Induction of G_1 arrest and apoptosis by *Scutellaria barbata* in the human promyelocytic leukemia HL-60 cell line

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Abstract. *Scutellaria barbata* has been used to treat cancer in Chinese medicine. The responsible anticancer mechanism, however, is not clear. Here we demonstrated an inhibitory mechanism due to a *Scutellaria barbata* extract (SBE) on a human promyelocytic leukemia cell line (HL-60) that has a mutation in the tumor suppressor gene p53. HL-60 cells were incubated with various concentrations of SBE. After a 24-h incubation, cytotoxicity and apoptosis were determined by MTT and DNA fragmentation assay, respectively. After treatment with SBE, cell cycle arrest was determined by measuring the cell number stained by 5'-bromo-2'-deoxyuridine (BrdU) and 7-amino-actinomycin D (7-AAD). Treatment of cells with SBE resulted in a concentration- and time-dependent inhibition of growth and a G_1 phase arrest of the cell cycle. This effect was associated with a marked decrease in the protein expression of cyclin A, D1, D2, D3, and E and their activating partners, cyclin-dependent kinases (CDK) 2, 4, and 6 with concomitant upregulation of p21, cyclin-dependent kinase inhibitor. Downstream of the CDK inhibitory protein-CDK/cyclin cascade, SBE decreased phosphorylation level of retinoblastoma protein. SBE treatment also resulted in apoptosis evidenced by an increase of sub-G_1 phase cells, DNA fragmentation and degradation of the inhibitory protein for the caspase-activated deoxyribonuclease. The molecular mechanism during SBE-mediated growth inhibition in HL-60 cells may be due to modulation of the cell-cycle machinery and the induction of apoptosis.

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Introduction

Many antiproliferative agents have been shown to interfere with the cell cycle machinery and to arrest cells in the G_1 phase of the cell cycle (1). Cell cycle transitions are largely governed by a family of cyclin-dependent kinases (CDK). The activities of CDK are regulated by various factors including: i) levels of various CDK, ii) levels of the cyclins which interact with CDK, and iii) levels of CDK inhibitory proteins (CDKI). Alterations in the formation of CDK/cyclin complexes could lead to increased cell growth and proliferation, and decreased cell growth and proliferation followed by differentiation and/or cell death by apoptosis (2,3). In mammalian cells, cyclin D and cyclin E are synthesized sequentially during the G_1 phase of the cell cycle, with both being rate-limiting for S phase entry. The major catalytic partners of cyclin D are CDK4 and CDK6 (4), while cyclin E is expressed periodically at maximum levels near the G_1/S transition and associates with CDK2 (5). During progression of the G_0/G_1 phase and transition of the G_1 to the S phase, a complex of cyclin D and CDK phosphorylates retinoblastoma protein (Rb), leading to the release of transcription factors such as E_2F (6). The released E_2F then promotes gene expression which is required for cellular proliferation (7). Additionally, cyclin A is produced in late G_1 and expressed during S and G_2 phases and primarily associates with and activates CDK2 (8). On the other hand, the CDKI play important roles in cell cycle control by coordinating internal and external signals to impede proliferation at several key checkpoints (9). Of the several CDKI, p21/WAF1/CIP1 is an important mediator of cell cycle arrest. Several studies have demonstrated that levels of p21 may be critical in determining the threshold kinase activity of various cyclin/CDK complexes, suggesting that finite levels of p21 may be critical in the regulation of cell growth (10,11).

In addition to the regulation of the cell cycle, apoptosis plays an important role in the maintenance of tissue homeostasis. Apoptosis is important for removal of damaged cells, and impaired apoptosis is theorized to contribute to the development of cancer (12). Apoptosis is a physiologic marker

of cell death and is characterized by chromatin condensation, membrane blebbing, cell shrinkage, and DNA fragmentation (13). The process is tightly regulated by the balance of pro- and anti-apoptotic proteins in the cell, which include members of the Bcl-2 family and inhibitors of apoptosis (IAP) (14). It also results from the coordinated actions of several caspase enzymes (15). Caspases cleave many structural proteins such as actin, fodrin, and lamin to generate characteristic apoptotic morphology, and ICAD, an inhibitory protein for the caspase-activated deoxyribonuclease (CAD) (16). Activated CAD cleaves the genomic DNA within the internucleosomal regions, thereby generating multimers of nucleosomal domain-sized fragments (16).

