Danshen: a Chinese herb that can trigger apoptosis in Human Promyelocytic Leukemia cells

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Abstract

Danshen, isolated from the Chinese medicinal herb Salvia Miltiorrhiza Radix, has recently been shown to exhibit antitumor activity in human cancer cell lines in vitro; however, the underlying mechanisms are not yet well defined. By using a variety of flow cytometric methods such as DNA-flow cytometry (DNA-FCM); Annexin V assay; and bivariate Bcl-2/ DNA flow cytometry, this study aimed to investigate the antineoplastic mechanisms of Danshen on Human Promyelocytic Leukemia HL-60 cells. Our data from DNA-FCM and Annexin V assay strongly indicated that treatment with Danshen triggered apoptotic death of HL-60 cells. In addition to flow cytometric analysis, morphological changes in HL-60 cells and DNA ladder formation on gel electrophoresis were used to confirm the apoptosis induced by Danshen. Furthermore, the expression of the anti-apoptotic gene, Bcl-2, was downregulated in HL-60 cells by Danshen. We conclude that the exposure of HL-60 cells to Danshen downregulated Bcl-2 expression likely to be associated with increased apoptosis and decreased clonal growth. Together, these findings suggest that induction of apoptosis may contribute to the mechanisms of antitumor activity of Danshen, which merit investigation as potential chemo-prevention agents in humans.

Key words: Danshen. HL-60. Apoptosis. Cancer.

Introduction

In spite of great success in diagnosis and treatment of cancer, total cancer incidence is going up. In reviewing the treatment of malignancies, chemotherapy is the classical means. However, one major problem is resistance of tumor cells to anti-cancer agents among which only a few can selectively prevent tumor growth without affecting that of normal tissues. Yet, a number of naturally occurring products from vegetables and herbs exert chemopreventive properties against carcinogenesis. Danshen, (Latin: *Radix Salviae Miltiorrhizae*), is the dry root and rhizome of *Salvia miltiorrhiza* Bge. (Labiatae), collected in seasons of spring and fall (Diagram 1). It is officially listed in the Chinese Shennong's Pharmacopoeia in the Han Dynasty and has been widely applied in traditional Chinese medicine for treatment of coronary heart diseases, particularly angina pectoris and myocardial infarction[1-3]. It has also been recommended for menstrual disorder, insomnia as well as against inflammation.

Studies on chemical components of the roots of *S. miltiorrhiza* began more than 50 years ago. Over twenty diterpene quinones known as "tanshinones" have been isolated from this herb[1-5]. The planar phenanthrene quinone of tanshinones resembles to a certain extent to that of antitumor agents like Anthracyclines and Anthracenediones. Earlier experimental reports indicated that the crude extracts of Danshen could markedly prolong the survival period of tumor-bearing mice; and tanshinone IIA sodium sulfonate could potentiate the cytotoxic action of hydroxy-camptothecine against Ehrlich ascites carcinoma in mice[3]. Recent studies demonstrated that Danshen exhibited a significant cytotoxicity against human tumor cell lines *in vitro* and displayed a significant increase of life-span *in vivo* when administered to mice bearing sarcoma-180 cells[1].

In addition to the non-toxic effects demonstrated in mice[2], as a potent antitumor substances with diverse biological activities, Danshen has attracted the particular attention of many chemists and clinicians. Yet, the underlying mechanisms of anticancer effects exerted by Danshen remained largely unclear. By using a variety of apoptosis assays including microscopy, DNA gel electrophoresis, DNA content, Annexin V and Bcl-2 expression, the aim of the

Diagram 1. Danshen (Salvia Miltiorrhizae Radix) Inset: Danshen root in dark reddish brown color and round slender shape. (Diagram adapted from Tsui et al., 1997).

present study is to investigate whether the anticancer mechanism of Danshen involves the apoptotic death of Leukemia HL-60 cells.

Materials and Method

Danshen Preparation

Danshen, composed mainly of the extract from top-quality natural roots of *Salvia Miltiorrhiza* Bge., was manufactured by modern scientific technology (obtained in bottles of 50 tablets from Winsor Health Products Ltd., Kowloon, Hong Kong). Stock solution was made by dissolving powder of Danshen extract in RPMI 1640 (GIBCO BRL Life Technologies, Inc., Grand Island; NY) in a concentration of 2mg/ml and kept frozen at -20°C until use.

HL-60 Cell Cultivation

Human Promyelocytic Leukemia HL-60 cells (ATCC, Rockville; Maryland) were grown in suspension in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 2mM L-glutamine; 100units/ml Penicillin; 100µg/ml Streptomycin; and 100µg/ml Fungizone (all from GIBCO BRL Life Technologies, Inc., Grand Island; NY). To maintain asynchronous and exponential growth, the cells were passaged by dilution every third day to a concentration of 2 x 105 cells/ml which were diluted 1:2 one day before each experiment. Cells were maintained at 37°C in a 5% CO_2 humidified atmosphere.

Danshen Treatment

10ml of 1x 10^5 cells/ml of HL-60 cells were incubated in the absence or presence of Danshen in three different concentrations: 0.05, 0.1 and 0.2mg/ml for 72hr before being harvested for subsequent apoptosis analysis. Experiments were repeated at least three times.

Microscopic Examination of HL-60 Cells

A cytospin preparation of an aliquot of cells was stained with Haematoxylin-Eosin solution after fixation in 100% methanol for 10 min. Slides were examined under 100X magnification.

