Mitochondrial-dependent caspase activation pathway is involved in baicalein-induced apoptosis in human hepatoma J5 cells

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Received May 28, 2009; Accepted July 15, 2009

Abstract. Baicalein has been reported to induce growth-inhibitory activity in vitro in human cancer cells; however, the molecular mechanism of action is not completely understood. A pharmacological dose (10-100 μM) of baicalein exerted a cytotoxic effect on human hepatoma J5 cells resulting in G2/M arrest and apoptosis. In addition to cytotoxicity in J5 cells, several apoptotic events including mitochondrial cytochrome c release, activation of caspase-9 and -3 occurred. Baicalein induced AIF and Endo G release from mitochondria indicating that baicalein stimulates apoptosis through the caspase-independent pathway, while undergoing apoptosis, there was a remarkable accumulation of G2/M cells. Also, the ratio of Bax/Bcl-2 was increased leading to changes in mitochondria membrane potential (ΔΨm) and release of cytochrome c, whereas the baicalein-induced apoptosis was partially abrogated by pretreatment with the pan-caspase inhibitor z-VAD-fmk, the accumulation of G2/M cells remained. These results demonstrate that the cytotoxicity of baicalein in J5 cells is attributable to apoptosis mainly involving G2/M-arrest in an ER-dependent manner, via a mitochondria-dependent caspase pathway and as well as contributions of AIF and Endo G pathways.

Introduction

Hepatoma is the second leading cause of death and most common malignancy in Taiwan with ~175.9 persons per 100,000 dying per year (People Health Bureau of Taiwan). However, effective treatment of human hepatoma in Taiwan is not satisfactory. Several studies reported that increasing consumption of a plant-based diet reduces the risk of cancer such as colon cancer (1,2). There are however no reports showing that such a dietary approach can decrease hepatoma in vivo. Herbal based dietary supplements contain a large array of phytochemicals which might mediate physiological functions related to cancer suppression and prevention in vivo.

Flavonoids exhibit a variety of biological activities such as anti-inflammation, antioxidant, anti-tumor and anti-viral actions (3). Baicalein (5,6,7-trihydroxyflavone), a bioactive flavonoid extracted from the root of Scutellaria baicalensis Georgi is used in Chinese herbal medicine (4) and exerts anti-tumor activity (5,6) through cell cycle arrest, suppression of proliferation and induced apoptosis in a variety of human cancer cell lines (7-10). Baicalein has free radical scavenging and antioxidant activities (11-13) and cytoprotective effects (14-16). Baicalein is an anti-inflammatory agent (17), an inhibitor of prostaglandin E2 (18) and was also protective against benzo[a]pyrene- and aflatoxin B1-induced genotoxicities (19). However, the precise mechanism of baicalein-induced apoptosis is not well-understood. Therefore, the purpose of the present experiments was to determine this in more detail.

Materials and methods

Chemicals and reagents. Baicalein, RNase, Tris-HCl, Triton X-100, propidium iodide and N-acetyl cysteine (NAC) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) and potassium phosphates were...
purchased from Merck Co. (Darmstadt, Germany). DMEM, penicillin-streptomycin, trypsin-EDTA, L-glutamine and fetal bovine serum (FBS) were obtained from Gibco BRL (Grand Island, NY, USA).

Cell culture. J5 cells were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were immediately placed onto 75 cm² tissue culture flasks at one atmosphere in DMEM medium supplemented with 10% FBS, 1% penicillin-streptomycin (100 U/ml penicillin and 100 μg/ml streptomycin) and 1% L-glutamine.

Cell viability determinations. J5 cells were plated onto 12-well plates at a density of 2x10⁵ cells/well and grown for 24 h. For dose-response experiments, different concentrations of baicalein (0, 10, 25, 50, 75 and 100 μM) were added to cells while only DMSO (solvent) was used for controls and grown at 37°C, 5% CO₂, and 95% air for 24 h. For time-dependent experiments, 50 μM baicalein was added to J5 cells for 24, 48 and 72 h. Cell viability was determined by flow cytometry as described previously (20-22).

Flow cytometric determination. J5 cells (2x10⁵ cells/well) were plated on 12-well plates and incubated with 0, 10, 25, 50, 75 and 100 μM baicalein for 48 h and in separate experiments 50 μM baicalein for 0, 6, 12, 24 and 48 h. Cells of each well were isolated and fixed gently by 70% ethanol in ice overnight. Each sample was then resuspended in PBS containing 40 μg/ml PI, 0.1 mg/ml RNase and 0.1% Triton X-100 in a dark room. After 30 min at 37°C, the cells were analyzed with a flow cytometer (Becton-Dickinson FACS Calibur, San Jose, CA, USA). The cell cycle was determined and analyzed as described previously (21.22).

