figure 3, M1).

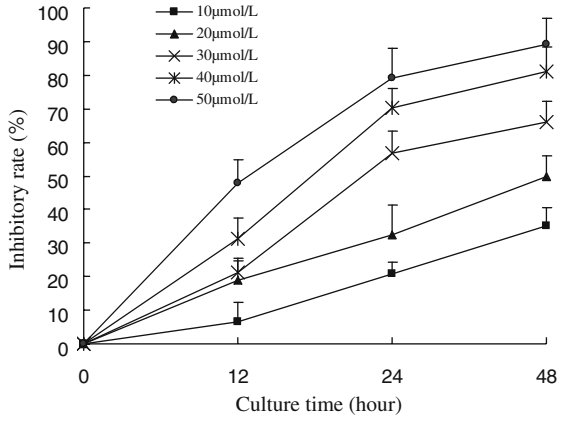

Figure 2. Cell growth inhibition caused by tanshinone IIA. After NB4 cells were treated with different concentrations of tanshinone IIA, MTT assay was used to detect cell growth inhibition as described in the 'Methods'. The inhibitory rate of tanshinone IIA between 30 and 50 μmol/l is much higher than that of lower concentrations of tanshinone IIA ($p < 0.01$).

Hoechst 33258 staining

After cells treated with 40 μmol/l tanshinone IIA for 0, 12, 24 and 48 h, marked morphological changes of cell apoptosis such as condensation of chromatin and nuclear fragmentations were found clearly using Hoechst 33258 staining (Figure 4). Apoptotic cells gradually increased in time-dependent manner in NB4 cells.

Expression of apoptosis related genes

To clarify the mechanism of tanshinone IIA induced apoptosis in NB4 cells, the expressions of Bcl-2 family members were detected after the cells treated with 40 μmol/l tanshinone IIA for 0, 12, 24 and 48 h. The results revealed that the expression of bcl-2 and bcl-xl was down-regulated remarkably while bax expression was up-regulated concurrently after the cells were treated with tanshinone IIA, and the expressions of other Bcl-2 family members including bid, bak and bad remained constant before and after apoptosis occurred (Figure 5).

Tanshinone IIA inhibit NB4 cell adhesion and invasion

The ECM proteins to which tumor cells initially attach include laminin, fibronectin, type IV collagen, and vitronectin. Laminin, fibronectin, vitronectin, and collagen have been found in the basal lamina that promote the adhesion and invasion of tumor cells. To determine whether tanshinone IIA affects the integrin-mediated cell adhesion to ECM, NB4 cells were incubated with tanshinone IIA for 24 h, and then the integrin-mediated cell adhesion was examined. As shown in Figure 6, pretreatment of NB4 cells with tanshinone IIA inhibited their adhesion to laminin-, fibronectin-, collagen- and vitronectin-coated plates. Matrigel matrix-coated Boyden chambers were used to examine the ability of tanshinone IIA to inhibit the invasiveness of NB4 cells. The cells were treated with different concentrations of tanshinone IIA overnight and then placed in Matrigel matrix-coated Boyden chambers and allowed to invade for 24 h. We observed that pretreatment with different concentrations of tanshinone IIA inhibited the invasiveness of NB4 cells in a dose-dependent manner (Figure 7).

