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Tanshinone IIA, an ingredient of *Salvia miltiorrhiza* BUNGE, induces apoptosis in human leukemia cell lines through the activation of caspase-3

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Abbreviations: PI, propidium iodide; SM, size marker; PARP, poly(ADP-ribose)polymerase; DMSO, dimethylsulfoxide; UV, ultraviolet

Abstract

Tanshinone II-A is a derivative of phenanthrenequinone isolated from *Salvia miltiorrhiza* BUNGE, a traditional herbal medicine that is known to induce antiinflammatory, anti-oxidative and cytotoxic activity. We have examined cellular effects of Tanshione II-A on HL60 human promyelocytic leukemic cells and K562 human erythroleukemic cells. Tanshione II-A induced a dose- and time-dependent DNA fragmentation into the multiples of 180 bp and specific proteolytic cleavage of poly(ADP-ribose) polymerase in both cell lines. PI-staining and flow cytometry analysis of K562 cells following Tanshione II-A treatment showed an increase of the cells possessing hypodiploid DNA indicative of apoptotic state of cells. Caspase-3 activity was significantly increased during Tanshinone II-A treatment of both HL60 and K562 cells, whereas caspase-1 activity was not changed. These results suggest that Tanshione II-A induced HL60 and K562 cellular apoptosis that may be associated with the selective members of caspase family.

Keywords: apoptosis, caspase-3, leukemia, *Salvia miltiorrhiza* BUNGE, tanshinone II-A

Introduction

A correlation between induction of apoptosis and neoplasia has been documented in some carcinogenesis model. Increasing evidences suggest that the process of neoplastic transformation, progression and metastasis involve alteration of normal apoptotic pathways (Bold *et al*., 1997). Apoptosis also provides some important clues on effective anticancer therapy and many chemotherapeutic agents were reported to exert their anti-tumor effects by inducing apoptosis on the chemosensitive cancer cells (Kamesaki, 1998).

Salvia miltiorrhiza BUNGE is a traditional oriental medicinal herb, the root of which has been traditionally used for multiple therapeutic remedies. According to phytochemical reports, ingredients of the root of *Salvia miltiorrhiza* BUNGE can be classified into two groups (Tang and Eisenbrand, 1992). The first group is phenolics such as salvianolic acid and lithospermate B, and the other is a group of abietane type-diterpene quinone pigments such as tanshinone I, tanshinone IIA, tanshinone IIB and cryptotanshinone. Lithospermate B was shown to be effective on renal failure in rats (Yokozawa *et al*., 1989). Diterpene quinones have been reported to have an antiplatelet aggregation effect (Wang *et al*., 1989). Recently, the growth inhibitory effects of various diterpene quinones on five tumor cell lines were reported (Ryu *et al*., 1997).

In this study, it was found that Tanshinone IIA, the most abundant and structurally representative diterpene quinone of *Salvia miltiorrhiza* BUNGE, induced apoptosis in human leukemic cell lines.

Materials and Methods

Chemicals

Caspase-3 substrate (Ac-DEVD-pNA) and caspase-1 substrate (Ac-YVAD-pNA) were purchased from BACHEM AG, Hauptstrasse, Switzerland. Anti-poly(ADP-ribose) polymerase antibody was from Boeringer Mannheim, Mannheim, Germany. Tanshinone IIA was kindly provided by Dr. Hee-Juhn Park, Sangji University, Wonju 220-702, Korea. All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA.

Cell culture

HL60 human promyelocytic leukemic cell line and K562 human erythroleukemic cell line were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

Internucleosomal DNA fragmentation

Cells were harvested and suspended in 500 μl of lysis buffer containing 20 mM Tris-HCl (pH 7.4), 4 mM EDTA,

0.4% (v/v) Triton X100 and 10 μg/ml digitonin, and incubated on ice for 30 min. After centrifugation for 5 min at 13,000 rpm using Eppendorf tube centrifuge, supernatants were collected. Each supernatant was extracted with phenol three times and once with chloroform. Then, DNA was precipitated by incubating at -80°C for 30 min after the addition of 1 μg of glycogen, 100 μl of 5 M NaCl, and 700 ul of isopropanol to each sample. DNA was collected by centrifuging at 13,000 rpm for 5 min, and washed once with 70% ethanol. DNA pellets were dissolved in 30 μl of TE buffer containing 10 μg/ml RNase A, and incubated at 37°C for 30 min. 10 μl of each DNA samples were loaded on 1.8% agarose gel.