Recent advances in our understanding of the molecular events that drive tumor progression have provided an opportunity to identify effective therapeutic regimens for the treatment and management of malignant diseases. *Scutellaria barbata* has been used as a remedy for various cancers, inflammation and urinary disease in Chinese medicine. Recent studies have demonstrated that *Scutellaria barbata* extract (SBE) has antiproliferative properties towards U937 leukemia cells via the mitochondrial signaling pathway (17). The present study was carried out to investigate the mechanisms by which SBE inhibits the growth of human myeloid leukemia HL-60 cells at a biochemical level. We demonstrated that SBE induced cell cycle arrest at the G_1 phase and apoptosis through a coordinated mechanism involving the downregulation of CDK, cyclin and pRb, and the induction of p21.

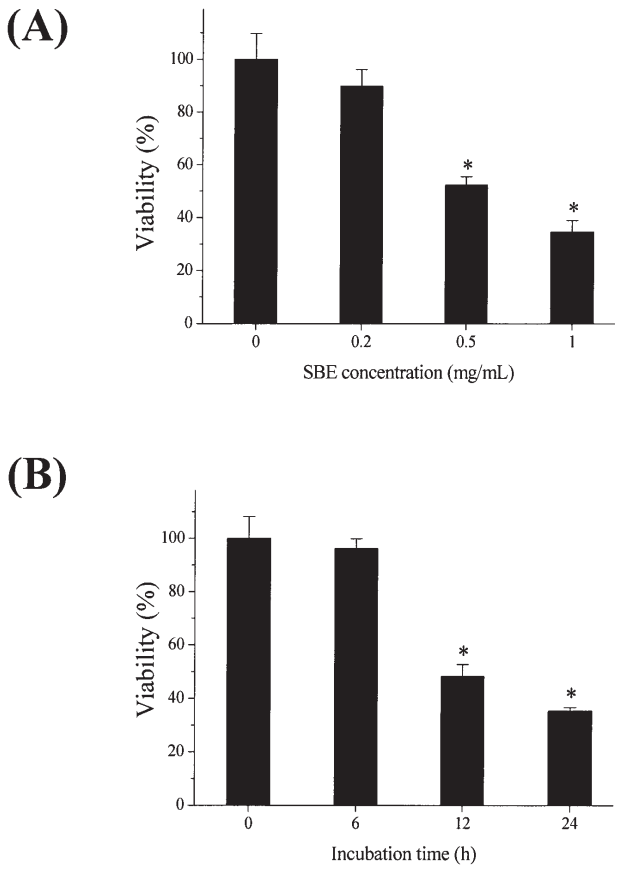

Figure 1. Cytotoxic effects of SBE in the human leukemia HL-60 cell line. Cells ($1x10^5$) were treated with various concentrations of SBE. Cell viability was determined by the MTT assay and was presented as a calculated percentage of viable cells between SBE-treated and -untreated control cells. Each point represents the mean ± SEM of three determinations. *P<0.01 vs. untreated control.

Materials and methods

Culture conditions. HL-60 cells, a promyelocytic leukemia cell line derived from the human, were purchased from the American Type Culture Collection. Cells were placed into 75-cm^2 tissue culture flasks and grown at 37˚C under a humidified, 5% CO_2 atmosphere in RPMI-1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum and 2 mM glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 2.5 μg/ml of amphotericin B.

Preparation of Scutellaria barbata extracts (SBE). For extraction, a mass of *Scutellaria barbata* (200 g) was ground and extracted with boiling water for 4 h. After centrifugation at 3,000 x g for 20 min, the supernatant was concentrated under reduced pressure to a final volume of 200 ml and lyophilized yielding 21.54 g of a sterile extract that was then stored at -70˚C.