DNA Gel Electrophoresis

3 x 10^6 untreated or Danshen treated cells were fixed in ice-cold 70% ethanol at -20°C for at least 24hrs. The cells were then centrifuged at 800g for 5 min and the ethanol was thoroughly removed. After two washes with PBS, the cell pellets were resuspended in 40µl of phosphate-citrate (PC) buffer at room temperature for 30 min. After centrifugation at 1000g for 5 min, the supernatant was transferred to new tubes and concentrated by vacuum in a SpeedVac concentrator (Savant Instruments inc., Farmingdale, NY) for 1hr. After the addition of 3µl 0.25% Nonidet NP-40 (Sigma) and 5µl of 1 mg/ml RNase A (Sigma) followed by incubation at 37°C for 30 min, 5µl of 1 mg/ml proteinase K (Boehringer Mannheim) was added and the extract was incubated for an additional 30 min at 37°C. After the incubation, 12µl of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol) was added and the entire content of the tube was transferred to the gel. Horizontal 2.0% agarose gel electrophoresis was performed at 80 voltage for 2.5hrs. The DNA in the gels was visualized under UV light after staining with 5µg/ml of ethidium bromide .

Detection of Apoptotic Cells by Cellular DNA Measurements

The harvested HL-60 cells (2 x 10^6) were washed twice with PBS and then fixed in 70% ice-cold ethanol at -20°C for overnight. After fixation, the cells were washed twice with PBS and the pellets were resuspended in 40µl of phosphate-citrate buffer at room temperature for 60 min. The cells were washed twice with PBS and then stained with 1ml PBS containing 1mg/ml ribonuclease and 10µg/ml PI for 30 min in the dark. The DNA stained cells were immediately subjected to flow cytometry measurement (Coulter EPICS, Miami, Florida). The channel number of the fluorescence intensity will be proportional to the DNA content of the cells.

Detection of Apoptosis and Necrosis by AnnexinV-FITC/PI Bivariate Staining

Phosphatidylserine translocation is an early event in apoptosis, thus, 48hrs cultivation was used for analysis instead of 72hrs. Unfixed cells were used in the TACS™ Annexin V- FITC staining (Trevigen, Inc., Gaithersburg, MD). As per manufacturer's instructions, 100µl Annexin V / PI reagent were added to 1ml of 2 x 10^5 washed HL-60 cells. After 15 min of incubation in the dark at room temperature, 400µl of binding buffer was added and then measured by bivariate flow cytometry for green fluorescence of FITC at 525nm and red fluorescence of PI at >625nm.

Positive Control for Annexin V Assay and Gel Electrophoresis

Inhibition of topoisomerase I by Camptothecin (CAM) arrests progression of the DNA replication forks and induces cell death[6]. Exponentially growing HL-60 cells were exposed to short (5hrs) treatment with 0.1µM/ml Camptothecin prior assays. Cells of HL-60 die by apoptosis shortly after administration of CAM.

Bivariate Bcl-2/DNA Measurement by Flow Cytometry

2 x 10^6 ethanol fixed HL-60 cells were centrifuged and washed with 10ml PBS followed by 2ml 1%BSA-PBS for better penetration. Then the cells were incubated for 1hr with 100µl 1:100 dilution of mouse monoclonal anti-human Bcl-2 antibody (Pharmingen, San Diego; CA). After washing twice with 2ml 1%BSA-PBS, the cells were incubated for 1hr with 100µl 1:40 diluted fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG secondary antibody (Sigma Chemical Co., St. Louis; MO). Negative controls were established with either 1:40 isotypic mouse IgG1 antibody (Sigma) or no primary antibody. The samples were counterstained by addition of 1ml 10µg/ml propidium iodide (Sigma) and 0.25mg/ml RNase A (Sigma) in PBS.

Cells were analysed on an EPICS flow cytometer (Coulter, Miami; Florida). FITC green fluorescence was measured through a 525nm band-pass filter, and PI fluorescence at >650nm. At least 10,000 events were analysed by using Coulter Elite version 4.0 software which were later analyzed by software "Winlist" version 1.04 and "Modfit" version 5.11 (Verity Software House, Topsham; ME). Percentages of variation in treated cells compared to control cells are expressed as the ratio of [MFC of labeled sample (MFC of negative sample] on [MFC of labeled control cells (MFC of negative control cells] x 100.

Statistics

Data was expressed as standard error of mean (S.E. M.). Statistical analyses were performed by Student's *t-test.* p-value <0.05 is considered significant.