Propidium iodide staining and flow cytometry

Cells were harvested and washed once with cold PBS. Then cell pellets were suspended in 500 μl of Propidium iodide (PI) solution containing 50 μg/ml of PI, 0.1% (w/v) sodium citrate, and 0.1% (v/v) NP-40. Cell samples were incubated at 4°C in the dark for at least 15 min, and analyzed using flow cytometer (FACSCalibur, Beckton Dickinson) and Cell Quest software.

Proteolytic cleavage of poly(ADP-ribose) polymerase (PARP)

Cells were harvested and washed once with cold PBS. Then cell pellets were lysed in 100 μl of lysis buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1% (v/v) Triton X100, and 1 mM PMSF for 30 min on ice. After centrifugation at 13,000 rpm at 4°C for 5 min, supernatants were collected and mixed with SDS sample buffer. Then, each samples were loaded on 8% polyacrylamide gel and transferred to nitrocellulose membrane. Western blotting experiment was done using rabbit polyclonal anti-PARP antibody and alkaline phosphatase-conjugated secondary antibody.

Measurement of caspase-1 and caspase-3 activity

Cells were harvested and washed once with cold PBS. Then cell pellets were lysed using lysis buffer containing 20 mM Hepes (pH 7.4), 100 mM NaCl, 0.5% (v/v) NP-40 and 10 mM DTT on ice for 30 min. After centrifugation at 13,000 rpm at 4°C for 5 min using Eppendorf centrifuge, supernatants were collected and added with Ac-YVAD-pNA (caspase-1 substrate) or Ac-DEVD-pNA (caspase-3 substrate) to the final concentration of 100 μM. Each samples were incubated at 37°C for 1 h and optical density at 405 nm was measured. A standard curve was made by measuring A_{405} of various amounts of pNA, and A_{405} value was converted to the amounts of pNA produced.

Results

Recently, growth inhibitory effect of the ingredients of *Salvia miltiorrhiza* BUNGE on tumor cell line was reported. Tanshinone IIA, the most abundant and structurally representative ingredient of *Salvia miltiorrhiza* BUNGE, inhibited the proliferation of several human tumor cell lines (Ryu *et al*., 1997). Since many anticancer drugs are apoptotic, the apoptosis-inducing activity of tanshinone IIA was investigated. Figure 1 shows the chemical structure of tanshinone IIA. Because tanshinone IIA can not be dissolved in water, DMSO was used. The final concentration of DMSO was adjusted to 0.1% (v/v) in cell culture medium, and 0.1% DMSO-treated cells were used as controls for all experiments. DMSO 0.1% itself had no effect on cells. Human promyelocytic leukemic cell line, HL60, was treated with various concentrations of tanshinone IIA for 4 h, and it was found that tanshinone IIA induced internucleosomal DNA fragmentation into the multiples of 180 bp at the concentration of 1 μg/ml (Figure 2). UV radiation was used as a positive control for the induction of apoptosis (Yoon *et al*., 1996). In time course experiment in which 3 μg/ml of Tanshinone IIA was treated, 180 bp DNA ladder was generated 2 h after the treatment (Figure 3). Tanshinone IIA induced internucleosomal DNA fragmentation in K562 human

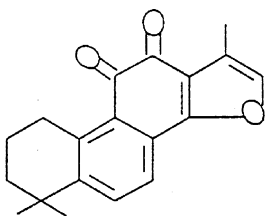

Figure 1. Chemical Structure of tanshinone IIA.