MTT assay for cell proliferation. The viability of cultured cells was determined by assaying the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan as described previously (18). After treatment, the cells in 96-well plates were washed twice with PBS and MTT (100 μg/100 μl of PBS) was added to each well. Cells were then incubated at 37˚C for 1 h, and DMSO (100 μl) was added to dissolve the formazan crystals. Absorbance was measured at 570 nm with a model Spectra MAX Plus (Molecular Devices, Sunnyvale, CA).

Cell cycle analysis. A 5'-bromo-2'-deoxyuridine (BrdU) flow kit (BD Pharmingen, San Diego, CA) was used to determine the cell cycle kinetics and to measure the incorporation of BrdU into DNA of the proliferating cells. The assay was performed according to the manufacturer's protocol. Briefly, cells ($1x10^6$/well) were seeded overnight in 6-well tissue culture plates and treated with various concentrations of SBE for 24 h, followed by the addition of 10 μM BrdU, and the incubation was continued for an additional 2 h. Cells were pooled from triplicate wells per treatment point, fixed in a solution containing paraformaldehyde and the detergent saponin, and incubated for 1 h with DNase at 37˚C (30 μg per sample). FITC-conjugated anti-BrdU antibody (1:50 dilution in wash buffer; BD Pharmingen) was added and incubation was continued for 20 min at room temperature. Cells were washed and DNA was stained using 7-amino-actinomycin D (7-AAD; 20 μl per sample), followed by flow cytometry analysis using FACSort (Becton Dickinson, Franklin Lakes, NJ). The BrdU content (FITC) and total DNA content (7-AAD) were determined using Cell Quest (Becton Dickinson) and FCS Express software (De Novo Software).

Western blot analysis. Cells were homogenized in 100 μl of ice-cold lysis buffer (20 mM HEPES, pH 7.2, 1% Triton