Results

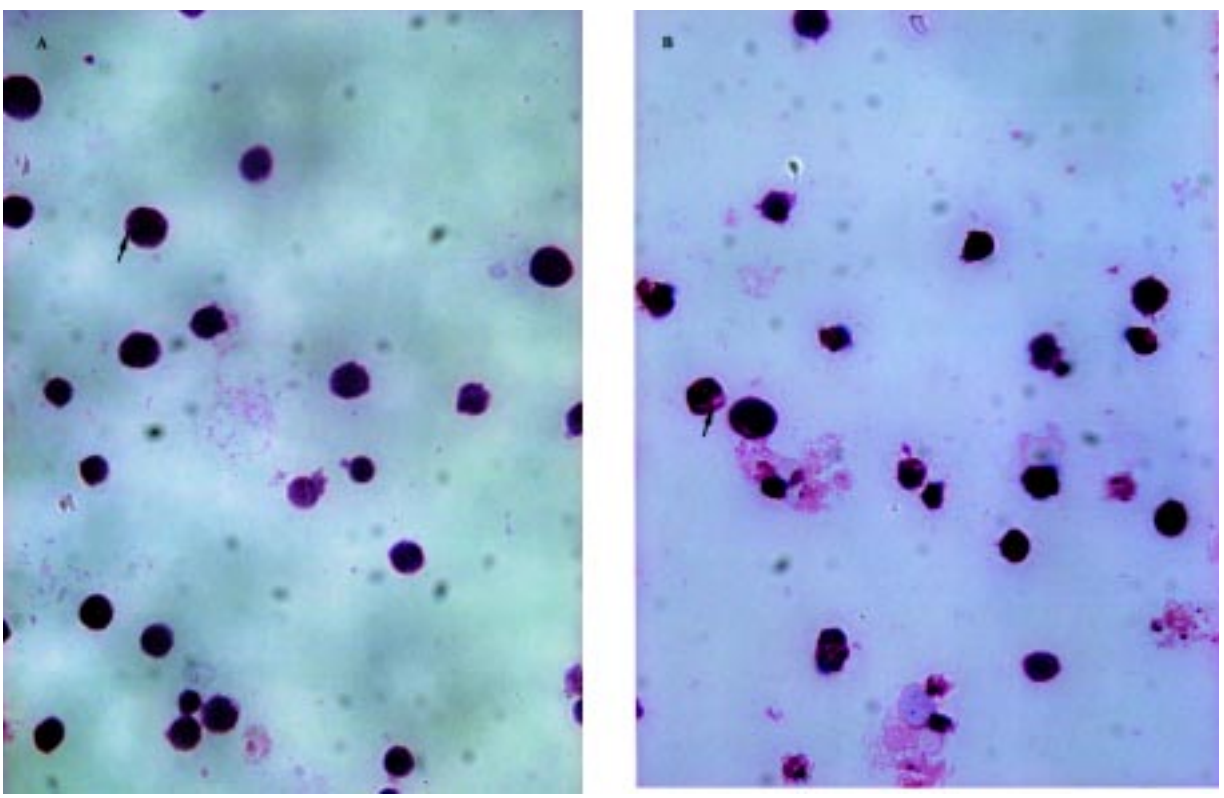
Microscopic Examination of HL-60 Cells

When H&E stained slides were examined under light microscope, untreated HL-60 cells remained ovoid shape with large intact nucleus (Diagram 2A) whereas distinct apoptotic features of condensed chromosomes and nuclear margination were observed in the 48hrs 0.1 mg Danshen-treated HL-60 cells (Diagram 2B).

Diagram 2. Normal and Apoptotic Morphology of HL-60 Cells

(A) Control HL-60 cells are majority premyelocytes which usually appear in ovoid shape with large nucleus (arrow). H&E stain, at x 1000

(B) After incubation at 0.1mg of Danshen for 48hrs, HL-60 cells exhibit characteristic apoptotic features with condensed chromatin along the margin of the nucleus (arrow). H&E stain, at x 1000



Gel Electrophoresis of DNA Fragmentation

Distinct internucleosomal DNA fragmentation ladder could be observed in the 0.1mg, 0.15mg and 0.2mg Danshen-treated HL-60 cells for 48hrs (Figure 1, lanes 5-8). Moreover, in comparison with 24hrs treatment, 0.2mg and even higher dose of 0.4mg of Danshen did not trigger DNA fragmentation (lanes 9-10).