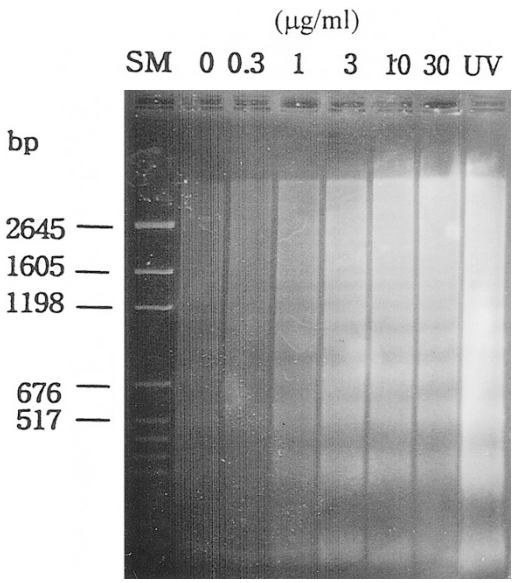

Figure 2. Tanshinone IIA-induced internucleosomal DNA fragmentation in HL60 cell line. Cells were treated with various concentrations of tanshinone IIA for 4 h. Ultraviolet (UV) treatment was used as a positive inducer of apoptotic cell death. SM represents DNA size marker.

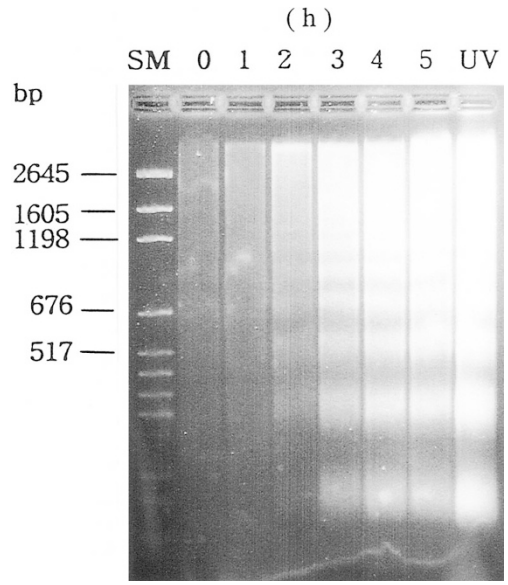

Figure 3. Time course experiment for tanshinone IIA-induced internucleosomal DNA fragmentation in HL60 cell line. Cells were treated with 3 μg/ml of tanshinone IIA for various time periods. SM represents DNA size marker.

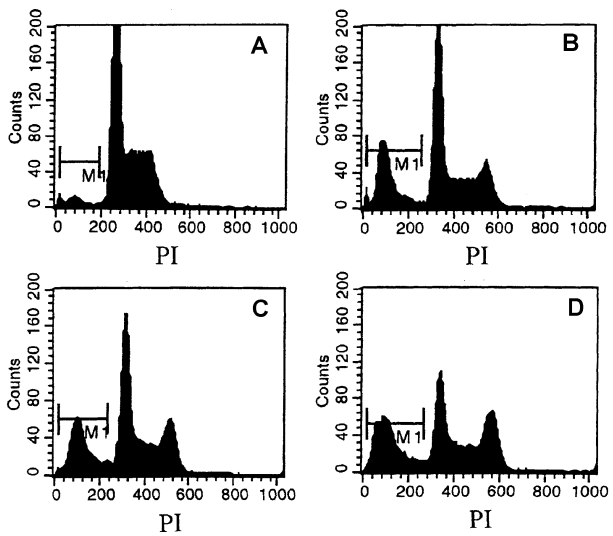

Figure 4. PI staining and flow cytometry analysis of tanshinone IIA-induced apoptosis in K562 cell line. Cells were treated with tanshinone IIA for 24 h at the concentration of 1 μg/ml (A), 3 μg/ml (B), 10 μg/ml (C) and 30 μg/ml (D). Apoptotic cell population which has hypodiploid DNA content was marked by M1.

erythroleukemic cell line at the concentration of 3 μg/ml (data not shown). Flow cytometry analysis of Tanshinone IIA-treated K562 cells showed the increase of hypodiploid apoptotic cells and G2/M phase of cell cycle, while the cell populations at G1 phase was decreased, suggesting that Tanshinone IIA induced an arrest of G2/M phase cell cycle and apoptosis (Figure 4). Apoptotic cell poputations were 4.34%, 26.95%, 26.53% and 34.53% in cells treated with 1, 3, 10, and 30 μg/ml of tanshinone IIA respectively, showing that apoptosis-inducing concentration was between 1 and 3 μg/ml. In control cells treated with 0.1% DMSO alone, 5.03% of apoptotic cell population was detected (data not shown).