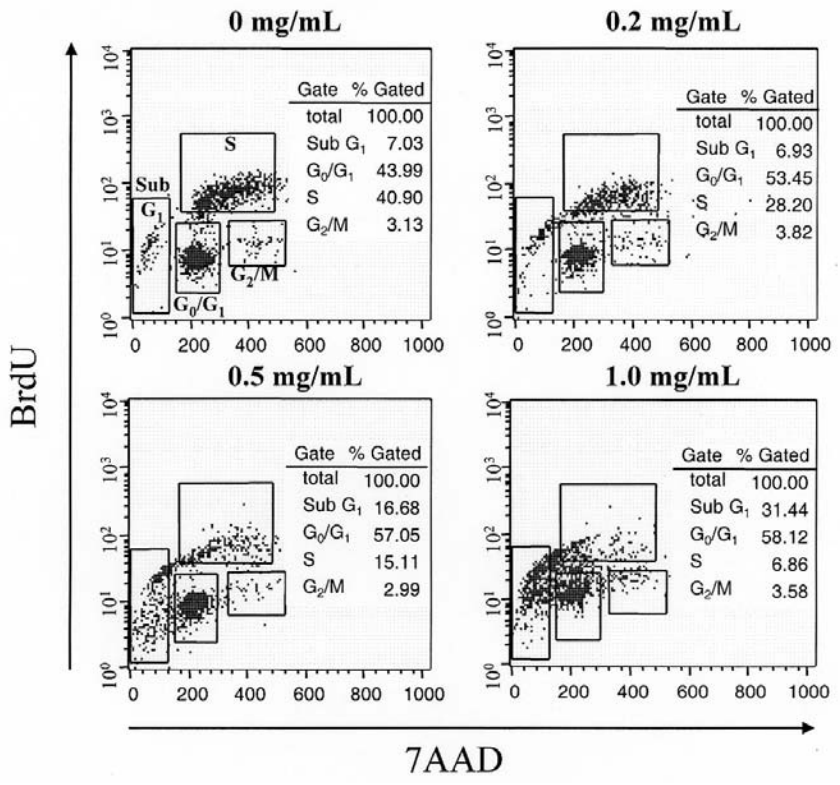

Figure 2. Effects of SBE on the cell cycle pattern of HL-60 cells by flow cytometry. Histogram patterns of HL-60 cells treated with various concentrations (0.2, 0.5, and 1.0 mg/ml) of SBE for 24 h by cell cycle analysis. Cell cycle distribution was analyzed by flow cytometry after coupled staining with fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody and 7-amino-actinomycin D (7-AAD) as described in Materials and methods. Representative histograms depict relative number of cells in the sub-G_1, G_0/G_1, G_2/M, and S phases of the cell cycle.

X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 10 μg/ml aprotinin). Homogenates containing 20 μg of protein were separated by SDS-PAGE with 10% resolving gel and 3% acrylamide stacking gel, and transferred to nitrocellulose membranes (Millipore, Bedford, MA). The nitrocellulose membranes were blocked with 2% bovine serum albumin and then probed overnight with primary antibodies for ICAD, cyclin A, D1, D2, D3, and E, CDK2, 4, and 6, p21, and pRb (Santa Cruz Biochemical, Santa Cruz, CA). Horseradish peroxidase-conjugated IgGs (Zymed, San Francisco, CA) were used as secondary antibodies and protein expression levels were determined by analyzing the signals captured on the nitrocellulose membranes using a Chemi-doc image analyzer (Bio-Rad, Hercules, CA).

Detection of DNA fragmentation by gel electrophoresis. Cell pellets ($3x10^6$ cells) were re-suspended in 500 μl of lysis buffer (0.5% Triton X-100, 10 mM EDTA, and 10 mM Tris-HCl, pH 8.0) at room temperature for 15 min and centrifuged at 16,000 x g for 10 min. DNA was then extracted twice with phenol/chloroform (1:1), precipitated with ethanol, and re-suspended in Tris/EDTA buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). DNA was analyzed after separation by gel electrophoresis (2% agarose).

Protein determination. Protein concentrations in the HL-60 cells were determined by the method of Bradford (19) with bovine serum albumin as the standard. All samples were assayed in triplicate.

Statistical analysis. Statistical analysis of the data was performed with ANOVA and the Student's t-test. Differences of $P<0.05$ were considered statistically different.

Results

SBE induces cell death of human leukemia HL-60 cells. The MTT conversion assay was performed to determine the cytotoxic effects of SBE on the HL-60 cells. As shown in Fig. 1, SBE treatment induced cell death of HL-60 cells in a concentration- and time-dependent manner. SBE treatment at 0.5 and 1.0 mg/ml concentrations for 24 h resulted in 47.7±3.2% ($P<0.01$) and 65.4±4.3% ($P<0.01$) inhibition of cell viability, respectively.

SBE induces G_1 arrest in HL-60 cells. Based on the cytotoxic response in HL-60 cells to SBE, we next examined the effects on cell cycle progression by flow cytometry. Arrest at G_1 can prevent the replication of damaged DNA and, therefore, helps to check the proliferation capacity of cancer cells. Consistent with an effect on cell viability, SBE treatment resulted in accumulation of cells in the G_0/G_1 phase (from 43.99 to 58.12%, Fig. 2). The increase in the G_0/G_1 phase cell population was accompanied by a decrease in the number of cells at the S phase (from 40.90 to 6.86%).