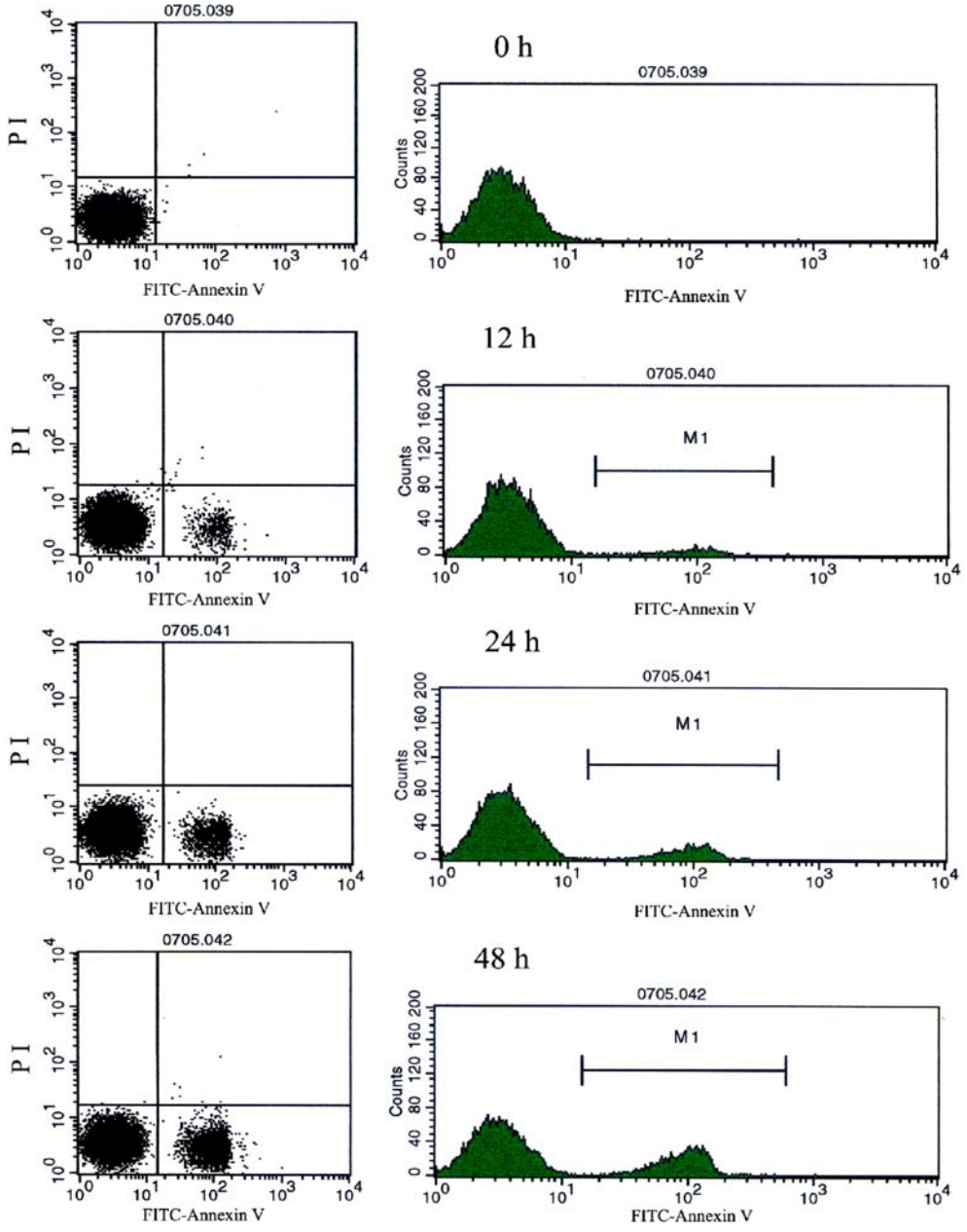

Figure 3. Cell apoptosis caused by tanshinone IIA. After NB4 cells were treated with 40 μmol/l tanshinone IIA for 0, 12, 24 and 48 h, cell apoptosis was analyzed by flow cytometry (double staining with annexin V and PI) as described in the 'Methods'. Tanshinone IIA could induce apoptosis when cultured with NB4 cells for 12–48 h, the apoptotic cells (positive for annexin V and negative for PI) were very higher, and the cell aoptotic rate (M1) gradually increased in a time-dependent manner.

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 24 h. The results showed that NB4 cells lost their mitochondrial membrane potential following tanshinone IIA treatment. After tanshinone IIA treatment for 24 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 (Figure 8). This suggests that tanshinone IIA

