J. Toxicol. Pub. Health
Vol. 17, No. 2, pp. 131-137, 2001

Induction of Apoptosis by Baicalein in Human Leukemia HL-60 Cells

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(Received May 9, 2001)
(Accepted May 30, 2001)

ABSTRACT *: Baicalein, a major flavonoid of extract from Scutellaria baicalensis Georgi, has been shown to exhibit antioxidant and antiproliferative effects. In the present study, we investigate the effects of baicalein on viability and induction of apoptosis in human promyelocytic leukemia HL-60 cells. Baicalein was found to induce apoptosis of HL-60 cells in a concentration-dependent and time-dependent manner. When HL-60 cells were exposed to 100 μM baicalein for 6h, the viability was decreased remarkably to 27% of control, whereas DNA fragmentation was significantly increased to 64%. Nucleosomal fragmentation of baicalein treated HL-60 cells, a hallmark of apoptosis, was further identified by agarose gel electrophoresis (DNA ladder). Flow cytometric analysis showed that apoptotic cells were increased to 66.6% after treatment with 100 μM baicalein for 6 h. Baicalein-induced apoptosis of HL-60 cells was reduced by 1 h pretreatment with inhibitor of caspases, z-Asp-CH_2-DCB. At 3 and 10 μM of z-Asp-CH_2-DCB, DNA fragmentation of HL-60 cells induced by baicalein (50 μM) was 36.8 and 17.1%, respectively, whereas, that of HL-60 cells treated by baicalein (50 μM) without pretreatment with inhibitor of caspases was 62.7%. These data suggest that baicalein induces apoptosis in human leukemia HL-60 cells, and that caspase enzymes might be involved in baicalein-induced apoptosis.*

Key Words *: Baicalein, Apoptosis, Caspase inhibitor, HL-60*

I. INTRODUCTION

The root of *Scutellaria baicalensis* Georgi is a conventional herbal medicine, which is widely used for traditional herbal preparations in Japan and China. It has been commonly used clinically in allergic and inflammatory diseases, liver cirrhosis, and arteriosclerosis. It is known that the active components of the root are flavonoids. Four major flavonoids, including baicalein, baicalin, wogonin, and wogonoside, have been isolated from the root (Gao *et al.*, 2001). Among these flavonoids, both of baicalein and baicalin have antioxidative activities (Hamada *et al.*, 1993; Gao *et al.*, 1996; Yoshino and Murakami, 1998; Gao *et al.*, 2001). It was reported that baicalein could effectively scavenge hydroxyl radicals, DPPH radicals and inhibit mitochondrial lipid peroxidation (Gao *et al.*, 1999). The antioxidative activities of baicalein and baicalin might also be involved in the antifibrogenetic effect of Sho-saiko-to in experimental hepatic fibrosis (Shimizu *et al.*, 1999). In contrast, baicalein and baicalin have antiproliferative effects on several cancer cell lines (Motoo and Sawabu, 1994; Matsuzaki *et al.*, 1996; Chan *et al.*, 2000).

Apoptosis is one of the most fundamental processes in eukaryotes, in which cell death occurs via intrinsic suicide mechanism triggered by extracellular as well as intracellular signals. Apoptotic cells exhibit morphologically distinct characteristics, such as cell shrinkage, membrane blebbing, and DNA fragmentation into nucleosomal size, which yields a ladder pattern in agarose gel, a hallmark of apoptotic cell death (Majno and Joris, 1995). The events leading to apoptosis are often mediated by activation of cascades of cysteine proteases called caspases (Wilson *et al.*, 1996). Therefore, inhibitors of caspases can partially or completely block apoptosis in many cases (Armstrong *et al.*, 1996; Glynn *et al.*, 1996; Farber *et al.*, 1999; Kim *et al.*, 2000).

In this paper, we present evidences that baicalein induces apoptotic cell death in human leukemia cells and that this effect may be mediated by caspases.