SBE modulates the protein levels of important G_1/S phase regulators. Perturbations in cell cycle regulation have been demonstrated as one of the most common characteristics of

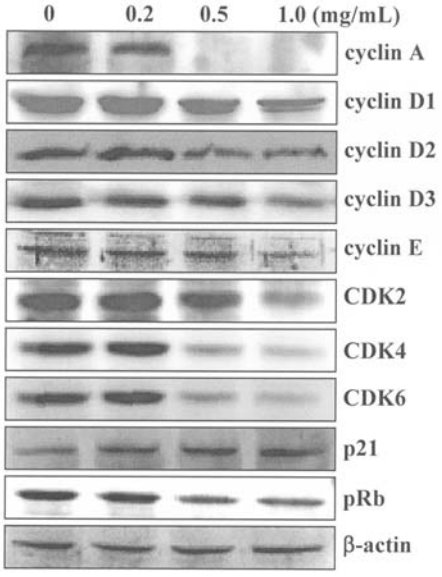

Figure 3. Concentration-dependent effects of SBE on cell cycle-related proteins. HL-60 cells ($5x10^6$) were treated with the indicated concentrations of SBE for 24 h and cyclin A, D1, D2, D3, and E, CDK2, 4, and 6, p21, and pRb were examined by Western blotting as described in Materials and methods. A representative result was presented from among at least three separate experiments yielding similar results.

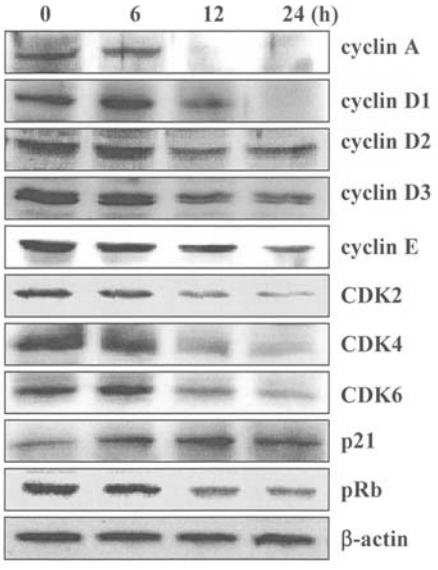

Figure 4. Time-dependent effects of SBE on cell cycle-related proteins. HL-60 cells ($5x10^6$) were treated with 1.0 mg/ml of SBE for the indicated times. Experimental conditions were the same as for Fig. 3. A representative result was presented from among at least three separate experiments yielding similar results.

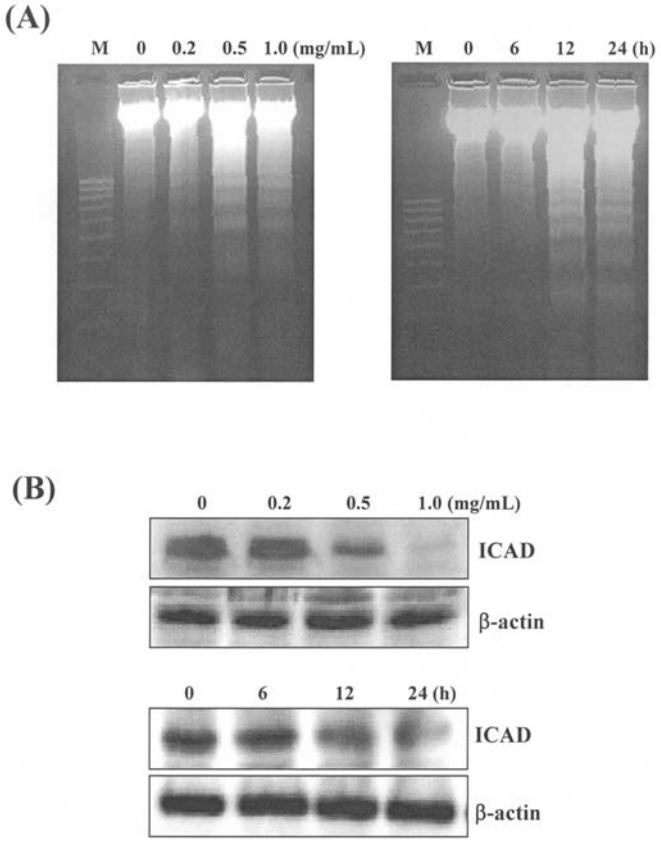

Figure 5. Induction of apoptotic cell death by SBE in HL-60 cells. (A) Induction of internucleosomal DNA fragmentation by SBE. HL-60 cells ($5x10^6$) were incubated with various concentrations of SBE as indicated. DNA was extracted and analyzed by 2% agarose gel electrophoresis in the presence of EtBr. (B) HL-60 cells ($5x10^6$) were treated with various concentrations of SBE as indicated and Western blot analysis was performed for ICAD.

