Activation of c-Jun N-Terminal Kinase Mediates Tanshinone IIA-Induced Apoptosis in KBM-5 Chronic Myeloid Leukemia Cells

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Aim of this study was to identify the molecular mechanisms of tanshinone IIA-induced apoptosis in chronic myelogenous leukemia (CML) cells. Cytotoxicity of tanshinone IIA was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Our data demonstrate that tanshinone IIA induced apoptosis by increasing the sub-G1 DNA contents and DNA fragmentation in KBM-5 CML cell line. In addition, tanshinone IIA significantly reduced mitochondrial membrane potential (MMP), mediated cytochrome c release from mitochondria and activated caspase-3 and 9, indicating mitochondria-dependent apoptosis by tanshinone IIA. Tanshinone IIA attenuated expression of several apoptosis-related proteins such as c-inhibitor of apoptosis protein (IAP), Mcl-1, and Bcl-2. Interestingly, although tanshinone IIA notably enhanced the phosphorylation of both c-Jun N-terminal protein kinase (JNK) and p38, JNK inhibitor, but not p38 inhibitor, reversed tanshinone IIA-induced apoptosis. Our findings suggest that tanshinone IIA induces mitochondria-dependent apoptosis via activation of JNK in KBM 5 cells as a potent anti-cancer agent for CML therapy.

Key words apoptosis; chronic myelogenous leukemia; c-Jun N-terminal kinase; mitochondria; tanshinone IIA

Leukemia, one of blood or bone marrow cancers, is characterized by an abnormal proliferation of white blood cells and classified into lymphoblastic leukemia and myelogenous leukemia.10 Of myelogenous leukemias, chronic myeloid leukemia (CML) is a myeloproliferative disorder resulting from the clonal expansion of transformed hematopoietic stem cells due to the translocation of chromosomes 9 and 22, leading to constitutively activated Bcr-abl tyrosine kinase, which may cause resistance to cancer therapy.2,3 Although various chemotherapeutic agents have been developed such as hydroxyurea (Hydrea), busulfan (Myleran) or imatinib mesylate (Gleevec) for the treatment of CML, their therapeutic efficacies were constrained due to undesirable side effects, for example, nausea, fluid retention and diarrhea restrict.4

Salvia miltiorrhiza Bunge (Danshen) is an Oriental medicinal herb that has been utilized for cardiovascular diseases and malignant tumors.5,6 Tanshinone IIA, a major active constituent of Danshen extracts, possesses various biological activities such as anti-inflammatory,7 anti-oxidant8 and neuron-protective effects.9 Tanshinone IIA also exhibited anti-cancer activity in many cancer cells such as liver,10 colorectal,11 breast,12 lung13 and prostate cancers14 by targeting the multiple signal transduction pathways such as p53,15 reactive oxygen species (ROS),15 signal transducer and activator of transcription 3 (STAT3)16 and phosphoinositide 3-kinase/AKT17 pathways. Furthermore, several groups reported that tanshinone IIA induced apoptosis in HL-60 and K562 leukemia cells through caspase-3 activation.17,18 Nonetheless, the underlying molecular mechanisms responsible for anti-cancer activity of tanshinone IIA in CML are not clearly established yet.

Mitogen-activated protein kinase (MAPK) family including extracellular signal-related kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 are important intermediates in a variety of signaling pathways implicated in cell growth, differentiation, proliferation and apoptosis. ERK usually mediates proliferation, differentiation and anti-apoptosis while JNK and p38 promote apoptotic cell death.20 In the present study, the role of c-Jun N-terminal kinase (JNK) was chiefly elucidated in tanshinone IIA induced apoptosis in CML KBM-5 cells.

MATERIALS AND METHODS

Isolation of Tanshinone IIA Tanshinone IIA (Fig. 1A) was isolation from Salvia miltiorrhiza Bunge as previously described.21

Cell Culture KBM-5 cells (human chronic myelogenous leukemia) were purchased from American Type Culture Collection (ATCC, Rockville, MD, U.S.A.) and maintained in IMDM medium supplemented with 15% fetal bovine serum (FBS), 2µM L-glutamine and penicillin/streptomycin. K562 (human chronic myelogenous leukemia) and U937 (human histiocytic lymphoma) were obtained from ATCC and maintained RPMI 1640 medium supplemented 10% FBS, 2µM L-glutamine and penicillin/streptomycin.

