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Regulation of Caspase Activity During Apoptosis Induced by Baicalein in HL60 Human Leukemia Cell Line

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Abstract Baicalein, one of the major flavonoid in *Scutellaria baicalensis*, has been known for its effects on proliferation and apoptosis of many tumor cell lines. Most biological effects of baicalein are thought to be from its antioxidant and prooxidant activities. In this report, baicalein was found to induce apoptosis in HL60 human promyelocytic leukemia cell line. Baicalein treatment induced DNA fragmentation and typical morphological features of apoptosis. To elucidate the mechanism of baicalein-induced apoptosis, the activities of the members of caspase family were measured. Interestingly caspase 2, 3, and 6 were significantly activated whereas caspase 1, 8, and 9 were not activated, suggesting selective involvement of specific caspases. Further, treatment with caspase inhibitors also supports the involvement of caspase 2 in apoptosis process. Although it has been reported that baicalein can induce apoptosis through many caspase pathways, the present study indicates that caspase 2 not caspase 9 pathway may be the important step in apoptosis on HL60 cell line.

Keywords: apoptosis, caspase, *Scutellaria baicalensis*, baicalein

Introduction

Apoptosis, an evolutionarily conserved form of cell suicide, requires specialized machinery. The central component of this machinery is a proteolytic system involving a family of proteases called caspases. These enzymes participate in a cascade that is triggered in response to apoptosis-inducing signals and cleave a set of cellular proteins, resulting in disassembly of the cell. Several members of the caspase family were identified so far, and they are classified into different functional groups. Caspase 2, 8, and 9 are known as apoptosis activator because they function at the early stage of apoptosis. Effector caspases including caspase 3 and 6, which were activated by activator caspase, degrade cellular structural components and lead to the final stage of cell death (1).

Scutellaria baicalensis (SB) is a medicinal herb, the root of which has been traditionally used to remove heat and toxic material from human body (2). Recent investigations have shown that SB can inhibit cancer cell growth or induce apoptosis in breast, hepatocellular, pancreatic, and prostatic carcinoma cell lines (3-6). Baicalein, a main ingredient of SB, has shown many biological effects such as antiviral, anti-inflammation, anti-hepatotoxicity, and anti-tumor properties (7-9). Most biological effects of baicalein are thought to be from its antioxidant and prooxidant activities. Many studies also indicated that baicalein induced apoptosis in tumor cell lines (10,11). Especially it was reported that baicalein induced apoptosis through generation of reactive oxygen species (ROS) in human promyelocytic leukemia cells (HL 60) and this process of apoptosis involved activation of caspase 3, 8, and 9 through mitochondrial dysfunction pathway (12,13). This result was also supported by similar studies showing that ROS plays a key role in apoptosis of HL 60 (14) and caspase 3 and 9 was activated during apoptosis in human myeloma cell lines (U266) (10). Interestingly, inhibitory effects of baicalein on ROS induced toxicity were also reported (9,15). In addition to induction of apoptosis, many diverse effects of baicalein have been reported including inhibition of lipoxygenase (16) and regulation of cyclooxygenase expression (17). Although several biological activities of baicalein are attributed to its antioxidant and prooxidant capacities, the exact mechanism by which baicalein induce apoptosis is still unclear. In this study, some members of caspases in HL60 human leukemia cell line were investigated to identify what kinds of caspases would be involved in apoptosis induced by baicalein. Surprisingly caspase 2, 3, and 6 were activated while caspase 1, 8, and 9 were not. This result is not consistent with previous reports that baicalein activates caspase 9 by way of mitochondrial pathway.