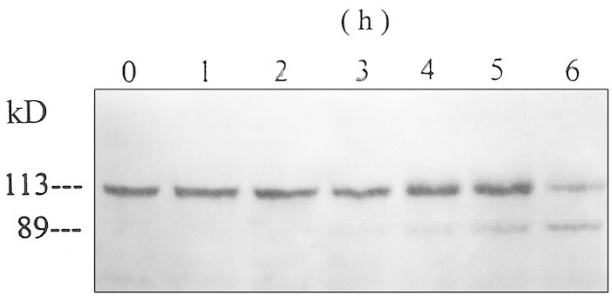

Figure 5. Proteolytic cleavage of PARP during the time course of tanshinone IIA-induced apoptosis. HL60 cells were treated with 3 μg/ml of tanshinone IIA for various time periods, and analyzed by western blotting using anti-PARP antibody. 113 kD PARP protein is specifically cleaved into 89 kD fragment after 4 h.

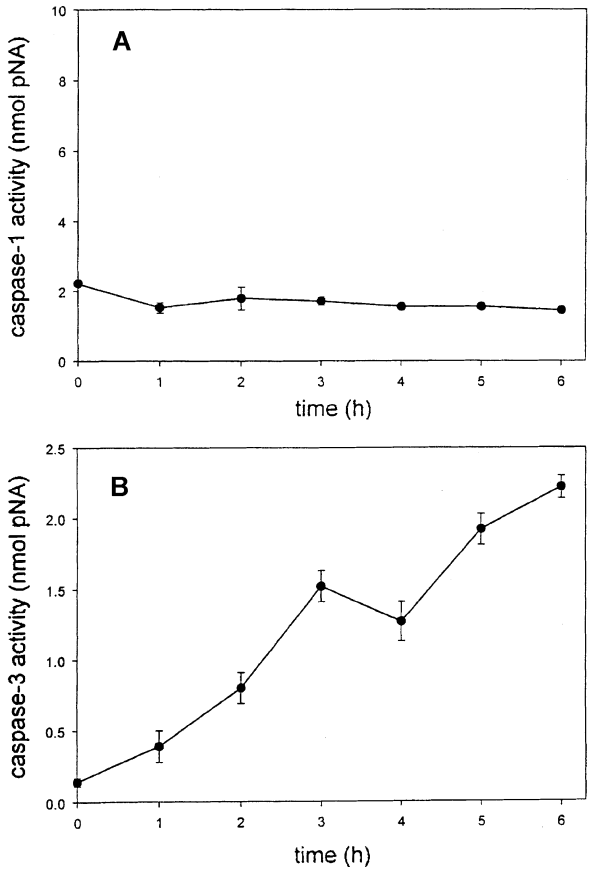

Figure 6. Change of caspase-1 (A) and caspase-3 (B) activities during tanshinone IIA-induced apoptosis in HL60 cells. Cells were treated with 3 μg/ml tanshinone IIA for various time periods, and each enzyme activity was measured using specific tetra-peptide substrate. Each data represent the mean and standard deviation of 4 experiments.

PARP is a nuclear enzyme which is involved in DNA repair process, and recently, it was found that 113 kD PARP protein is cleaved into 89 kD and 24 kD fragments by the action of CPP32, a protease recently named as