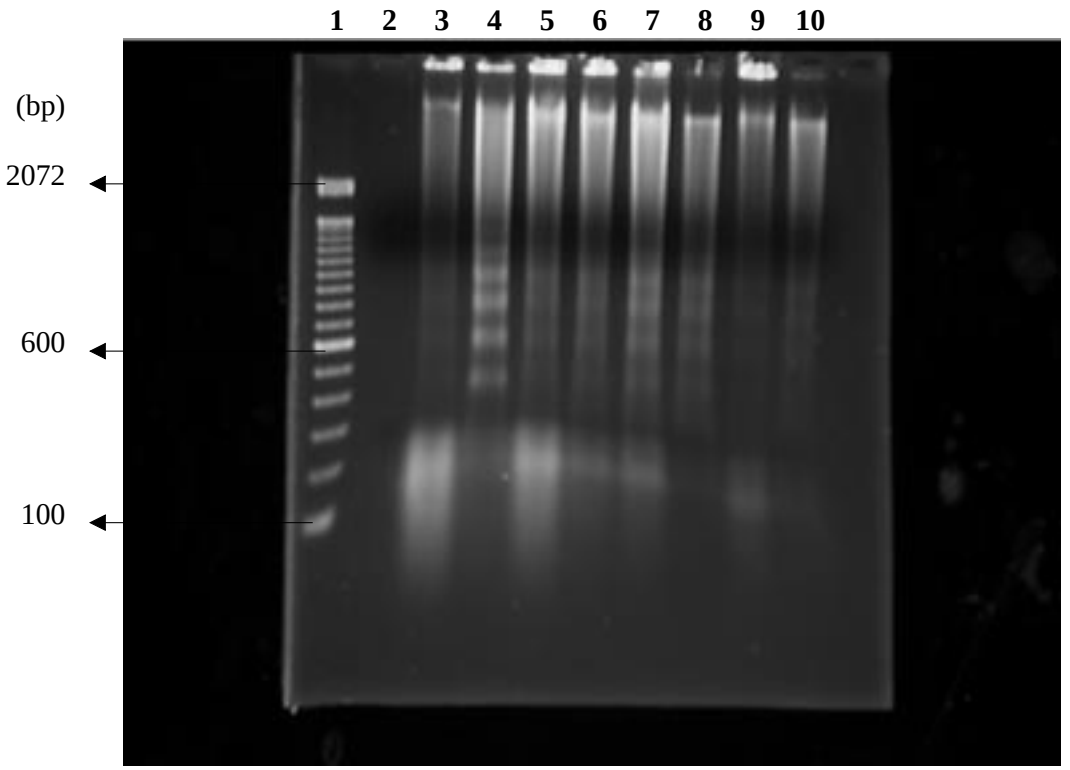


Figure 1. Gel Electrophoresis of HL-60 Cellular DNA Extract

DNA extracts of the HL-60 cells were subjected to gel electrophoresis. Lane1) MW marker in bp; 2) PC buffer; 3) Control HL-60 cells; 4) 1μM CAM positive control; lanes5-8) HL-60 cells after 48 hours incubation at concentrations of 0.1, 0.1, 0.15 and 0.2mg of Danshen showed a ladder pattern characteristic for apoptosis; lanes 9-10) HL-60 cells after 24 hours incubation at 0.2 and 0.4 mg of Danshen did not produce any DNA ladder.

Effects of Danshen on HL-60 Cells Measured by DNA Flow Cytometry

Generally in flow cytometric measurement, apoptotic cells show diminished staining below the G0/G1 population of normal diploid cells. The DNA specific fluorochrome PI, identified this distinct hypo-diploid cell population, designated apoptotic (A0) cells.

The DNA distribution of control cells in G0/G1, S, G2/M phases and A0 was measured as 40.58±1.48%, 45.56±1.81%, 13.86±0.93% and 0.58±0.53%, respectively. When these control cells were treated with 0.1mg of Danshen for 72 hrs, the proportion of cells at G0/G1 phase was relatively decreased from 40.58% to 32.69±2.48% (Figure 2). On the other hand, the proportion of cells in S and G2/M phases were increased to 50.06±2.76% and 17.25±2.62%. However, upon 0.05mg treatment, no significant effect of Danshen on the HL-60 cell cycle could be observed. Diagram 3C shows a representative DNA-histogram of HL-60 cells as observed 72hrs after incubation with 0.1mg Danshen. The percentages of hypo-diploid nuclei (A0 cells), as measured by DNA-FCM, was 8.83(3.76%. When the dose of Danshen increased to 0.2mg, total cells were undergoing death completely (data not shown).

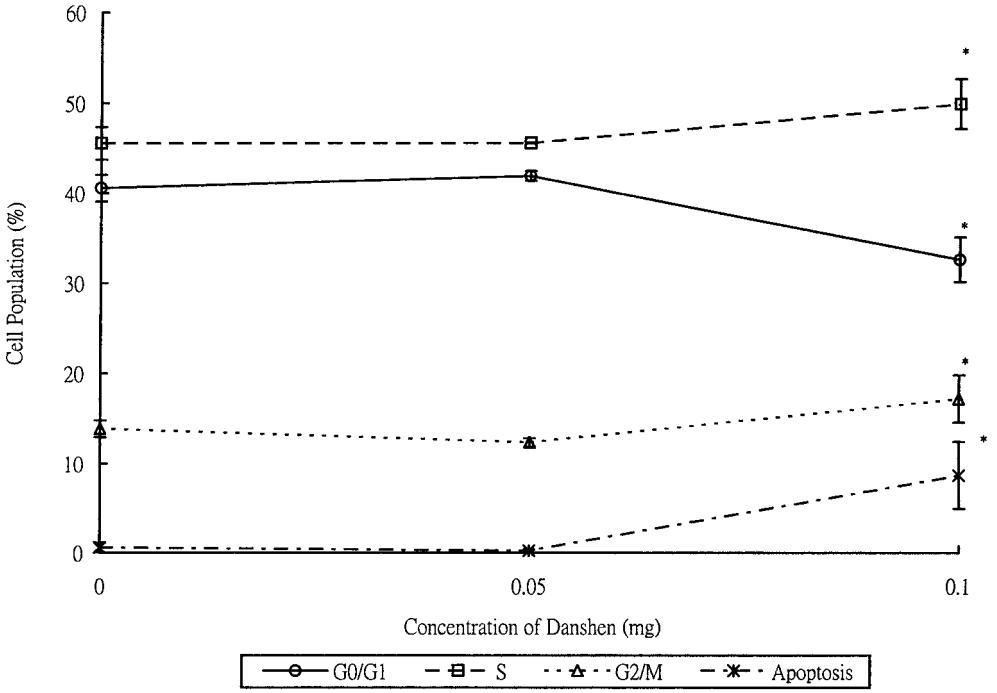


Figure 2. Effects of Danshen on HL-60 cellular DNA Distribution
Values are expressed as mean ± S.E.M. *p<0.05 vs. control