cancer therapy (20-25). These alterations are generally associated with uncontrolled cell growth and involve the downregulation of CDK and cyclin expression or the increased expression of CDKI. Based on our data showing that SBE induces robust G_1 arrest in the HL-60 cells, we assessed the effect of SBE on cell cycle regulatory molecules that play important roles in the G_1/S transition phase such as CDK and cyclin. Expression of cyclin A, D-type cyclins (cyclin D1, D2, and D3), and cyclin E were decreased by SBE treatment in a concentration- and time-dependent manner (Figs. 3 and 4). SBE treatment of HL-60 cells decreased the expression of CDK2, 4 and 6 in a concentration- and time-dependent manner as well (Figs. 3 and 4). The observed downregulation of cyclin and CDK protein levels by SBE was not due to overall changes in protein levels as confirmed by probing the membranes with ß-actin antibodies.

Since SBE showed strong decreases in the expression of CDK and cyclin, we chose to investigate p21 because enhanced levels of p21 have been shown to be associated with arrest at G_1 (10,11). Western blot analysis showed that SBE treatment (0.2, 0.5 and 1.0 mg/ml SBE for 24 h) of HL-60 cells markedly induced protein levels of p21 in a concentration- and time-dependent manner (Figs. 3 and 4).

The SBE-induced accumulation of cells in the G_0/G_1 phase indicated a potential effect on Rb, whose growth-suppressive hypophosphorylated form is characteristic for cells in the early G_1 phase (26,27). Furthermore, Rb serves as substrate for CDK2, 4 and 6. The phosphorylation levels of Rb protein in cells treated with SBE were markedly decreased; it changed to the hypophosphorylated or nearly unphosphorylated form as the concentration and time were increased (Fig. 4).

SBE causes apoptotic death of HL-60 cells. We investigated the possible involvement of apoptosis induction by SBE that

might have contributed to the potent G_1 arrest effect. By flow cytometric analysis, SBE increased the apoptotic portions of sub-G_1 peaks to 6.93, 16.68, and 31.44% at concentrations of 0.2, 0.5, and 1.0 mg/ml, respectively (Fig. 2). Treatment with SBE also resulted in internucleosomal DNA fragmentation which was seen as a characteristic DNA pattern ('ladder') on agarose gels (Fig. 5A). To further confirm the apoptotic effect of SBE, Western blot analysis was performed to analyze ICAD degradation. As shown in Fig. 5B, SBE increased the degradation of ICAD in a concentration- and time-dependent manner.

Discussion

Numerous studies have demonstrated an association between cell-cycle regulation and cancer, and the inhibition of the cell cycle has become an appropriate target for the treatment of cancer (20-25). In our experiments, SBE induced G_1 arrest in a concentration-dependent manner in the HL-60 cell line. In eukaryotes, passage through the cell cycle is governed by a family of protein kinase complexes that are controlled in part by CDK and its essential activating partner, cyclin (21). These complexes are activated at specific intervals under normal conditions, and through a series of events caused the progression of cells through the different phases of the cell cycle, thereby ensuring normal cell growth. Any defect in this machinery causes an alteration in cell cycle regulation that may result in unwanted cellular proliferation culminating ultimately in the development of cancer (20,28). Cyclins D and E, along with CDK2, 4, and 6 are driving forces for the G_1/S phase of the cell cycle (29). Overexpression of cyclin D is associated with various cancers and tumor-derived cell lines that are linked with dysregulated cell growth (29,30). Therefore, significant decreases in protein expression of the D-type cyclins, cyclin A and E, and CDK2, 4, and 6 by SBE suggest the potential, through cell cycle regulation, to inhibit HL-60 cell growth.