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Materials and Methods

Chemicals Caspase-1 substrate (Ac-YVAD-pNA), caspase-2 substrate (Ac-VDVAD-pNA), caspase-3 substrate (Ac-DEVD-pNA), caspase-6 substrate (Ac-VEID-pNA), caspase-8 substrate (Ac-IETD-pNA), caspase-9 substrate (Ac-LEHD-pNA), caspase-2 inhibitor (Ac-VDVAD-CHO), caspase-3 inhibitor (Ac-DEVD-CHO), and caspase-9 inhibitor (Ac-LEHD-CHO) were purchased from Bachem AG (Hauptstrasse, Switzerland). Baicalein was purchased from Sigma-Aldrich (Milwaukee, WI, USA). Cell proliferation reagent WST-1 was purchased from Roche Diagnostics (Mannheim, Germany) and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture, viability, and morphological change HL60 human promyelocytic leukemia cell line was cultured in

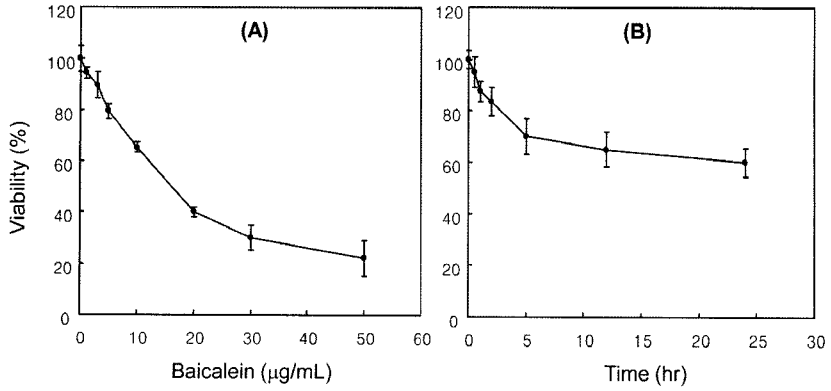

Fig 1. Cytotoxicity of baicalein. (A) Cells were treated with various concentrations of baicalein for 24 hr or (B) for various time at 10 μg/mL of baicalein before mitochondrial dehydrogenase activity was measured as a proliferative parameter.

RPMI 1640 medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cell viability was measured using a colorimetric assay (modified MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide], assay) based on the cleavage of the tetrazolium salt (WST-1) by mitochondrial dehydrogenases in viable cells (18,19). In brief, cells (5,000 cells/well) in 96-well plates were treated with baicalein to make a final volume of 100 μL/well culture medium. After a further incubation period, 10 μL/well of WST-1 was added and incubation continued for a further 0.5-3 hr. After shaking thoroughly for 1 min, absorbance was measured at 450 nm using a microplate reader (Model 550; Bio-Rad, Richmond, CA, USA). Morphological change of cells by the treatment with baicalein was investigated with phase contrast microscope (Inverted Axovert; Carl Zeiss, Inc., Thornwood, NY, USA) at the magnitude of 200.

Internucleosomal DNA fragmentation DNA fragmentation assay was performed as described previously (20). Briefly, cells were suspended in a buffer consisting of 20 mM Tris-HCl (pH 7.4), 4 mM 2-[2-(bis(carboxymethyl)amino)ethyl-(carboxymethyl)amino] acetic acid (EDTA), 0.4%(v/v) Triton X-100, and 10 μg/mL digitonin, and incubated at 4°C for 1 hr. The cells were centrifuged for 5 min at 10,000 ×g, and the supernatants were moved to new tubes. Each supernatant was extracted with phenol and chloroform. Cellular DNA was aggregated by incubating at −80°C for 1 hr after adding 1 μg of glycogen, 100 μL of 5 M NaCl, and 700 μL of isopropanol. Aggregated DNA was gathered by centrifuging at 10,000×g for 5 min. DNA pellets were dissolved in 20 μL of TE buffer containing 10 μg/mL RNase A. Each DNA samples were loaded on 1.8% agarose gel and examined under ultraviolet (UV) light after ethidium bromide staining.