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II. MATERIALS AND METHODS

1. Chemicals

Baicalein and diphenylamine were purchased from Sigma Co. Baicalein was dissolved in DMSO. The final culture concentration of DMSO in all experiments was 0.1% (v/v) or less. RPMI 1640 was procured from Gibco Co, and FBS from HyClone. Caspase inhibitor (z-Asp-CH_2-DCB) was obtained from Peptide Institute, Inc. (Japan).

2. Cell culture and drug treatment

Human promyelocytic leukemia HL-60 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 0.15% $NaHCO_3$, and penicillin (100 units/ml) and streptomycin (100 μg/ml), and grown at 37°C with 5% CO_2 in an air atmosphere. Stock cultures of exponentially grown cells, which were cultured for 2 days before drug treatment, were plated in 24 well plates (Costar, Cambridge, MA) at a density of 1×10^7 cells/well in 1 ml of culture medium. Baicalein solutions were directly added to the wells.

3. Cell viability

Cell viability was measured by hemocytometry using trypan blue exclusion. One volume of trypan blue (0.4%, Sigma) was added to 5 volumes of cell suspension harvested from cultures. The cells were examined by inverted light microscopy. Cell viability was expressed as percent of control.

4. DNA extraction and agarose gel electrophoresis

DNA ladder pattern, a typical feature of apoptosis, was visualized on agarose gel electrophoresis as previously reported in detail (Yoo and Kim, 1997). Briefly, HL-60 cells were harvested and centrifuged at 200×g for 10 min. Cell pellets were lysed with 400 μl of lysing buffer (0.2% Triton X-100, 10 mM Tris, and 1 mM EDTA, pH 8.0). The supernatant containing small DNA fragments was separated from the pellet containing intact DNA; half was used for agarose gel electrophoresis, and the other half, as well as the pellets were used for quantitative analysis of fragmented DNA by diphenylamine reaction.

The supernatants (200 μl) were extracted with an equal volume of absolute isopropyl alcohol at -20°C overnight. The pellets were completely dried and then resuspended in 100 μl of TE solution (10 mM Tris HCl, 1 mM EDTA, pH 7.4) and 50 μl of loading buffer (15 mM EDTA, 2% SDS, 50% glycerol, 0.5% bromophenol blue, and 10 μg/ml RNase). The samples were then heated at 65°C for 10 min and analyzed by electrophoresis at 50 V for 40 min on a 1.5% agarose gel with TBE buffer (90 mM Tris, 90 mM boric acid, and 2 mM EDTA, pH 8.0).

5. Quantitation of fragmented DNA

The quantitation of fragmented DNA was carried out by the diphenylamine (DPA) method. The pellet containing intact DNA and the supernatant containing fragmented DNA were prepared as described above. The pellet was resuspended in 200 μl of lysing buffer. After addition of perchloric acid to the pellet suspension and the supernatant (remaining 200 μl) at the final concentration of 0.5 N, sample tubes were heated at 95°C for 15 min. Two volumes of DPA reagent (0.088 M DPA, 98% v/v glacial acetic acid, 1.5% v/v sulfuric acid and 0.5% v/v of 1.6% acetaldehyde solution) were added to the sample. After overnight at room temperature, OD at 595 nm was measured on ELISA reader (Molecular Devices). The percentage of DNA fragmentation was expressed as follows :

$$\text{DNA fragmentation (\%)} = \frac{2\,(\text{OD of supernatant})}{\text{OD of pellet} + 2(\text{OD of supernatant})} \times 100$$

6. Flow cytometry

Cells were harvested by centrifugation at 1000 rpm for 10 min, washed with ice-cold PBS, and fixed in 50% ice-cold ethanol at 4°C for 1 h. After centrifugation, cells were washed twice with cold PBS, and incubated at the room temperature in the presence of RNase and propidium iodide at final concentration of 0.2 mg/ml and 50 μg/ml, respectively. Cells were then washed and resuspended in PBS. Fluorescence was measured using a FACScan flow cytometer (Coulter

Co.) in the FL3 channel. At least 10^4 cells were analyzed for each sample, at a flow rate of about 200 cells/s.