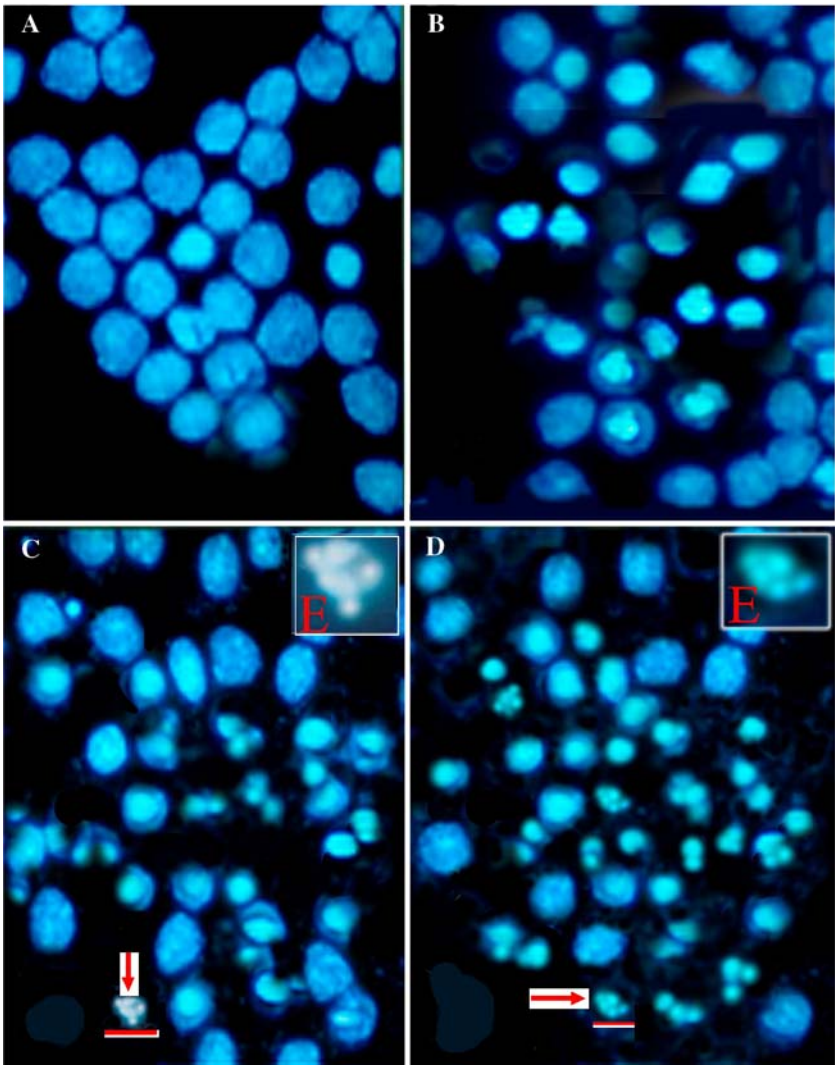

Figure 4. Cell apoptosis observed by Hoechst 33258 staining (200× magnification). After NB4 cells exposed to 40 μmol/l tanshinone IIA for 0, 12, 24 and 48 h, Hoechst 33258 staining was used to observe the apoptotic cells as described in the 'Methods'. Apoptotic cells gradually increased in a time-dependent manner, and marked morphological changes of cell apoptosis including condensation of chromatin and nuclear fragmentation were found clearly after the cells treated for different times. (A) 0 h; (B) 12 h; (C) 24 h; (D) 48 h; (E) 400× magnification. Arrow: the apoptotic cell displaying apoptotic bodies and nuclear fragmentation. Bar = 20 μm.

induced apoptosis involves mitochondrial signaling pathway.

Caspase-3 activities in tanshinone IIA-induced apoptosis

To understand the activation of caspase cascade during tanshinone IIA induced apoptosis in leukemia cells, we first investigated various caspase specific activities after the cells were treated with 40 μmol/l tanshinone IIA for 0, 12, 24 and 48 h. As shown in Figure 9, the colorimetric assay of caspase activities revealed that caspase-3 activities were increased remarkably in a dose-dependent manner.

After the cells exposed to 40 μmol/l tanshinone IIA for 24 h, Western blot analysis was also used to detect the variation of caspase-3. As shown in Figure 10A, Caspase-3 was activated by the loss of caspase-3 proenzyme (32-kD) and the appearance of its 20-kD subunit after the cells were incubated over 24 h.