Measurement of caspase activity Caspase activity was measured as previously described (21). Briefly, cells treated with baicalein for 4 hr were washed once with cold phosphate buffered saline (PBS). Cell pellets were lysed in a buffer consisting of 20 mM Hepes (pH 7.4), 100 mM NaCl, 0.5%(v/v) NP-40, and 10 mM dithiothreitol on ice for 1 hr. After centrifugation at 10,000×g for 5 min, supernatants were moved into new tubes and added with caspase substrate to the final concentration of 100 μM. Each sample was incubated at 37°C for 1 hr and absorbance at 405 nm was measured using a spectrophotometer. Absorbance values were converted to the amounts of pNA using a standard curve measured using various amounts of pNA. For caspase inhibitors, cells were treated with inhibitors up to final 100 μM for 1 hr prior to treatment of baicalein to completely block the activities of caspases.

Statistical analysis Each data represent the mean of 4 experiments. Statistical difference of the mean between groups was determined using the Student's *t*-test by SPSS 10.0. Probability (p) values less than 0.01 were considered significant. Values are shown as the mean±standard deviation (SD).

Results and Discussion

SB has been used as traditional herbal medicine to reduce inflammation, high blood pressure, and allergic actions in China (2,22,23). The major flavonoids contained in SB are baicalin, baicalein, and wogonin, all of which are known to be important for pharmacologic actions of SB (15).

Cell viability assay Because of poor solubility of baicalein in water, dimethyl sulfoxide (DMSO) was used as a solvent in all experiments. The final concentration of DMSO was adjusted to 0.1%(v/v) in cell culture medium. In first, toxic effect of baicalein was measured with various concentrations of baicalein. As shown in Fig. 1A, above concentration of 10 μg/mL of baicalein, significant toxic effect appeared with concentration dependent manner. The viability was 65.5% at 10 μg/mL of baicalein. This concentration of baicalein was used for the following experiments. This pattern of cell viability was also measured with time dependent experiment (Fig. 1B). After 5 hr of treatment with 10 μg/mL baicalein, cell viability decreased with time dependent pattern.

Apoptosis measurement To identify whether the decrease of viability would be from apoptosis, cellular morphology and DNA cleavage were investigated. At concentration of 10 μg/mL for 4 hr, baicalein induced membrane blebbing and apoptotic body formation, typical morphological

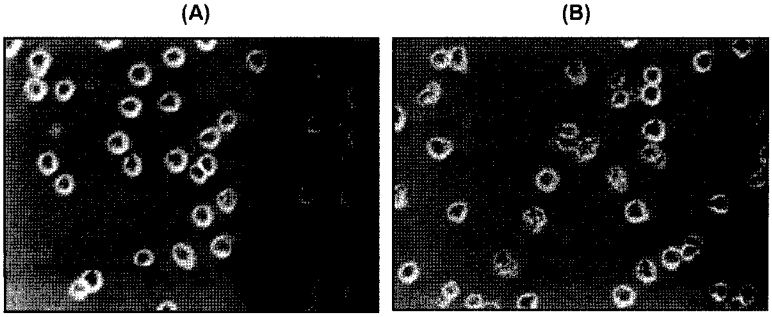

Fig 2. Morphological change of HL60 cells after baicalein treatment. Cells were treated with vehicle alone (A) or 10 μg/mL of baicalein (B) for 4 hr and analyzed by phase contrast microscope.