During cell cycle progression, the CDK/cyclin complexes are inhibited via binding to CDKI. Numerous studies have shown that CDKI regulates the progression of cells in G_1 phase and that induction of these molecules causes a blockade of the G_1/S transition resulting in a G_1 arrest of the cell cycle (9-11,20,21,28,29). The important CDKI include p21, a universal inhibitor of CDK whose expression is primarily regulated by the p53 tumor suppressor protein (31). Thus, we focused our study on the effects of SBE on p21 expression. The results obtained provide convincing evidence that SBE exerts its effects on cell cycle progression mainly via the upregulation of p21.

Many investigations have demonstrated that exogenous stimuli may result in p53-dependent as well as p53-independent induction of p21, which may cause a blockade of the G_1/S phase transition with the outcome being an arrest at G_1 and apoptosis (31,32). Since HL-60 cells are p53-negative (33), SBE promotes the expression of p21 in a p53-independent pathway followed by cell cycle arrest at the G_1 phase. The Rb family members are nuclear phosphoproteins which are regulated in a cell cycle-dependent manner by phosphorylation and, therefore, are critical targets for inactivation by transforming oncoproteins (34,35). SBE was shown to induce decreased phosphorylation of Rb protein in a concentration- and time-dependent manner (Figs. 3 and 4). The p21 protein binds to cyclin/CDK complexes resulting in inhibition of the G_1/S phase transition by inhibiting phosphorylation of Rb protein (21). The decrease in phosphorylated Rb proteins, therefore, should be due to SBE-triggered p21 expression and the decreased expression of cyclin-CDK.

The p53-independent arrest at G_1 is tightly associated with apoptosis (36) at least in p53-negative cells. This observation prompted our next series of experiments wherein we determined the extent of apoptosis in HL-60 cells caused by SBE. It is well established that apoptosis and the associated signaling pathways and cellular events controlling the process have a profound effect on the progression of the benign to malignant phenotype and as such can be targeted for interventional therapy (37). Our results indicated that SBE caused apoptotic cell death that was evidenced by the increase of sub-G_1 phase cells, the ladder pattern of DNA fragmentation and the degradation of ICAD. Since induction of CDKI has been reported in anticancer agent-induced apoptosis in cancer cells from the prostate of the human (38), the SBE-related increase in p21 could be responsible, in part, for the observed death of HL-60 cells. Degradation of ICAD suggests that caspase activation could have an important role in SBE-induced apoptosis induction, however, more detailed studies are needed to better understand an SBE-induced apoptotic death mechanism for the HL-60 cells. Our results demonstrating the induction of apoptotic death of HL-60 cells by SBE could be of considerable significance in identifying another anti-cancer mechanism for SBE in HL-60 cells.

Acute promyelocytic leukemia is a rare disease that accounts for nearly 10% of acute myeloid leukemia. The mean age at diagnosis is ~40 years, which is considerably younger compared to other subtypes of acute myeloid leukemia (39,40). The search for promising agents that could reduce the incidence and the burden of cancer has become increasingly important in recent years. Naturally occurring dietary supplements and herbs have stimulated great interest because of their chemopreventive and chemotherapeutic properties as well as their relatively non-toxic effects, low cost and availability as oral formulations (41-44). *Scutellaria barbata* used in Chinese medicine has been shown to provide protection against several major liver, lung, and rectal cancers (17,45). In this regard, we focused our studies on the effects of SBE extracts on cell proliferation in a human promyelocytic leukemia cell line with the objective of elucidating the signaling mechanism through which they exert their effects. We have demonstrated that SBE strongly induces G_1 arrest and apoptosis through the induction of p21 expression and the downregulation of CDK/cyclin complexes followed by a decrease in phosphorylated levels of Rb. Based on our data, it may prove useful to develop *Scutellaria barbata* as a cancer preventive or therapeutic agent against acute leukemia.

Acknowledgements

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