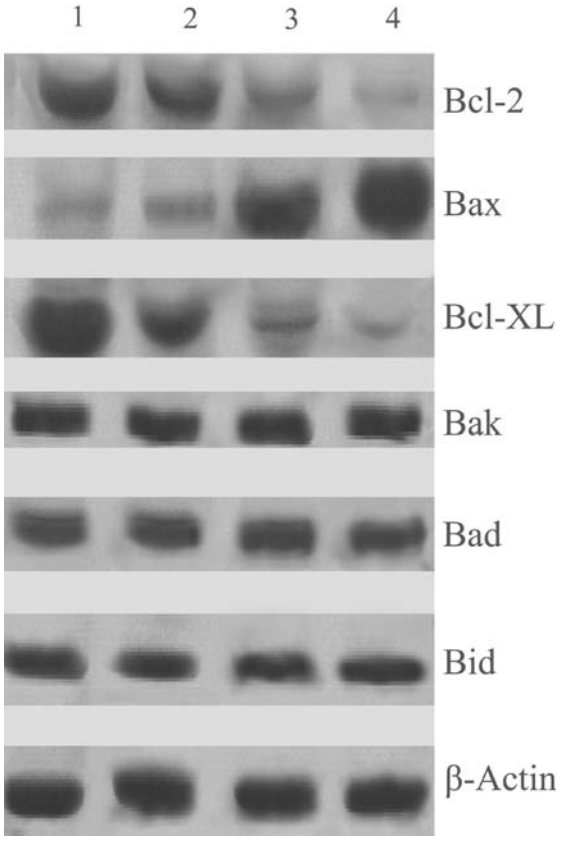

Figure 5. Western blot analysis of apoptosis related genes. After NB4 cells treated with 40 μmol/l tanshinone IIA for 0, 12, 24 and 48 h, Western blot analysis was used to detect the variation of Bcl-2 family as described in the 'Methods'. The expressions of bcl-2 and bcl-xl were down-regulated while Bax expression was up-regulated, and the expressions of other Bcl-2 family members including bid, bak and bad remained constant before and after apoptosis occurred. Lane 1, 2, 3 and 4 were: 0, 12, 24 and 48 h. *β*-actin as an internal control.

To estimate the contribution of caspase-3 to tanshinone IIA induced cell apoptosis, the cells were pretreated with a caspase-3-specific inhibitor. As shown in Figure 10B, 1 h pretreatment of NB4 cells with z-DEVD-FMK could block tanshinone IIA induced activation of caspase-3, indicating that tanshinone IIA caused apoptosis was mediated via the activation of caspase-3.

Discussion

Herbal medicines have always held an attraction for patients. Approximately 25% of prescription medicines are derived from plants. It is currently estimated that over 50% of all patients diagnosed with cancer explore complementary and alternative medicine especially herbal medicine. 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 [4, 11]. Tanshinone IIA (as shown in Figure 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 [12], inhibition of NO synthase [13] and dose-dependent inhibition on the basic fibroblast growth factor (bFGF)-induced human Smooth muscle cell (SMC) proliferation [14]. Recent data have demonstrated that tanshinone IIA has anti-cancer activities on a large variety of cancer cells including solid tumor [9, 10] as well as lymphocytic leukemia cells [15].

In this study, we found that tanshinone IIA could inhibit cell growth, induce apoptosis by activation of caspase-3 and disruption of the mitochondrial membrane potential, as well as reduce cell adhesion to and invasion through ECM on leukemia NB4 cells *in vitro*. Western blotting analysis demonstrated that anti-apoptotic protein bcl-2 was downregulatd while pro-apoptotic protein bcl-xl upregulated remarkably in a time-dependent manner when apoptosis occurred. We therefore conclude that tanshinone IIA has significant anti-proliferation effect by induction of apoptosis via activation of caspase-3 and disruption of mitochondrial membrane potential, as well as by downregulation of anti-apoptotic protein bcl-2 and bcl-xl and upregulation of pro-apoptotic protein bax. Furthermore, tanshinone IIA demonstrate significant inhibition effects on cell adhesive and invasive ability in leukemia NB4 cells *in vitro*.

The dissemination of cancer occurs via shedding of cells from the primary tumor with subsequent seeding, followed by invasion and proliferation at the secondary site [16]. The extracellular matrix (ECM) serves as a scaffold to promote the cellular responses of adhesion, proliferation, and migration during tumor cell growth [17]. ECM is a prerequisite for the structural and functional homeostasis of bone marrow (BM)