7. Statistical analysis

Data were analyzed by anlysis of variance (ANOVA) followed by Turkey's test for multiple comparisons. $P < 0.05$ was considered to be significant in all cases.

III. RESULTS

HL-60 cells were maintained in culture medium containing 10% FBS and exposed to 0~100 μM baicalein for 6h. Cell viability was assayed by trypan blue exclusion method. Cell viability of baicalein-treated cells was expressed as a percentage of the control cells in order to show the extent of cytotoxicity. Baicalein showed a concentration-dependent cytotoxicity on HL-60 cells (Fig. 1). It showed a strong cytotoxic effect on HL-60 cells by 27% at concentration of 100 μM baicalein, but a weak effect at the concentration of 10 μM baicalein.

Quantitative analysis of DNA fragmentation induced by baicalein was performed using diphenylamine method. Cells treated with 10~100 μM baicalein for 6h also showed increasing DNA fragmentation (%) in a concentration dependent manner, and and DNA fragmentation (%) at 30 and 100 μM baicalein was significantly higher than the control level (Fig. 1).

Apoptosis of HL-60 cells induced by baicalein was also confirmed by agarose gel electrophoresis analysis, which showed ladder bands of DNA fragments, typical of apoptotic cell death (Fig. 2). DNA ladder bands were clearly detectable in HL-60 cells treated with 30, 100 μM (Fig. 2, lane 4 and 5, respectively). Fig. 3 shows the time course of DNA fragmentation in HL-60 cells treated with 50 μM baicalein. When the cells were exposed to 50 μM baicalein for 4 h, a significant increase in the amount of DNA fragmentation was apparent (Fig. 3).

To further examine the cell death induced by baicalein, we used flow cytometry to quantify the apoptotic cells. The percentages of apoptotic cells among HL-60 cells exposed to 10, 30, and 100 μM baicalein were

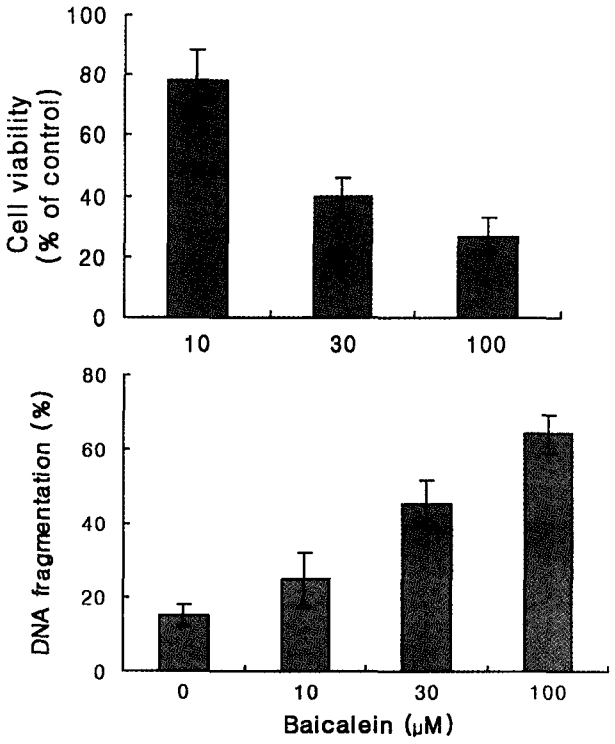

Fig. 1. DNA fragmentation and cell viability of HL-60 cells as a function of baicalein concentration after treatment with baicalein for 6 h. DNA fragmentation and cell viability were assessed as described in the Materials and Methods. Data are means±SE for three independent experiments performed in duplicate.