DAPI staining for apoptosis. J5 cells at a density of 2x10⁵ cells/well were plated on 12-well plates and incubated with 0, 10, 25, 50, 75 and 100 μM baicalein for 48 h, then cells in each well were stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), then examined and photographed using a fluorescence microscope as described previously (22).

DNA damage assay (Comet assay). J5 cells at a density of 2x10⁵ cells/well were plated on 12-well plates and grown for 24 h. Cells were incubated with 0, 10, 25, 50, 75 and 100 μM baicalein and grown at 37°C in 5% CO₂ and 95% air, then isolated for the examination of DNA damage using the Comet assay as described elsewhere (23).

Caspase-3 activity. J5 cells at a density of 2x10⁵ cells/well were plated on 12-well plates and grown for 24 h. Baicalein (50 μM) or DMSO (solvent control) were grown at 37°C in humidified 5% CO₂ for 24 and 48 h. Cells from each well were harvested and 50 μl of 10 μM substrate solution (Phosphorim is a unique class of substrates for caspase-3) was then added to cell pellets containing 1x10⁵ cells. Cells were then incubated at 37°C for 60 min and washed once by adding 1 ml of ice cold PBS and re-suspend in fresh 1 ml PBS. Caspase-3 activity was analyzed by flow cytometry (Becton-Dickinson FACS Calibur) as described previously (23,24).

Detection of ROS, Ca²⁺ production levels and mitochondrial membrane potential (ΔΨm). J5 cells (2x10⁶ cells/ml) were plated onto 12-well plates and treated with 75 μM of baicalein for various time periods. Cells were harvested and washed twice, re-suspended in 500 μl of 2,7-dichlorodihydrofluorescein diacetate (10 μM) (DCFH-DA, Sigma) (dye for staining of ROS), Indo 1/AM (3 μg/ml) (dye contains fluorescence for staining of Ca²⁺) and DiOC₆(4 μmol/l/˚C) (dye contains fluorescence for staining of mitochondrial membrane potential) then incubated at 37°C for 30 min for detecting changes in ROS, Ca²⁺ and mitochondrial membrane potential levels by using flow cytometry as described previously (Becton-Dickinson FACS Calibur) (25,26).

Cell viability was also used to examine the role of ROS on the cytotoxicity of J5 cells after pre-treatment with NAC. Cells (2x10⁶ cells/well) were plated onto 12-well plates, pretreated with NAC for 3 h then incubated with 50 μM of baicalein for various time periods. Cells were harvested and ROS production and percentage of viable cells were determined as previously reported (25,26).

Western blotting. Cells (1x10⁶ J5 cells/ml) were plated onto 6-well plates and treated with 75 μM baicalein for 0, 6, 12, 24, 48 and 72 h. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting as previously described (25,27-29). It was used to determine all abundance of the following proteins: p53, p27, p21, Chk2, Wee1, Cdc25c, cyclin B1, Cdc2, Bax, Bcl-2, cytochrome c, Apaf-1, pro-caspase-9, AIF, Endo G, caspase-3, GRP78, GADD153, calalase, and Mn-SOD.

Confocal laser microscopy. J5 cells (5x10⁴ cells/well) plated onto 4-well chamber slide were treated with 75 μM baicalein for 24 h. Cells were fixed in 4% formaldehyde in PBS for 15 min and permeabilized with 0.3% Triton X-100 in PBS for various time periods. Cells were harvested and washed twice, re-suspended in 500 μl of 2,7-dichlorodihydrofluorescein diacetate (10 μM) (DCFH-DA, Sigma) (dye for staining of ROS), Indo 1/AM (3 μg/ml) (dye contains fluorescence for staining of Ca²⁺) and DiOC₆(4 μmol/l/˚C) (dye contains fluorescence for staining of mitochondrial membrane potential) then incubated at 37°C for 30 min for detecting changes in ROS, Ca²⁺ and mitochondrial membrane potential levels by using flow cytometry as described previously (Becton-Dickinson FACS Calibur) (25,26).

Statistical analysis. Statistical calculations of the data were performed using unpaired Student’s t-test and ANOVA analysis. Statistical significance was set at *p<0.05; **p<0.01; ***p<0.001.

Results

Morphological changes and percentage of viable J5 cells after treatment with baicalein. J5 cells were treated with various doses of baicalein for different time periods. Cell morphological changes and percentage of viable cells are presented in Fig. 1. Baicalein induced morphological changes in J5 cells (Fig. 1A) and decreased the percentages of viable cells and those effects were dose- and time-dependent (Fig. 1B).