Human Peripheral Blood Lymphocyte (PBL) Isolation PBL was isolated from blood samples of healthy donor by Ficoll-Hypaque (Amersham Pharmacia, Piscataway, NJ, U.S.A.) gradient centrifugation.

Cytotoxicity Assay Cytotoxicity of tanshinone IIA was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded onto 96-well microplates at a density of 2×104 cells per well and exposed to various concentrations of tanshinone IIA (0, 10, 20, 40 or 80µM) for 24h. The cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (1mg/mL) (Sigma Chemical Co., St. Louis, MO, U.S.A.) for 2h and then...
Fig. 1. Tanshinone IIA Induces Apoptosis in KBM-5 Cells

(A) Chemical structure of tanshinone IIA. Molecular weight=294.3. (B) Cytotoxicity of tanshinone IIA was evaluated by MTT assay in human leukemic cell lines KBM-5, K562 and U937, and human normal peripheral blood lymphocytes (PBLs). Cells were treated with various concentrations of Tan IIA (0, 10, 20, 40 or 80 µM) for 24h. (C, D) KBM-5 cells were treated with various concentrations of tanshinone IIA (0, 10, 20, 40 or 80 µM) for 24h. (C) After fixing in 75% ethanol, cells were stained with propidium iodide (PI) and cell cycle was analyzed by flow cytometry. Graphs represent the percentages of sub-G1 DNA contents. Data are presented as means±S.D. *p<0.05 vs. untreated control. (D) DNA fragmentation was analyzed by 2% agarose gel and visualized with ethidium bromide under UV light. (E) KBM-5 cells were treated with or without tanshinone IIA (80 µM) for 24h. TUNEL staining was performed by using Dead End™ fluorometric TUNEL assay kit. The stained cells were visualized under Axio vision 4.0 fluorescence microscope at ×630 of original magnification. DAPI was utilized for counter staining of nucleus.
with MTT lysis solution overnight. Optical density (OD) was measured using a microplate reader (Molecular Devices Co., Sunnyvale, CA, U.S.A.) at 570 nm. Cell viability was calculated as a percentage of viable cells in tanshinone IIA-treated group versus untreated control by following equation. Cell viability (%)=\(\frac{\text{OD (tanshinone IIA)} - \text{OD (blank)}}{\text{OD (control)}}\times 100\).

**Cell Cycle Analysis** Cell cycle analysis was performed by propidium iodide (PI) staining. KBM-5 cells were treated with Tan IIA for 24 h, collected and fixed in 70% ethanol. The cells were then incubated at 37°C with 0.1% RNase A in phosphate buffered saline (PBS) for 30 min and suspended in PBS containing 25 µg/mL PI for 30 min at room temperature. The stained cells were analyzed for DNA content in FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) using the Cell Quest program (Becton Dickinson).

**Detection of Oligonucleosomal Fragmentation by DNA Gel Electrophoresis** The pattern of DNA cleavage was analyzed by agarose gel electrophoresis. Briefly, KBM-5 cells were homogenized in lysis buffer (20 mM Tris–HCl, pH 8.0, 10 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0, 0.2% Triton X-100) and sequentially incubated for 10 min at room temperature and 20 min on ice. The supernatant containing the fragmented DNA was collected and incubated at 50°C overnight with proteinase K (20 mg/mL). The DNA was then precipitated for 3 h at −70°C in 100% ethanol and 0.1 volumes of 3 M sodium acetate. The precipitates were dissolved in distilled water containing 10 mg/mL RNase A (Sigma Chemical Co., St. Louis, MO, U.S.A.) and incubated for 30 min at 37°C. DNA fragmentation was analyzed on 2% agarose gels.