Fig. 2. Agarose gel electrophoresis of DNA extracted from HL-60 cells treated with baicalein at the indicated concentration for 6 h. After extraction, DNA samples were separated on a 1.5% agarose gel, stained with ethidium bromide. Lane 1, 1 kb DNA size marker; lane 2, control; lane 3, 10 μM baicalein; lane 4, 30 μM baicalein; lane 5, 100 μM baicalein.

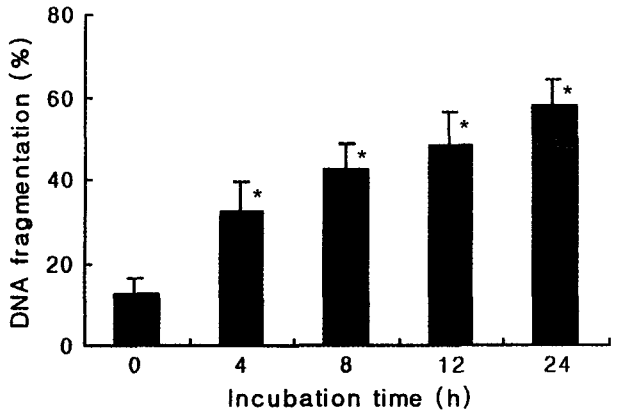

Fig. 3. Time course of DNA fragmentation of HL-60 cells treated with 50 μM baicalein for indicated times. DNA fragmentation was assessed as described in the Materials and methods. Data are meansSE for three independent experiments performed in duplicate. *$P < 0.01$ compared to untreated cells.

16.6, 47.7, and 66.6%, respectively (Fig. 4). These results were consistent with the results of DNA fragmentation (%) and agarose gel electrophoresis of HL-60 cells treated by baicalein.

To examine whether the caspases are involved in baicalein-induced apoptosis in HL-60 cells, we tested z-Asp-CH_2-DCB, an inhibitor of caspases. As shown in Fig. 5, z-Asp-CH_2-DCB was effective at preventing HL-60 cells from baicalein-induced apoptosis when added to the culture medium 1 h before baicalein treatment at the concentration of 50 μM. The amount of DNA fragmentation treated with 50 μM baicalein for 6 h without z-Asp-CH_2-DCB was 62.7%. However, in the case of treating with 3 and 10 μM z-Asp-CH_2-DCB, DNA fragmentation rate of HL-60 cells induced with baicalein was reduced by 36.8 and 17.1%, respectively (Fig. 5A). These results was also confirmed in agarose gel electrophoresis (Fig. 5B).

IV. DISCUSSION

The present studies have demonstrated that baicalein exhibited a concentration- and time-dependent cytotoxic effect on HL-60 cells. The results showed that cytotoxic effect induced with baicalein resulted