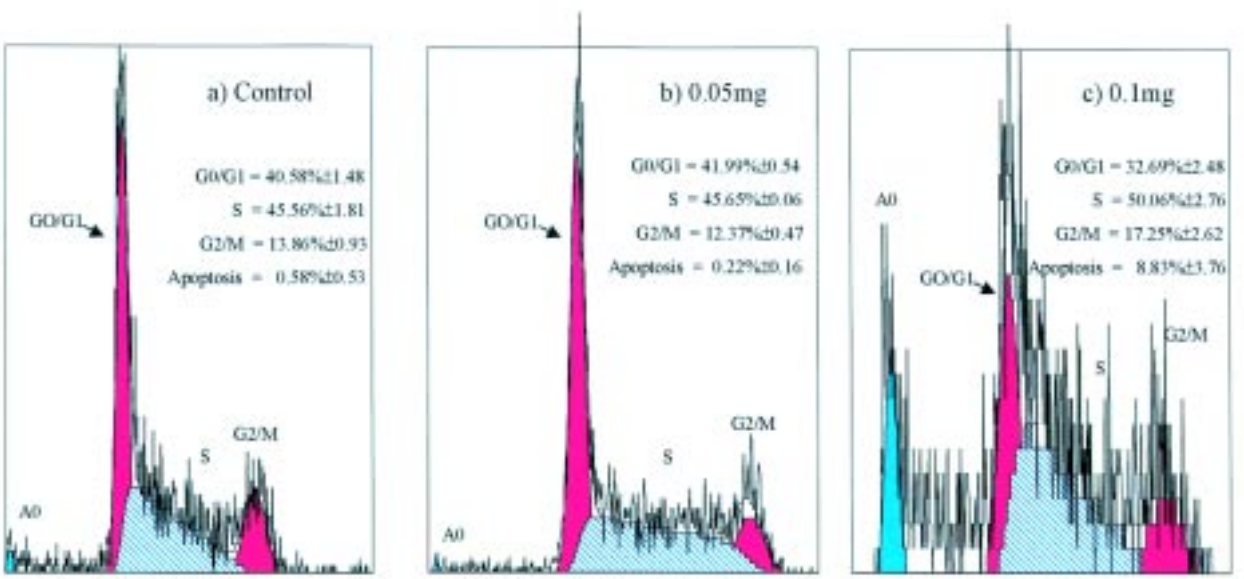


Diagram 3. DNA Histograms
The effects of a) control cells b) Danshen 0.05mg and c) Danshen 0.1mg on HL-60 cell cycle distribution. Proportions of cells (%) in G0/G1, S and G2/M phases of the cell cycle was analysed by software “Modfit” program. A0 is a sub-G1 peak which represents the proportions of apoptotic cells.

Apoptotic Measurement by Annexin V-FITC/PI Bivariate FCM

Diagram 4 shows the apoptotic effects of Danshen on HL-60 cells. After 48hrs incubation, HL-60 control cells contained 90.75(1.16% viable cells, 6.62(0.94% underwent apoptosis and 2.27(0.30% were necrotic. Whereas for the cells treated with 0.1mg and 0.15mg Danshen, viable cells drop to 56.75(2.08% and 23.22 (1.95%. On the other hand, the percentages of apoptotic cells increased gradually from 29.61(2.70% to 61.16(2.50% with the drug concentration increased from 0.1mg to 0.2mg. Simultaneously, necrotic cells also increased with increasing doses of Danshen, rises from 7.51(0.59% to 15.32(1.66%. Danshen reduced the viability and increased the apoptosis and necrosis in HL-60 cells in a dose dependent manner (Figure 3).

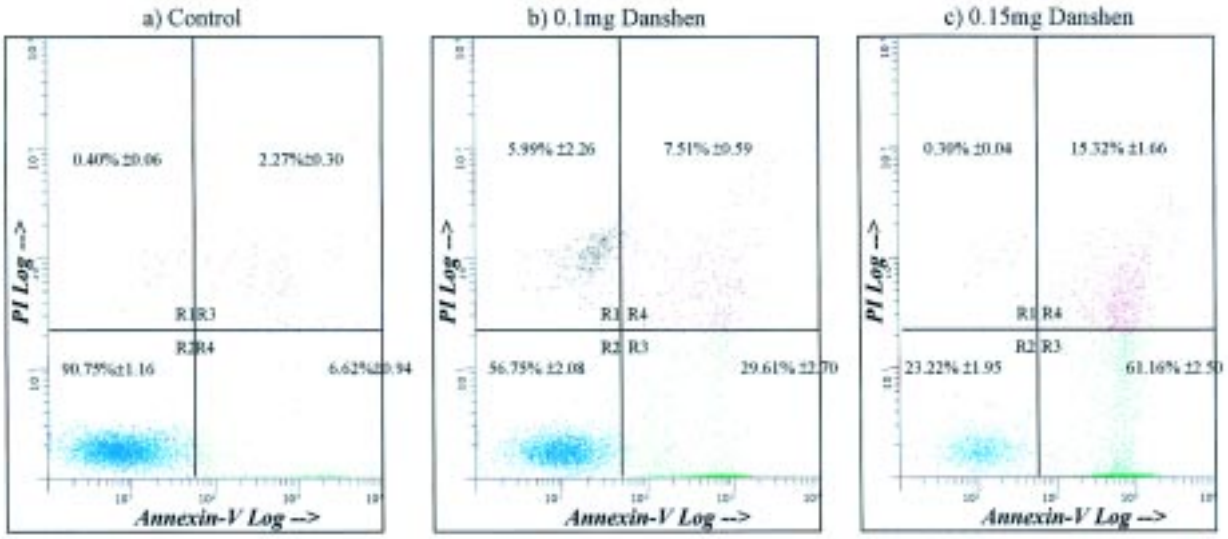


Diagram 4 Bivariate Annexin V/PI analysis of Danshen treated HL-60 cell line. Dot plot of a) Untreated HL-60 cells; b) HL-60 cells treated with 0.1mg Danshen; and c) 0.15mg. Apoptosis was induced by treating cells with Danshen for 48h. The cells were incubated with annexin V-FITC in the dark for 15 min. PI was added to the obtained cell suspension before flow cytometric analysis. The different labelling patterns identify the different cell populations, i.e., region R1: damaged cells (Annexin V-apoptotic or necrotic cells (Annexin V-positive/PI-positive).