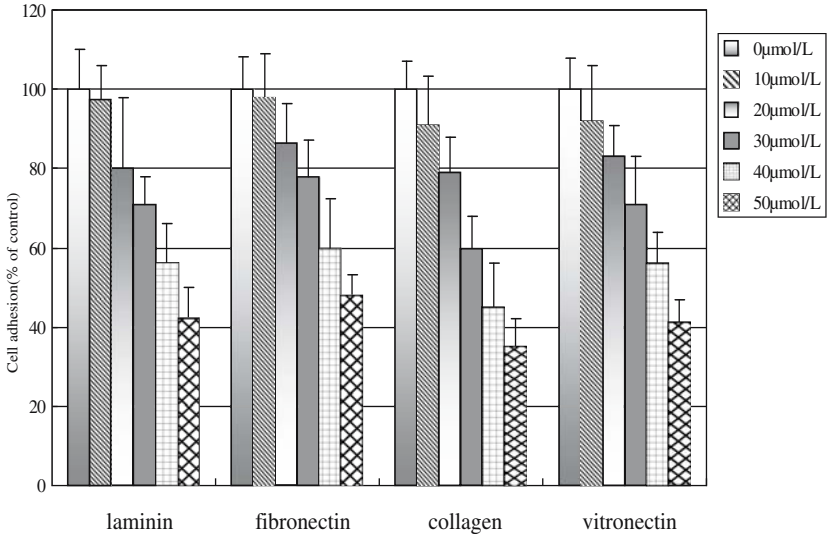

Figure 6. Tanshinone IIA inhibited adhesive property of NB4 cells. NB4 cells were incubated with different concentrations of tanshinone IIA for 24 h, and then processed for adhesion assays using laminin-, fibronectin-, collagen type IV, or vitronectin-coated 96-well plates as described in "Materials and Methods". The results showed that cell adhesive cells were gradually reduced in a dose-dependent manner.

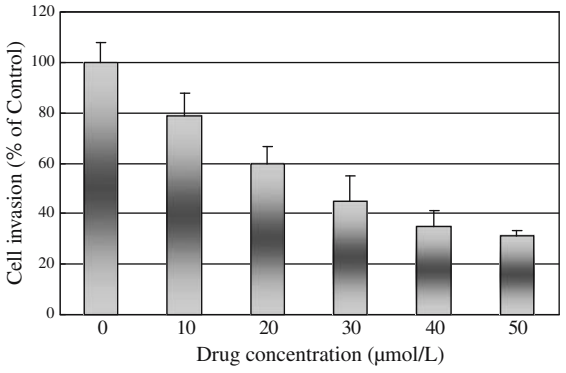

Figure 7. Tanshinone IIA inhibited invasive property of NB4 cells. NB4 cells were treated with different concentrations of tanshinone IIA overnight and then placed in Matrigel matrix-coated Boyden chambers and allowed to invade for 24 h as described in "Materials and Methods". The results showed that tanshinone IIA inhibited the invasiveness of NB4 cells in a dose-dependent manner, the amount of invasive cells gradually decreased after treatment with different concentrations of tanshinone IIA.

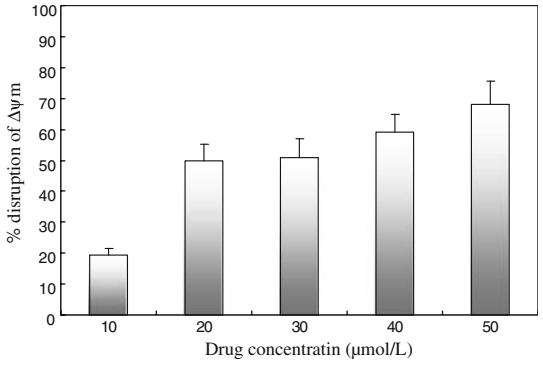

Figure 8. Disruption of the $\Delta\psi$m. The changes in the membrane potential of the mitochondria in tanshinone IIA treated cells were detected as described in "Materials and Methods". After tanshinone IIA treatment for 24 h, the NB4 cells exhibited a significant alterations in $\Delta\psi$m, and the percentage of disruption of $\Delta\psi$m gradually increased in a dose-dependent manner.

microenvironment. The role of ECM in physiologic hematopoiesis and its pathologic change are very important in the development of leukemia, and cell–ECM interactions may trigger leukemia cell proliferation, survival and dissemination [18]. Therefore, adhesion to and invasion through ECM contributes to enlarged leukemia cell dissemination, proliferation, and may cause the poor clinical outcome in leukemia patients. In this study, our results demonstrated that tanshinone IIA could inhibit the effects on cell adhesive and invasive ability in leukemia NB4 cells *in vitro*, indicating tanshinone IIA may serve as a potential reagent in the treatment of APL.