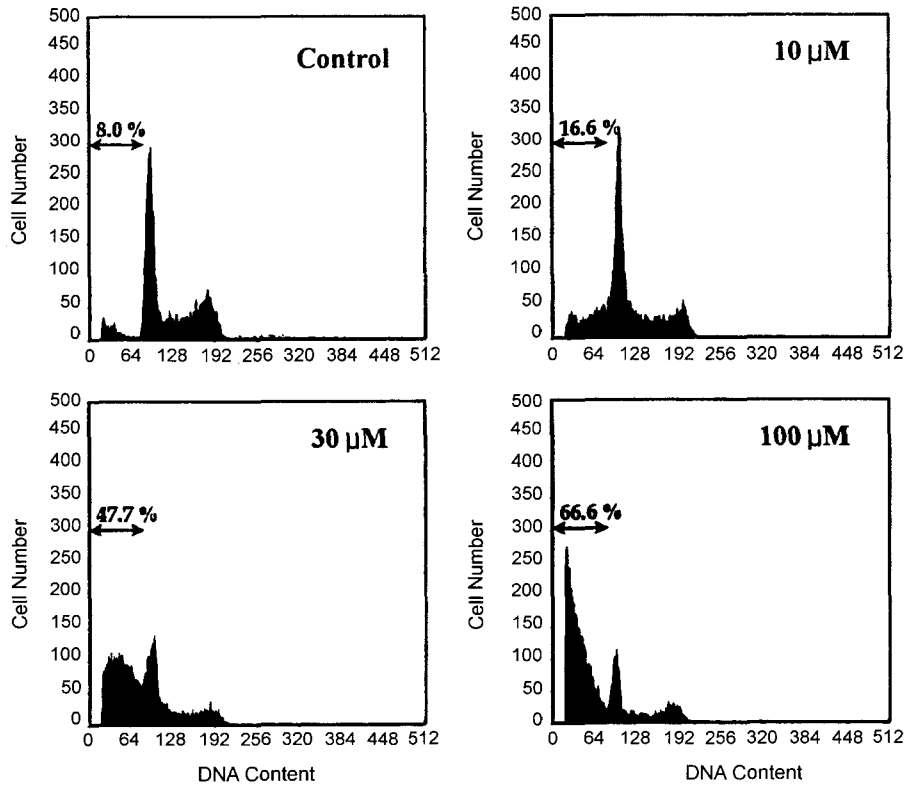

Fig. 4. Flow cytometric analysis of HL-60 cells treated with baicalein. HL-60 cells were treated with baicalein at the indicated concentration for 6h, then washed and harvested. The cells were fixed and stained with propidium iodide, and the DNA content was analyzed by flow cytometry. The number of apoptotic cells is expressed as a percentage of the total number of cells.

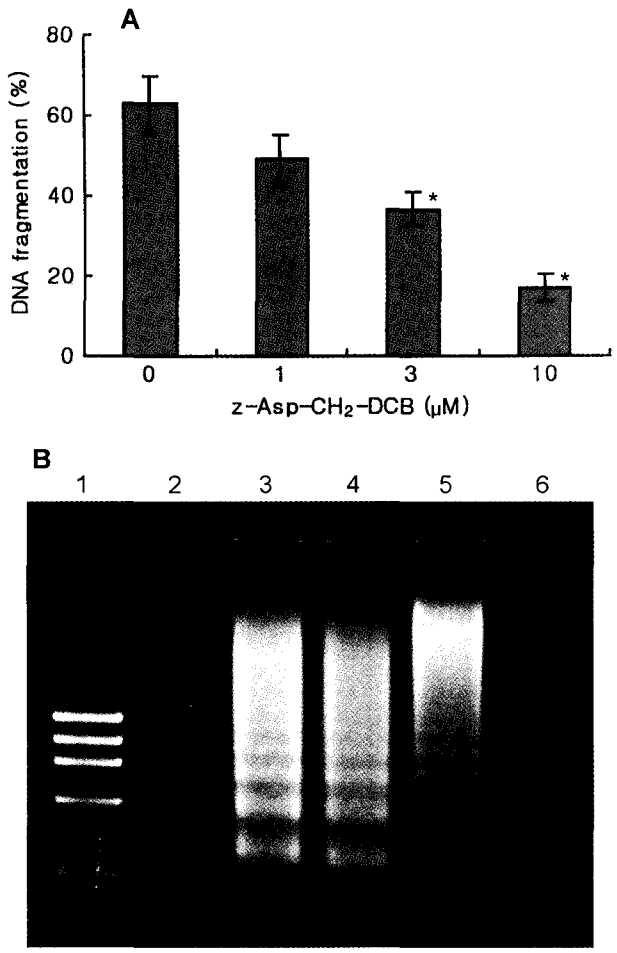

Fig. 5. Inhibition of baicalein-induced apoptosis in HL-60 cells by a caspase inhibitor (z-Asp-CH_2-DCB). HL-60 cells were treated with the caspase inhibitor at the indicated concentration for 1h prior to the addition of baicalein (50 μM). Incubation was continued for 6 h. (A) DNA fragmentation was calculated as described in the Materials and Methods. Data are meansSE for three independent experiments performed in duplicate. *$P < 0.05$ compared to HL-60 cells treated with only baicalein. (B) Agarose gel electrophoresis of DNA extracted from HL-60 cells treated with 50 μM baicalein and various concentrations of the caspase inhibitor. After extraction, DNA samples were separated on a 1.5% agarose gel and stained with ethidium bromide. Lane 1, 1 kb DNA size marker; lane 2, control; lane 3, 50 μM baicalein; lane 4, 50 μM baicalein and 1 μM caspase inhibitor; lane 5, 50 μM baicalein and 3 μM caspase inhibitor; lane 6, 50 μM baicalein and 10 μM caspase inhibitor.