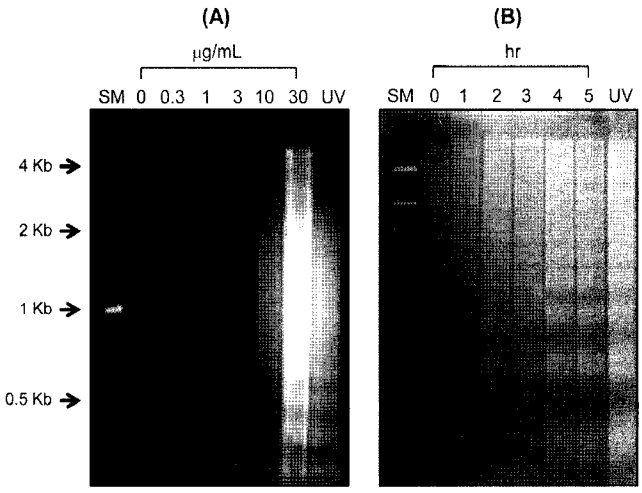

Fig 3. Baicalein-induced internucleosomal DNA fragmentation in HL60 cell line. (A) Cells were treated with various concentrations of baicalein for 4 hr. (B) Cells were treated with 10 μg/mL of baicalein for various time periods. Ultraviolet (UV) treatment was used as a positive control of apoptotic cell death. SM represents DNA size marker.

features of apoptosis, in HL60 cell line (Fig. 2). When HL60 cells were treated with various concentrations of baicalein for 4 hr, it was found that baicalein induced internucleosomal DNA fragmentation into the multiples of 180 bp at the concentration of 10 and 30 μg/mL (Fig. 3A). UV radiation was used as a positive control for the induction of apoptosis (20). In time course experiment in which 10 μg/mL of baicalein was treated, 180 bp DNA ladder was generated 4 hr after the treatment (Fig. 3B).

Caspase activity measurement Although it was already reported that baicalein induced apoptosis in HL-60, the proposed mechanism of apoptosis involves activation of caspase 9 through mitochondrial dysfunction pathway induced by ROS (12,13). To elucidate the mechanism of baicalein-induced apoptosis, the activities of various members of caspase family were measured using specific peptide substrate of caspase. Figure 4 shows that caspase 2, 3, and 6 activities were increased by baicalein at the concentrations above 10 μg/mL. Caspase 1, 8, and 9 activities, however, were not significantly changed by baicalein treatment. The role of caspase on baicalein-induced apoptosis was further investigated with various types of caspase inhibitors. Because caspase 3 is the key component of apoptosis, caspase 3 activity was measured as indications of apoptosis. The result in Fig. 5 shows that inhibitor of caspase 2 could down-regulate the activation of caspase 3 induced by baicalein. In accordance with result of Fig. 4, caspase 9 inhibitor did not show significant effects on caspase 3 activity. Unlike report of Wang *et al.* (12), in our study, caspase 9 was not activated and further caspase 9 inhibitor did not show significant effect on baicalein induced activation of caspase 3. Although more direct evidences such as cytochrome release from mitochondria and detection of ROS were not presented in our study, it is obvious that caspase 9 did not confer to apoptotic process in experimental conditions. This different result for the role of caspase 9 might come from different experimental condition in each study. Interestingly, caspase 2 was highly activated in our experiment, which has not been reported yet in other studies (Fig. 4). Although the exact role of caspase 2 has not been clearly defined, it was reported that ROS can activate caspase 2 (24). As shown in Fig. 5, inhibitor of caspase 2 blocked the activation of caspase 3, which support the important role of caspase 2 in baicalein induced apoptotic signal pathway. In this experiment the effect of caspase 6 inhibitor was not measured on the activity of caspase 3 induced by baicalein, because caspase 6 has been known as effector caspase related with caspase 3 (1). However, to elucidate the detailed mechanism of apoptosis induced by baicalein, effector caspase as well as initiator caspase should be investigated. We are now studying whether caspase 2 or caspase 6 acts as initiator or effector caspase in baicalein induced apoptosis and whether mitochondrial pathway are involved in this apoptotic process.

In conclusion, it was observed that baicalein could activate caspase 2 not caspase 9 by selective activation among the initiator caspases. Although many effects of baicalein have been known, the exact pathway of apoptosis by baicalein has not been clearly identified, because baicalein can act pro- or anti-oxidant simultaneously according to experimental conditions.