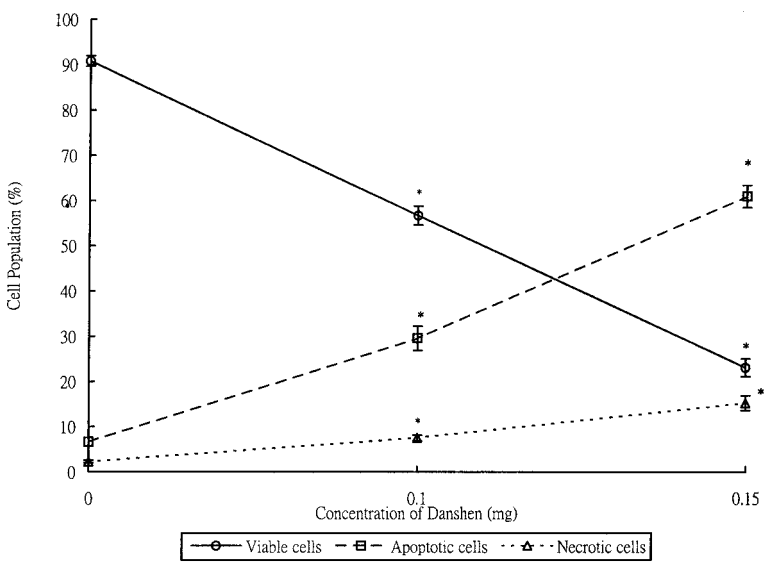


Figure 3. Effects of Danshen on HL-60 Cells Viability, Apoptosis and Necrosis
Values are expressed as mean ± S.E.M. *p<0.05 vs. control

Effects of Danshen on Bcl-2 Expression in HL-60 Cells Measured by Bivariate Bcl-2/DNA Flow Cytometry

Bcl-2 protein was immunocytochemically stained and measured by bivariate flow cytometry that allows the correlation of Bcl-2 expression in the positive cells with their cell cycle phases. Diagram 5 shows the dot plot bivariate measurement of Bcl-2 (Y-axis) which are immunocytochemically stained positive (green) after subtraction of the non-specific fluorescence (blue) given by the same population of cells; and simultaneously measured the proportion of individual DNA content in each specific phases of the cell cycle stained by PI (X-axis). Bcl-2 positivity expressed in HL-60 cells was significantly lowered after treatment with 0.1 mg Danshen as compared to the control cells.