**Terminal Deoxynucleotidyl Transferase Deoxyuridine Triphosphate (dUTP) Nick End Labeling (TUNEL) Assay** TUNEL assay was performed by using Dead End™ fluorometric TUNEL assay kit (Promega, Madison, WI, U.S.A.) according to the manufacturer’s instructions. KBM-5 cells were treated with various concentrations of tanshinone IIA (0, 10, 20, 40 or 80 µM) for 24 h, plated onto poly-L-lysine-coated slide and fixed in 4% methanol-free formaldehyde solution for 25 min at 4°C and washed with PBS twice for 5 min. The cells were then permeabilized in 0.2% Triton X-100 for 5 min and equilibrated in equilibration buffer. After removing equilibration buffer, terminal deoxynucleotidyl transferase (TdT) enzyme buffer containing fluorescein-12-dUTP for 1 h at 37°C in the humidified chamber in dark and terminated by 2× SSC for 15 min at room temperature. The slides were mounted with mounting medium containing 4,6-diamidino-2-phenylindole (DAPI) (VECTOR, Burlingame, CA, U.S.A.) and visualized under an Axio vision 4.0 fluorescence microscope (Carl Zeiss Inc., Weimar, Germany).

**Measurement of Mitochondria Membrane Potential** KBM-5 cells were treated and stained with tetramethylrhodamine ethyl ester (TMRE), a fluorescent potential-dependent indicator, for 30 min at 37°C. Mitochondrial membrane potential was determined by flow cytometry (FACS Calibur, Becton Dickinson) at 582 nm.

**Western Blot Analysis** KBM-5 cells were lysed in lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 1 mM Na3VO4, 1 mM NaF, protease inhibitors cocktail). The extracts were incubated on ice for 30 min and supernatants were centrifuged at 14000×g at 4°C. The protein contents in the supernatant were measured by using a Bio-Rad DC protein assay kit II. Proteins were separated by electrophoresis on 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) gel and electrotransferred onto a Hybond enhanced chemiluminescence (ECL) transfer membrane with transfer buffer (25 mM Tris, 250 mM glycine, 20% methanol) at 300 mA for 90 min. The membrane was blocked in 5% nonfat skim milk, and probed with primary antibodies for cleaved caspase-9, cleaved caspase-3, phospho-JNK, JNK, phospho-p38, p38 (Cell Signaling Tech., Danvers, MA, U.S.A.), poly(ADP- ribose) polymerase (PARP), survivin, c-IAP-1, c-IAP-2, Bel-2, Bax, Mel-1 (Santa Cruz Biotechnologies, Santa Cruz, CA, U.S.A.), and β-actin (Sigma-Aldrich, St. Louis, MO, U.S.A.) and horseradish peroxidase (HRP)-conjugated secondary antibodies. Protein expression was detected by using ECL) system (Amersham Pharmacia, Piscataway, NJ, U.S.A.).

**Caspase-3 Activity Assay** Caspase-3 activity was measured by using caspase-3 colorimetric assay kits (R&D Systems, Inc., Minneapolis, MN, U.S.A.) according to manufacturers’ instructions. In brief, KBM-5 cell lysates were reacted with 50 mM synthetic substrate chromophore p-nitroanilide (DEVDF-pNA) in the reaction buffer containing 10 mM di-thiothreitol (DTT) at 37°C for 2 h. Caspase-3 activity was determined by measuring changes in absorbance at 405 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices Co., Sunnyvale, CA, U.S.A.) and calculated on the basis of a standard curve prepared using p-NA (p-nitroanilide). The relative levels of p-NA were normalized against the protein concentration of each extract.

**Immunofluorescence Assay** KBM-5 cells were exposed to tanshinone IIA (80 µM), plated onto poly-L-lysine coated slide glass and fixed in 4% (v/v) methanol free formaldehyde solution (pH 7.4) at 4°C for 25 min. The cells were permeabilized in 0.2% (w/v) Triton X-100, blocked in 5% (w/v) bovine serum albumin (BSA), 0.5% (v/v) Tween-20 in humidified chamber and incubated with cytochrome c antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, U.S.A.) containing 100 nm of a mitochondrion-specific dye (Mitotracker green FM: Molecular probes, Inc., Eugene, OR, U.S.A.) and probed with primary antibodies for cleaved caspase-9, cleaved caspase-3, phospho-JNK, JNK, phospho-p38, p38 (Cell Signaling Tech., Danvers, MA, U.S.A.), poly(ADP- ribose) polymerase (PARP), survivin, c-IAP-1, c-IAP-2, Bel-2, Bax, Mel-1 (Santa Cruz Biotechnologies, Santa Cruz, CA, U.S.A.), and β-actin (Sigma-Aldrich, St. Louis, MO, U.S.A.) containing 100 nm of a mitochondrion-specific dye (Mitotracker green FM: Molecular probes, Inc., Eugene, OR, U.S.A.) and visualized under an FLUOVIEW FV10i confocal (Olympus, Tokyo, Japan).