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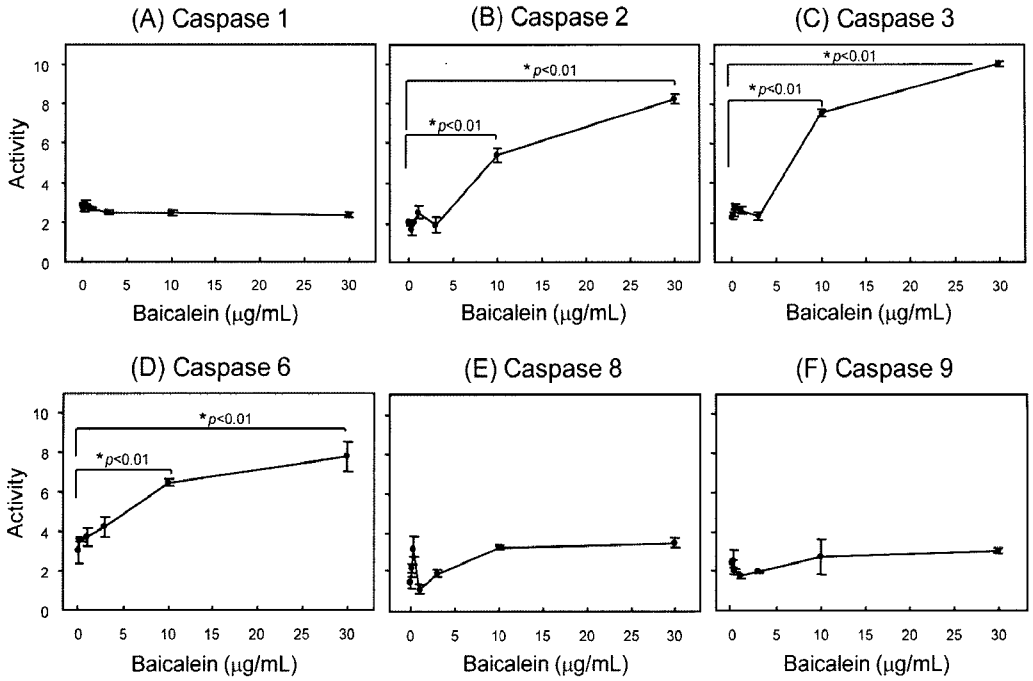

Fig 4. Change of caspase activities during baicalein-induced apoptosis. HL60 cells were treated with various concentrations of baicalein for 4 hr and caspase activities were measured using specific peptide substrates.

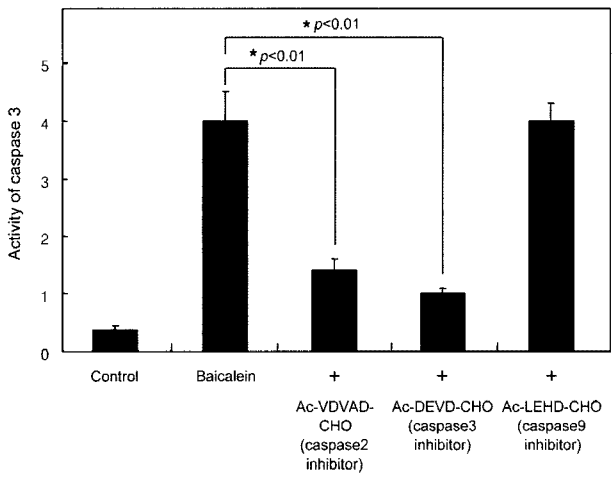

Fig 5. Effects of caspase inhibitors on activity of caspase 3. HL60 cells were pre-treated with caspase inhibitors for 1 hr (Ac-VDVAD-CHO for caspase 2, Ac-DEVD-CHO for caspase 3, and Ac-LEHD-CHO for caspase 9) before treatment with baicalein. Caspase 3 activity was measured using specific peptide substrate.

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