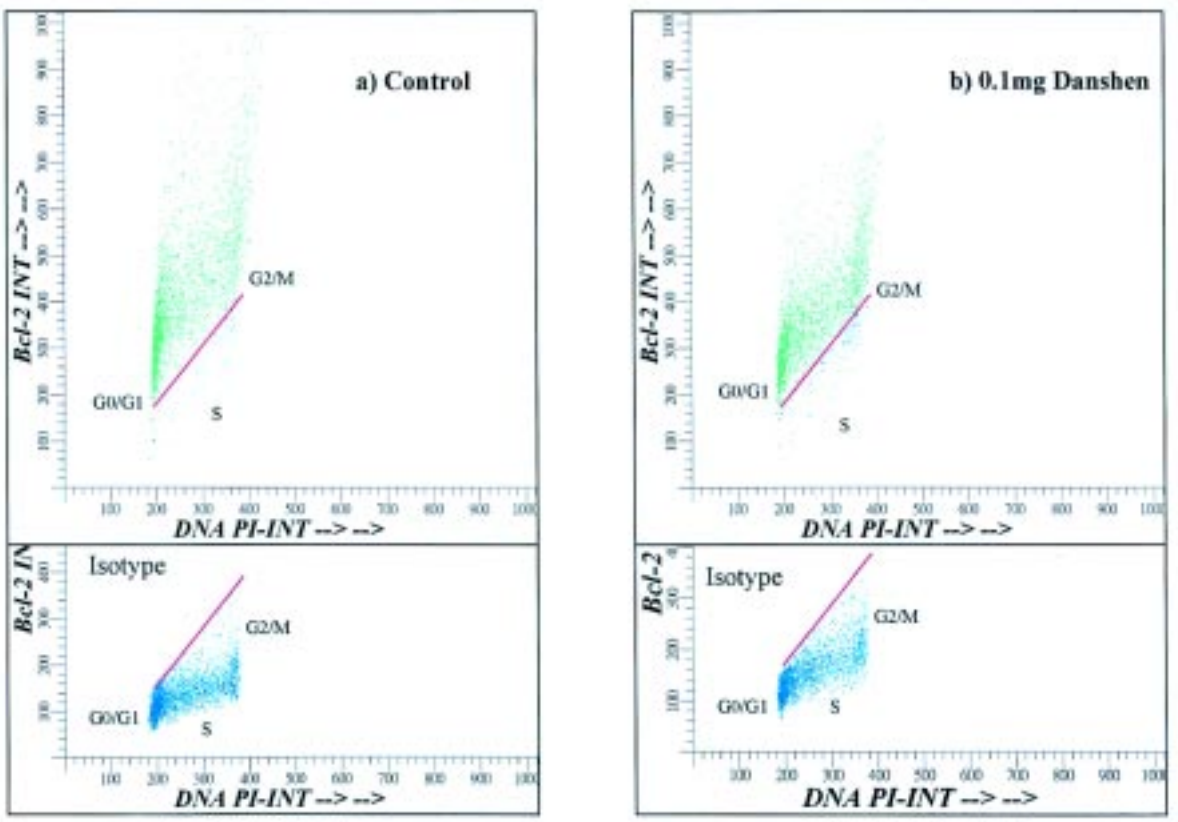


Diagram 5. Effects of Danshen on Bel-2 Expression in HL-60 cells

Dot plot bivariate of a) control cells b) Danshen 0.1mg on Bcl-2/DNA expression measured by flow cytometer. Data were analysed by soft ware "Modfit"

Figure 4 shows the Bcl-2 positivity obtained in the G0/G1, S and G2/M phases of the cell cycle with 88. 70±3.67%, 82.36±11.17% and 83.43±5.60% in control cells and 36.36±3.81%, 40.64±4.49% and 38. 21±3.11% in 0.1mg Danshen treated HL-60 cells, respectively. Obviously, Bcl-2 level was downregulated after Danshen treatment and its effect was not cycle phase specific.

Discussion

The search for novel antitumor drugs has reached a plateau phase. The carcinomas remain almost as stubborn as they did during the last few decades and the need for effective therapy is urgent. Danshen roots (*Salviae Miltiorrhizae Radix*), with "tanshinones" being the main active components, is an effective medicine widely used in Chinese communities for treatment of coronary heart disease, menstrual problems as well as insomnia. Recently, several

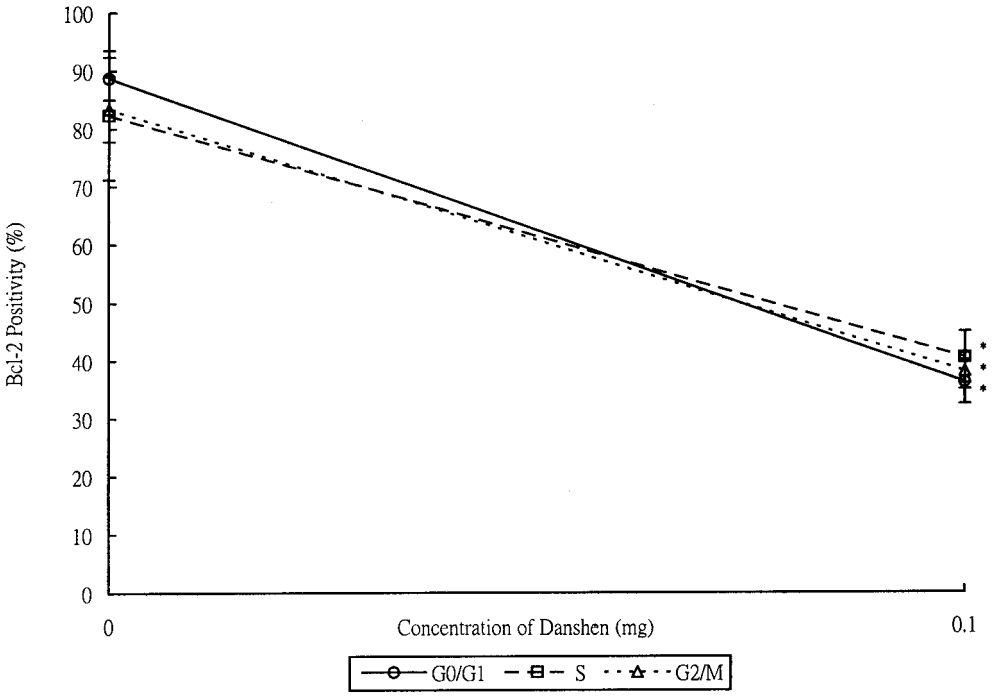


Figure 4. Effects of Danshen on Bcl-2 Positivity Measured in HL-60 Cells at Different Cell-cycle Phases
Values are expressed as mean ± S.E.M. *p<0.05 vs. control

studies provide abundant evidence showing that tanshinones were active against a variety of tumor cell lines in in vitro tests[1,5]. Still and all, the mechanisms by which Danshen destroy the tumor cells are largely unclear. The present study, with the advanced technology from flow cytometer, is trying to tackle these unanswered questions.

By the use of DNA-fluorochrome— propidium iodide (PI) the DNA contents in each phase of the cell cycle as well as the lower DNA content of a "sub G1" peak, a characteristic of apoptotic cells that have lost fragments of DNA, was determined in the DNA histogram by flow cytometry. Our data obtained from the DNA histogram (Diagram 3) exhibited an obvious apoptotic (A0) peak occupying 8.83% of the cell population as HL-60 cells exposed to 0.1mg Danshen while control cells and cells at 0.05mg Danshen demonstrated very low percentages of apoptosis, with 0.58% and 0.22% respectively.