**Statistical Analyses** All data were presented as means± standard deviation (S.D.). Statistical significance was verified by Student’s t-test using Sigmaplot software (Systat Software Inc., San Jose, CA, U.S.A.).

## RESULTS

**Tanshinone IIA Induces Apoptotic Cell Death in KBM-5 Cells** To evaluate the cytotoxicity of tanshinone IIA, MTT assay was performed in human leukemic cell lines such as KBM-5, K562 and U937, along with human normal peripheral blood lymphocytes (PBLs). Cells were treated with various concentrations of tanshinone IIA (0, 10, 20, 40 or 80 µM) for 24 h. Tanshinone IIA decreased the viability of KBM-5, K562 and U937 cells, while it did not hurt PBLs (Fig. 1B).

To test whether or not the cytotoxic effect of tanshinone IIA is associated with induction of apoptosis, we performed several cell-based apoptosis assays. As shown in Fig. 1C, tanshinone IIA increased the sub-G1 DNA contents undergoing
apoptosis in a dose-dependent manner. Consistently, tanshinone IIA remarkably induced oligonucleosomal DNA fragmentation in DNA gel electrophoresis (Fig. 1D) and TUNEL staining (Fig. 1E).

**Tanshinone IIA Induces Mitochondria-Dependent Apoptosis in KBM-5 Cells**

Alteration of mitochondria membrane potential (MMP) is involved in an initial and irreversible step of apoptosis pathway. Thus, we measured MMP in tanshinone IIA-treated KBM-5 cells using tetramethylrhodamine (TMRE) staining. As shown in Fig. 2A, tanshinone IIA significantly reduced fluorescence intensity reflecting MMP in a dose-dependent manner, since MMP loss is closely associated with cytochrome c release from the mitochondria into the cytosol. As shown in Fig. 2B, confocal microscopic observation revealed that localization of cytochrome c was almost in the area stained by Mitotracker, a fixable mitochondria-selective dye, in control cells (top panel), while diffuse distribution of cytochrome c was observed in tanshinone IIA-treated cells (bottom panel). Consistent with immunofluorescence data, tanshinone IIA clearly increased expression level of cytochrome c in cytoplasmic fraction in a dose-dependent manner (Fig. 2C). These results indicate that tanshinone IIA induces apoptosis through mitochondria-dependent pathway (intrinsic pathway) in KBM-5 cells.

**Tanshinone IIA Regulates Apoptosis-Related Protein Expression in KBM-5 Cells**

Apoptosis is regulated by various protein families such as caspase, c-inhibitor of apoptosis protein (IAP) and bel-2. Tanshinone IIA activated caspase-3 and -9, and cleaved PARP, a caspase-3 substrate, in dose- (Fig. 3A) and time-dependent manner (Fig. 3B). Tanshinone IIA also significantly enhanced caspase-3 activity in a dose-dependent manner (Fig. 3C). Additionally, tanshinone IIA suppressed expression of anti-apoptotic proteins such as survivin, c-IAP, Bel-2 and Mcl-1L in dose- (Fig. 3D) and time-dependent manner (Fig. 3E), implying tanshinone IIA induces apoptosis through the intrinsic apoptosis pathway in KBM-5 cells.

**Activation of JNK Mediates Apoptosis Induced by Tanshinone IIA in KBM-5 Cells**

Activation of JNK and p38 MAPK is involved in a variety of physiological processes such as proliferation, differentiation and apoptosis. Therefore, whether tanshinone IIA can induce JNK and p38 activation was examined by Western blotting. Tanshinone IIA significantly increased phosphorylation of both JNK and p38 in a dose-dependent manner (Fig. 4A). However, to confirm the involvement of JNK or p38 in tanshinone IIA-induced apoptosis, cell cycle analysis was performed using their specific inhibitors SP600125 for JNK and SB203580 for p38. As shown in Fig. 4B, SP600125, but not SB203580, significantly decreased the sub-G1 cell population, implying the important role of JNK in tanshinone IIA-induced apoptosis in KBM-5 cells.