from apoptosis, as demonstrated by DNA fragmentation, DNA ladder pattern in agarose gel, and flow cytometric analysis. Involvement of caspases in baicalein-induced apoptosis of HL-60 cells was also demonstrated by the inhibition of baicalein-induced apoptosis by z-Asp-CH_2-DCB, an inhibitor of caspases.

Flavonoids are widely found within the plant kingdom. Several studies revealed that baicalein, a major flavone component in *Scutellaria baicalensis* Georgi, possesses antioxidant properties, like many other flavonoids (Gabrielska *et al.*, 1997; Shao *et al.*, 1999; Shimizu *et al.*, 1999; Gao *et al.*, 2001). An extract of *S. baicalensis* consisting of 75% baicalein reduced lipid peroxidation in phosphatidyl choline liposomes (Gabrielska *et al.*, 1997), and baicalein directly scavenged superoxide, hydrogen peroxide, and hydroxyl radicals (Shao *et al.*, 1999). Baicalein prevented human dermal fibroblast cell damage by reducing hydroxyl and superoxide radicals (Gao *et al.*, 1999), and attenuated oxidant stress to protect cells from lethal oxidant damage in an ischemia-reperfusion model (Shao *et al.*, 1999). These findings suggest that antioxidant properties of baicalein might play a role in many protective effects.

Although baicalein may be a powerful antioxidant, it also showed antiproliferative activities (Kuntz *et al.*, 1999; Matsuzaki *et al.*, 1996; Kuo 1996; So *et al.*, 1997). Baicalein, a flavonoid derived from of *Scutellaria radix* in Sho-saiko-to, induced cell-specific apoptosis in three different hepatocellular carcinoma cell lines (Matsuzaki *et al.*, 1996), and showed strong antiproliferative effect in hepatic stellate cells (Inoue and Jackson, 1999). Baicalein also induced apoptosis in several cell lines, such as HT 29, Caco-2 cells (Kuntz *et al.*, 1999), LLC-PK_1 (Hagar *et al.*, 1997), and MCF-7 (So *et al.*, 1997). Although the antiproliferative activity of baicalein might be mediated by apoptosis induction, the mechanisms by which baicalein exert its cellular effects remain to be determined. It is not clear whether caspase activation may involve in the intracellular events leading to apoptosis induced by baicalein. Caspase-3 activation has been shown in the renal cell line to be important for apoptosis induced by baicalein (Maccen *et al.*, 1998). Caspase-3 activation also was involved in baicalin-induced apoptosis in human prostate cancer cells (Chan *et al.*, 2000). However, baicalein did not activate caspase-3 in LLC-PK1 cells in spite of its potent growth inhibitory activity, suggesting that the effects of baicalein on cell growth might be most likely based on an arrest in cell cycle progression (Kuntz *et al.*, 1999).

In summary, the results from this study demonstrated that baicalein was cytotoxic for HL-60 cells, and its inhibitory effect was mediated through the induction of apoptosis. Baicalein-induced apoptosis of

HL-60 cells was almost completely prevented by z-Asp-CH_2-DCB, an inhibitor of caspases, suggesting the involvement of caspases in apoptotic processes. The exact mechanism of apoptosis induced with baicalein remains to be evaluated in further experiments.

ACKNOWLEDGEMENTS

This research was supported by grants from the Korea Science and Engineering Foundation (KOSEF grant) (1999-2-214-001-5).

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