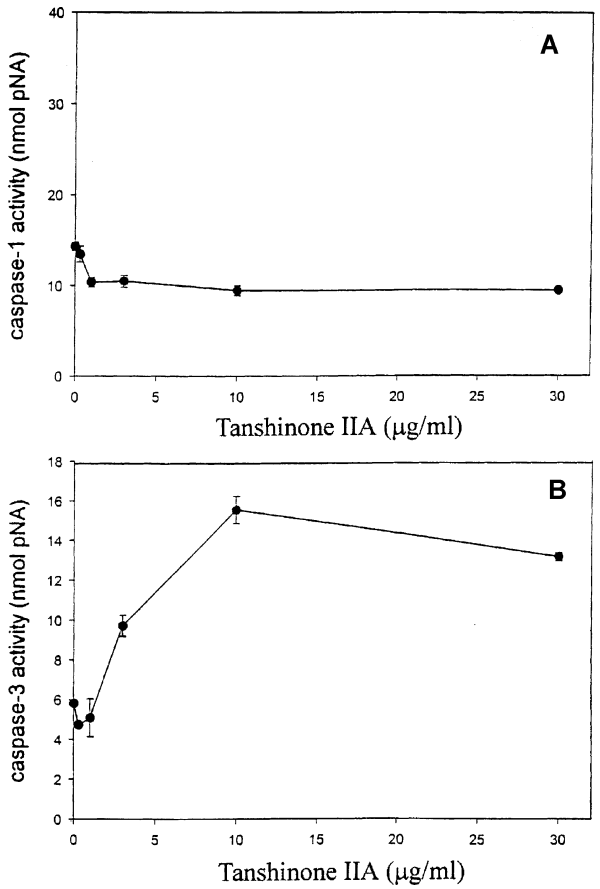

Figure 7. Change of caspase-1 (A) and caspase-3 (B) activities during tanshinone IIA-induced apoptosis in K562 cells. K562 cells were treated with various concentrations of tanshinone IIA for 24 h, and each enzyme activity was measured using specific tetra-peptide substrate as described in 'Materials and Methods'. Each data represent the mean and standard deviation of 4 experiments.

caspase-3 (Nicholson *et al.*, 1995). Since the specific proteolytic cleavage of PARP is considered to be a biochemical characteristic of apoptosis (Nicholson *et al.*, 1995), we did western blotting experiment using the antibody against PARP. Figure 5 shows that PARP is cleaved into 89 kD fragment by the treatment of Tanshinone IIA in HL60 cells, indicating that caspase-3 was activated. PARP cleavage was also detected in K562 cells after 3 μg/ml of tanshinone IIA treatment (data not shown). To measure the caspase-3 activity directly and quantitatively, we used Ac-DEVD-pNA, a specific colorimetric substrate of caspase-3 (Datta *et al.*, 1996). Figure 6 shows that caspase-3 activity began to increase 2 h after the Tanshinone IIA treatment in HL60 cells. Caspase-1 activity was also measured using a specific tetrapeptide substrate, Ac-YVAD-pNA, and it was found that there was no change in its activity during tanshinone IIA-induced apoptosis, suggesting that there is a selective involvement among the caspase family members. The selective activation of caspase-3 was also detected in K562 cells after tanshinone IIA treatment (Figure 7).

Discussion

In this study, it was found that HL60 and K562 human leukemic cell lines showed the features of apoptotic cell death after tanshinone IIA treatment. Among the various features of apoptosis, a ladder-like pattern of DNA fragmentation into the multiples of 180 bp has been considered as a biochemical hallmark of apoptosis. Recently, however, there are some controversial reports that internucleosomal DNA fragmentation also occurs in some necrotic cell death, suggesting the possibility that internucleosomal DNA fragmentation may not be an essential indicator of apoptotic cell death (Cohen *et al.*, 1992; Schulze-Osthoff *et al.*, 1994).

Caspase activation is now considered as a most reasonable criterion for a distinction between apoptosis and necrosis, because the central mechanism of apoptosis is evolutionarily conserved from nematode to mammals and caspase activation is an essential step in this complicated pathways (Thornberry and Lazebnik, 1998). The data shown in Figure 6 and Figure 7, therefore, represent the most important evidence that tanshinone IIA-induced cell death is apoptosis.

Tanshinone IIA treatment induced apoptotic cell death both in HL60 and K562 cell lines at concentrations ranging 1~3 μg/ml with a different responding time. Tanshinone IIA-treated HL60 and K562 cells became apoptotic after 2 h (Figure 3 and Figure 6) and 24 h (data not shown) respectively. A similar time difference between HL60 and K562 cell lines, 2-3 h and 24-48 h respectively, was also reported in etoposide-induced apoptosis (Martins *et al.*, 1997), suggesting that there is some intrinsic difference between two leukemic cell lines.

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