**DISCUSSION**

We here report that tanshinone IIA induces apoptosis in association of JNK signaling pathway in human chronic myeloid leukemia KBM-5 cells. Tanshinone IIA induced typical
apoptotic morphological changes including DNA fragmentation, condensed chromatin and pyknosis and increased the population of sub-G1 phase cells.

Mitochondrion plays a pivotal role in the regulation of apoptosis,22) by releasing cytochrome c from the mitochondrion into the cytosol,23) which is linked to loss of mitochondria membrane potential (MMP).22) In our study, tanshinone IIA effectively induced loss of MMP by flow cytometric analysis and the release of cytochrome c by confocal microscopy observation. Also, we found that tanshinone IIA activated...
caspase-3, a key mediator of apoptosis, and caspase-9 and mediated the proteolytic cleavage of PARP, a major caspase-3 substrate, from 116kDa intact form into 85kDa fragment, implying tanshinone IIA induces apoptosis via intrinsic mitochondria-dependent pathway.

Bcl-2 is localized in the mitochondria outer membrane to block the release of cytochrome c from the mitochondria, leading to preventing “apoptosome,” a complex of cytochrome c, caspase-9 and Apaf-1. Thus, Bcl-2 overexpression inhibits apoptosis induction in response to various apoptosis stimuli. Similarly, IAPs as functionally- and structurally-related anti-apoptotic protein family have been reported to bind to caspases to inhibit apoptosis signaling. In the present study, tanshinone IIA down-regulated the expression of Bcl-2 and Bcl-xL of Bcl-2 family, and Survivin and c-IAP2 of IAP family, indicating tanshinone IIA suppresses anti-apoptotic proteins such as Bcl-2, Mcl-xL, Survivin and c-IAP2 in KBM-5 cells.

Activation of JNK and/or p38 plays significant roles in the regulation of apoptosis signaling pathways. In human CML K562 cells, taxol induced apoptosis by inducing intracellular oxidative stress and JNK activation pathway. Likewise, irisiniatistatin A induced JNK activation involved in caspase-8-dependent apoptosis via the mitochondrial dependent pathway in human leukemia Jurkat cells. There are also evidences that p38 mitogen-activated protein kinase is involved in apoptosis induction. Tyrphostin AG825 induced p38 MAPK dependent apoptosis in androgen-independent prostate cancer cells, as p38 is involved in caspase-8-dependent diallyl trisulfide (DATS)-induced apoptosis in human CNE2 cells. Likewise, melanotin induced apoptosis in LNCaP prostate cancer cells via JNK and p38 pathways, and 1-beta-o-arabinofuranosylcytosine induced apoptosis in HL-60 cells through JNK/p38 stress signaling pathway. However, although tanshinone IIA activated the phosphorylation of JNK and p38 in KBM-5 cells, specific JNK inhibitor SP600125, but not p38 inhibitor SB203580, blocked tanshinone IIA-induced sub G1 accumulation, suggesting that JNK activation mediates tanshinone IIA induced apoptosis in KBM-5 cells. Nevertheless, Jiao and colleagues recently reported that tanshinone IIA activated p38 to induce apoptosis in cisplatin-resistant ovarian cancer cells, which raises the possibility that tanshinone IIA may affect different signaling pathways according to cell type or culture condition.

In summary, tanshinone IIA induced apoptotic morphological features, increased sub G1 apoptotic portion, activated caspase-9 and -3, released cytochrome c from mitochondria into cytosol, down-regulated Bcl-2, Mcl-xL, Survivin and c-IAP2 in KBM 5 cells. Furthermore, tanshinone IIA activated phosphorylation of JNK and JNK inhibitor SP600125 suppressed sub G1 accumulation induced by tanshinone IIA in KBM-5 cells. Taken together, our findings suggest that tanshinone IIA induces mitochondria-dependent apoptosis via activation of JNK in KBM 5 cells.

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