Effects of baicalein on cell cycle arrest and induction of apoptosis in J5 cells. J5 cells were treated with various doses...
of baicalein for 48 h or treated with 75 μM for different time periods. Cells were harvested for cell cycle and sub-G1 (apoptosis) population analysis. The results shown in Fig. 2 indicated that baicalein induced G2/M arrest and sub-G1 group and effects were dose- (Fig. 2A) and time-dependent (Fig. 2B).

Baicalein-induced apoptosis and DNA damage in J5 cells were examined by using DAPI staining and Comet assay. J5 cells were incubated with various doses of baicalein for 48 h. Apoptosis was determined using DAPI staining and DNA damage examined using Comet assay and the results can be seen in Figs. 3 and 4. Baicalein induced apoptosis based on the light nuclei stained by DAPI indicating an increase in apoptotic cells (Fig. 3A). Baicalein-induced DNA damage was noted by the extension of the comet tail in the baicalein treated cells as shown in Fig. 3B. Effects on DAPI staining and Comet assay were dose-dependent.

Baicalein affects production of reactive oxygen species (ROS) and Ca" and ΔΨm levels in J5 cells. J5 cells treated with 75 μM for 2-, 6- and 12-h incubation were harvested for ROS, Ca" and ΔΨm determinations. It can be seen in Table I that baicalein promoted ROS and Ca" production and also decreased the levels of ΔΨm in J5 cells, these effects were time-dependent.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>ROS</th>
<th>Ca&quot;</th>
<th>ΔΨm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10.44±0.17</td>
<td>54.77±0.98</td>
<td>51.82±1.25</td>
</tr>
<tr>
<td>2</td>
<td>23.28±1.73b</td>
<td>62.31±1.16b</td>
<td>31.26±4.20b</td>
</tr>
<tr>
<td>6</td>
<td>29.75±0.49b</td>
<td>76.47±2.10b</td>
<td>24.63±1.41b</td>
</tr>
<tr>
<td>12</td>
<td>31.16±0.95b</td>
<td>77.33±1.20b</td>
<td>34.68±2.18b</td>
</tr>
</tbody>
</table>

The J5 cells (5x10^5 cells/ml) were treated with 0, 2, 6 and 12 h of baicalein. The zero concentration was defined as control. The percentage of ROS, Ca" and ΔΨm were stained by DCFH-DA, Indo-1/AM and DiOC6 respectively. The stained cells were determined by flow cytometry as described in Materials and methods. Values are means ± SD (n=3). Significantly different from the 0-h treatment at *p<0.05; **p<0.01; ***p<0.001.
Baicalein induces caspase-3 activation and antioxidant (NAC) inhibits ROS release in J5 cells. J5 cells treated with 75 μM for 24- and 48-h incubation were harvested for caspase-3 activity determination. It can be seen in Fig. 4A that baicalein increased staining of cells by PhiPhilux kit (a unique class of substrates for caspase-3). Baicalein also promoted caspase-3 activity in J5 cells and longer periods of treatment time enhanced caspase-3 activity. NAC (antioxidant) reduced effects of baicalein on ROS production (Fig. 4B) and increased the percentage of viable J5 cells (Fig. 4C).

Effects of baicalein on the protein levels of cell cycle and apoptosis in J5 cells. Proteins levels associated with G2/M arrest such as p53, p27, p21, Chk2, Wee1, Cdc25c, cyclin B1, Cdc2 are presented in Fig. 5A. Baicalein promoted the levels of p53, p27, p21, Chk2, Wee1 and decreased the levels of Cdc25c, cyclin B1 and cdc2 and were associated with G2/M arrest. Baicalein also increased levels of cytochrome c, Apaf-1, pro-caspase-9, AIF, Endo G, caspase-3, Bax and decreased Bcl-2 levels (Fig. 5B) and ER stress associated proteins GRP78 and GADD153 (Fig. 5C) indicative of apoptosis. Baicalein also stimulated levels of Mn-SOD but decreased the levels of catalase (Fig. 5D) suggesting oxidant activity.

Effects of baicalein on cytochrome c, AIF and Endo G in J5 cells. J5 cells after treatment with 75 μM for 24 and 48 h, cells were harvested and the translocation of cytochrome c, AIF and Endo G were determined using Confocal laser microscope. It can be seen in Fig. 6 that baicalein promoted the release of cytochrome c, AIF and Endo G from mitochondria and that longer treatment time periods increased the release of the 3 mitochondrial proteins.