Although the quantitative analysis of hypo-diploid cells using DNA-FCM is easy to perform, it measures only cells at later stage of apoptosis[6]. Hence this method provides no information about the number of apoptotic cells which are in the initial apoptosis phase. Many studies indicate that loss of membrane asymmetry is an early event of apoptosis, started right after the caspase activation but preceding nuclear condensation and nuclear fragmentation. The dual-parameter FCM permits the detection of this early phase of apoptosis before the loss of cell membrane integrity. The quantitation of apoptosis in this method is based upon differences between live, apoptotic and necrotic cells with respect to phosphatidylserine (PS) translocation and membrane permeability[7]. Diagram 4 and Figure 3 show the results of applying the Annexin V assay, to indicate the numbers of apoptotic and necrotic cells in relation to treatment by Danshen of different concentration. HL-60 cells increased the production of apoptotic cells to 29.61% and necrotic cells to 7.51% at 0.1mg Danshen and even higher at 0. 15mg Danshen with 61.16% apoptotic and 15.32% necrotic cells. These data suggest that the apoptotic and necrotic effects exercised by Danshen is dose dependent. It also indicates that the range of concentrations for Danshen to be effective in inducing apoptosis is quite narrow, between 0.1mg to 0.15mg. Since Danshen at 0.05mg did not have inhibitory effect on HL-60 cells whereas majority of dead cells at 0.2mg are necrotic (data not shown).

Although flow cytometry can provide rapid and quantitative assays for enumeration of apoptotic cells, to exclude the pitfalls of apoptotic analysis, it should always be confirmed by the inspection of cells under the light or electron microscope, regardless of the method used. Many studies emphasized that morphological changes during apoptosis are unique and they should be the deciding factor when ambiguity arises regarding the mechanisms of cell death. Thus, a cytospin preparation on a slide of the HL-60 cells insulted with 0.1mg Danshen were stained and examined using light microscope. Characterized cell shrinkage, chromatin condensation in nuclear margin and apoptotic bodies were observed only among the Danshen treated HL-60 cells (Diagram 2B) so as to confirm the A0 peak found in DNA-FCM analysis are of apoptotic origin.

Additional evidence of drug induced apoptotic cell death can be obtained by gel electrophoretic analysis of the DNA degradation products. Studies indicated that an elevation of cellular calcium is a central event in the activation of a calcium-magnesium-dependent endonuclease which cleave DNA at regular internucleosomal linker sites resulting in 180-200 base pair integer of mono- and oligo-nucleosomal fragments, which generate a characteristic "ladder" pattern during agarose gel electrophoresis[8]. In our study, typical ladder was found in HL-60 cells treated with 0.1mg, 0.15mg and 0.2mg of Danshen for 48hrs. These results further support our postulation that HL-60 cell death is affected by the apoptotic signal evoked by Danshen. The question then arise is the observation of different results obtained at 0.2mg Danshen harvested at 24 and 48hr incubation. Several studies found internucleosomal cleavage was a late apoptotic event after DNA condensation and PS translocation, suggesting that 24 hrs incubation is too early for the detection of the late stage activity. That explain the absence of DNA ladder at insufficient time of incubation.

In contrast, necrosis which represents cell death induced by gross injury or an overdose of cytotoxic agents. DNA degradation is not so extensive during necrosis as in apoptosis, and the products of degradation are heterogenous in size, failing to form ladder on electrophoresis. This explain our findings that no DNA ladder was observed when HL-60 cells were exposed at higher concentrations to 0.4mg Danshen for 24 hrs, suggesting that these higher concentrations induced an overdose effect instead of causing apoptotic death of cells. It should be pointed out that prolonged cell arrest in the cell cycle induced by some drugs leads to growth imbalance that may dramatically alter cell biochemistry and morphology. Hence, in many cell types, DNA degradation in apoptosis does not proceed to nucleosomal sized fragments but rather results in 50-300-kb DNA fragments[9].

To understand better the apoptotic regulatory mechanisms is helpful in the exploration of how antitumor agent such as Danshen behaves on modulation of the cell's propensity to undergo apoptosis. The regulation system of apoptosis most often is controlled by the bcl-2/bax family of proteins as well as the cysteine-proteases[9]. Through several oncogenes and tumor suppressor genes such as p53, this two family of proteins interact with the machinery which regulate cell proliferation and DNA repair[10-12].

By using multiparameter analysis combining immunocytochemical detection of individual proteins with apoptotic markers and with DNA content analysis allows the study of the mechanism of cytotoxic drugs, and the effects of biological modifiers on target cells and other molecular interactions associated with cell death. Our data show that the Bcl-2 protein level, as represented by positivity measurement (Figure 4), are downregulated in HL-60 cells after the challenge with 0.1mg Danshen. These data correspond with the hypothesis that alterations in the expression of the oncogene *bcl-2* have been implicated in the regulation of apoptosis, and in the sensitivity of cells to a variety of cytotoxic drugs.

In conclusion, the results of our initial study proves our postulation that by downregulation of Bcl-2 expression, Danshen induces the apoptotic pathway in HL-60 cells and thus causes a dramatic inhibition in the proliferation of the leukemia cells. Although Danshen have potential inhibitory effects on tumor progression, further work should check for its anticancer effect on different cell lines as well as on normal cells so as to ensure that Danshen treatment is safe without any toxicity.

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