The caspases are a family of intracellular cysteine proteases with specificity for aspartic acid residues [19]. Two of these groups, named "initiator" and "effector" caspases, play an important

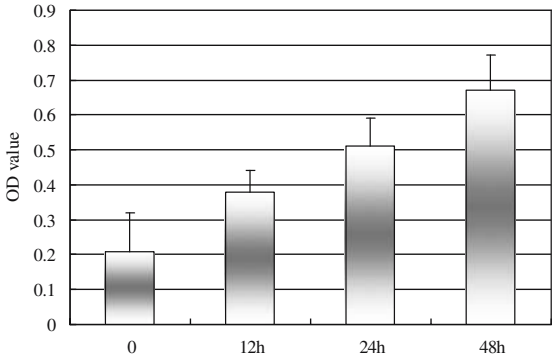

Figure 9. The variation of caspase-3 activities caused by tanshinone IIA. After NB4 cells were treated with 40 μmol/l tanshinone IIA for 0, 12, 24 and 48 h, caspase-3 activities were detected as described in "Materials and Methods". The results revealed that caspase-3 activities were increased remarkably in a time-dependent manner.

roles in the apoptotic process [20]. Caspases-3 is one of the most important executioner, which is capable of cleaving many important cellular substrates, and caspase-3 mediated cell death plays an important role in pathogenesis and therapy of a variety of hematological malignancies [21]. Our results demonstrated that tanshinone IIA induced apoptosis was related to activation of caspase-3, and caspase-3 specific inhibitor z-DEVD-FMK could block tanshinone IIA induced activation of caspase-3, indicating that tanshinone IIA caused apoptosis in NB4 cells was mediated via the activation of caspase-3.

The Bcl-2 family consists of about 20 homologs of important apoptotic regulators of programmed cell death. This family of proteins now includes both anti-apoptotic molecules such as Bcl-2 and Bcl-XL, and pro-apoptotic molecules such as Bax, Bak, Bid and Bad [22]. 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 anti-cancer drugs [23]. 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 preexisting channels [24]. In this study, our results revealed that treatment by tanshinone IIA in NB4 cells caused upregulation of bax as well as downregulation of bcl-2 and bcl-xl followed by disruption of mitochondrial membrane potential. This inidicates that

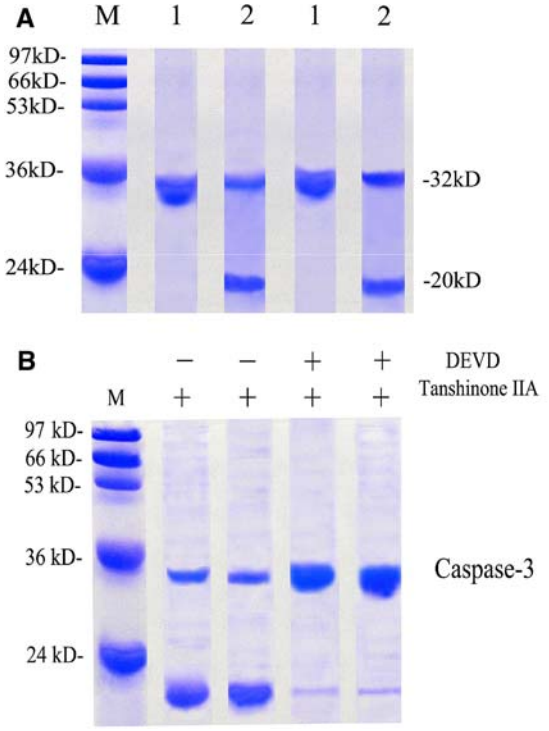

Figure 10. Western blot analysis of caspase-3. (A) Caspase-3 was activated by the loss of caspase-3 proenzyme (32-kD) and the appearance of its 20-kD subunit after the cells exposed to 40 μmol/l tanshinone IIA for 24 h. M, Molecular weight marker; lane 1, cells treated with 0 μmol/l tanshinone IIA; lane 2, cells treated with 40 μmol/l tanshinone IIA. (B) Tanshinone IIA caused activation of caspase-3 was blocked by caspase-3 specific inhibitor. The cells were firstly treated with z-DEVD-FMK for 1 h, then the cells were incubated with 40 μmol/l tanshinone IIA for 24 h. The results showed that pretreatment of NB4 cells with z-DEVD-FMK could block tanshinone IIA induced activation of caspase-3, indicating that tanshinone IIA caused apoptosis was mediated via the activation of caspase-3.

mitochondrial signaling pathway was involved in tanshinone IIA induced apoptosis in APL cells.

In summary, our data provide a potential mechanism for tanshinone IIA-induced apoptosis and cell growth inhibition in leukemia NB4 cells, suggesting that tanshinone IIA may serve as an effective adjunctive reagent for the treatment of leukemia, and that *in vivo* anti-cancer effects as well as its potential clinical effectiveness need further investigation.

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