Discussion

Effects of baicalein on cells were widely described. However, studies are limited on effects of baicalein on human hepatoma J5 cells. Human normal cells displayed low cytotoxicity when incubated with baicalein but opposite effects were observed in human cancer cells such as bladder, lung and breast cancer (30). Agents inducing cell cycle arrest and apoptosis are one of the major chemotherapy approaches used in treating cancer. In this study, we demonstrated that baicalein induced morphological changes and decreased the percentage of viable cells through G2/M arrest and induction
of apoptosis in J5 cells in a dose- and time-dependent manner. Baicalein also increased the expression of cyclin-dependent kinase inhibitor p21WAF1/CIP1 and p16, cdc25c and Wee1 which may contribute to the G2/M arrest). We also showed that baicalein promoted pro-apoptotic factor Bak and inhibited the ant-apoptotic factor Bcl-2 (the imbalanced expression of increased Bak and decreased Bcl-2) was observed in the cells exposed to baicalein which was associated mitochondrial dysfunction i.e., decreased the levels of Δψm. These findings suggest that the increased Bak level and the decreased Bcl-2 expression may play a role in the regulation of baicalein-triggered apoptosis in J5 cancer.

Baicalein promoted levels of p53, p53-independent p21WAF1/CIP1 repression or activation has been known to be involved in carcinogenesis of human tumors as well as anti-tumor treatment (31-33). In agreement with the baicalein-mediated cytotoxicity, apoptotic sub-G1 peak increased in J5 cells after baicalein treatment, demonstrating that the cytotoxicity was attributable to stimulation of apoptosis. It was reported that chemical agent-induced apoptotic signaling pathway involving activation of caspase cascade could be triggered either by cytochrome c release from mitochondria (34) or by the death receptor-mediated signal (35). In J5 cells exposed to baicalein, mitochondrial cytochrome c release and activation of caspase-9, -3, and -8 were detected, whereas Fas and FasL levels were unaffected. The cytotoxic effect of baicalein was not reduced by the anti-Fas neutralizing antibody ZB-4 (data not shown). This observation indicates that baicalein-induced apoptosis was through the mitochondrial-dependent caspase activation pathway.

There was an enhancement of G2/M cells prior to the induction of sub-G1 cells, suggesting that baicalein-mediated accumulation of G2/M cells might be an upstream event that leads to apoptosis in J5 cells. In the presence of hydroxyurea which blocked the cell cycle progression at the G1/S boundary and caused baicalein failed to induce the G2/M arrest (data not shown).

We showed that baicalein induced G2/M arrest and increased protein levels of p27 and cyclin B1 but reduced proteins levels of p21, Cdc25c, Wee1, cyclin B1 and Cdc2.
Up-regulation of cyclin B1 and alterations in cdc2 phosphorylation could render the cdc2/cyclin B1 kinase activation to prevent the cells from completing the M phase after being treated with baicalein. Cyclin B/cdc2 kinase has been reported to play a critical role in the G2/M transition and should be inactivated through the degradation of cyclin B1 by anaphase promoting complex in order to exit from the M phase (36). Baicalein decreased the levels of Bcl-2 which...
may cause the changes of the levels of ΔΨm. And it was reported that Bcl-2 could be phosphorylated at threonine 56 residue by cdc2 kinase at the G2/M phase (37).

Baicalein induced ROS and Ca2+ production and increased protein levels of GRP78 and GADD153 which are hallmarks of ER stress (38-40). In order to confirm that the mitochondrial cytochrome c release and subsequent activation of caspase cascade, and mitochondrial AIF and Endo G release were critical for the baicalein-mediated apoptosis, we have employed two experimental approaches; one was to take advantage of the anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bak both levels can affect the levels of ΔΨm, which can lead to mitochondrial cytochrome c release as well as endoplasmic reticulum stress-mediated Ca2+ release, and the other was to use caspase inhibitors, such as z-VAD-fmk, the broad caspase inhibitor (41), and z-DEVD-fmk, the caspase-3 inhibitor. We also found that pretreatment J5 cell with z-DEVD-fmk, the caspase-3 inhibitor then exposed cells to baicalein led to decrease the percentage of apoptosis (data not shown).

Taken together, these results demonstrate that baicalein induced cytotoxicity in J5 cells is due to the G2/M arrest of the cell cycle and subsequent induction of apoptosis via mitochondria-dependent activation of caspase-3 and -independent activation of caspase-3 by AIF and Endo G release which are summarized in a proposed model of baicalein induction of apoptosis in human hepatoma J5 cells shown in Fig. 7. These findings demonstrate the apoptogenic activity of baicalein against J5 cells, which is exerted by the ER stress mechanism at the pharmacological doses, and may expand our understanding of the benefits of the clinical application of baicalein therapy.

Acknowledgements

This work was supported by grants CMU95-110 from the China Medical University and NIH grants AG23524 and AG18357.

References


