Original Article

Baicalein 6-methy ether 7-glucoside induced apoptosis in HeLa cells via caspase dependent pathway

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Abstract: Background: Baicalin is a traditional herbal medicine and widely used in the pharmaceutical, cosmetic, and food industries due to its biological functions. In the present study, we report that an analogue of baicalin, baicalein 6-methy ether 7-glucoside (BG), is significantly more toxic to HeLa cells than baicalin and investigate the potential molecular mechanisms. Methods: The effects of baicalin and BG on cell viabilities in vitro were quantified by CCK-8 assay. Apoptosis of HeLa cells treated with BG was analyzed by Terminal deoxynucleotidyl transferase deoxy-UTP-nick end labeling (TUNEL), immunoblot, flow cytometry and transmission electron microscopy (TEM) as say. Results: BG induced obvious cell toxicity in HeLa cells which is evidenced by the induction of apoptosis with a dose- and time-dependent manner. We observed an increase in apoptotic bodies, DNA fragmentation and Annexin-V positive cells, as well as cleaved-caspase-3, -caspase-9 and poly-ADP-ribose polymerase (PARP) expression. We further demonstrated that BG-induced HeLa cell apoptosis involved down-regulating expression of B-cell lymphoma 2 (Bcl-2) and up-regulation of the expression of Bcl-2-associated X (Bax). Conclusions: Our data revealed that BG had anti-cancer properties through the induction of the caspase dependent apoptosis. As a potential agent for the treatment of human cervical cancer, further researches of BG are warranted.

Keywords: Baicalein 6-methy ether 7-glucoside, baicalin, apoptosis, cervical cancer

Introduction

Human cervical cancer is the second common cause of cancer-related death in women worldwide [1]. However, a significant proportion of patients with cervical cancer do not respond to chemotherapy due to drug resistance by tumors or significant side effects [1, 2]. We aim to derive and analyze novel chemotherapeutic agents from natural compounds.

As a predominant flavonoids, Baicalin (5, 6-dihydroxy-7-O-glucuronide flavone) was isolated from the roots of Scutellaria baicalensis Georgi [3]. It has been reported that baicalin possesses various pharmacological activities such as anti-inflammatory, anti-oxidative and anti-tumor properties [4-6]. Recent evidence has shown that baicalin induces HeLa cell apoptosis through caspase-3 activation, which involves intracellular mitochondrial pathways and surface death receptor pathways [7]. Baicalin administration has also been shown to ameliorate the severity of colon inflammation through the blockade of the TLR4/NF-kB-p65/IL-6 signaling pathway [8]. Although numerous evidence has shown that baicalin has a promising and potential role in developing novel chemotherapeutics for various diseases, therapeutic resistance against natural products limits the clinical application. It is attractive to explore new flavonoids with higher bioactivity. Indeed, certain analogues have even shown improved pharmacological activities in comparison to baicalin [9].

In this study, we elucidated that BG, an analogue of baicalin, induced significantly higher levels of apoptosis of HeLa cells than baicalin. BG may, therefore, act as a promising new therapeutic strategy for human cervical carcinoma.

Materials and methods

Reagents

Baicalin and BG were obtained from Ningbo Traditional Chinese Pharmaceutical Co., LTD
BG induced apoptosis in HeLa cells

The purity of baicalin and BG was over 99%, as analyzed by high performance liquid chromatography. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich (St. Louis, USA). Apoptosis Detection kit was obtained from BD Bioscience (Franklin Lakes, USA). RIPA Cell Lysis Buffer, BCA Protein Quantitation Kit and TUNEL Apoptosis Detection kit were purchased from KeyGEN BioTECH (Nanjing, China). Antibodies to poly-ADP-ribose polymerase (PARP), Caspase-3/Cleaved Caspase-3, Caspase-9/Cleaved Caspase-9, Bax and Bcl-2 were purchased from Cell Signaling Technology (Danvers, USA).

Cell lines

The cervical cancer cell line HeLa, gastric cancer cell line AGS, hepatic cancer cell line HepG2 and human oral epidermoid carcinoma cell line KB were purchased from Cell Bank of Chinese Academy of Science. AGS cell line was cultured in RPMI-1640 medium. HeLa, HepG2 and KB cell lines were cultured in DMEM (CORNING) medium. All of the culture media were added with 10% fetal bovine serum (Gibco), penicillin (100 IU/mL) and streptomycin (100 IU/mL) at 37°C in a 5% CO₂ atmosphere incubator.

Cell viability assay

Cell Counting Kit-8 (CCK-8) assay was used to detect cell viability. Briefly, cells were seeded in 96-well plates at a density of 4×10³ cells/mL and incubated 12 h in order to adhere, then treated with baicalin or BG for the indicated concentrations and time points. Thereafter, the cells were incubated with CCK-8 solution at 37°C for 1 h. The optical density (O.D.) was determined with a microplate reader at an absorbance wavelength of 450 nm.

TUNEL assay

TUNEL assay was used to detect apoptosis according to previously published work (10). Briefly, after treatment with 40 μM of BG for 0, 12, 24, 48 or 72 h, cells were cultured with 4% formaldehyde for 15 min and permeabilized with 0.2% Triton X-100 in phosphate buffer solution (PBS). Slides were washed three times with PBS and incubated with digoxigenin-labeled deoxy-UTP and terminal deoxynucleotidyl transferase at 37°C for 1 h. Samples were then washed three times with PBS and incubated with 5 μg/mL DNA binding dye (blue fluorescence) 4',6-diamidino-2-phenylindole (DAPI) for 15 min at incubator.

Immunoblot analysis

HeLa cells were washed with cold PBS twice, then re-suspended in RIPA Cell Lysis Buffer and kept on ice for 2 h. The supernatant of cell lysate was collected after centrifugation at 13,000 g and 4°C for 5 minutes. The protein concentration was determined by the bicinchoninic acid (BCA) method. Equal amounts of total protein were separated by SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were incubated with 5% nonfat milk for 1.5 h and incubated with primary antibodies for 4 h at room temperature. After incubation with peroxidase-conjugated secondary antibodies for 2 h, signals were detected with an enhanced chemiluminescent detection kit (KeyGEN BioTECH, Nanjing, China).

Flow cytometry analysis

Annexin V-FITC/PI Apoptosis Detection Kit was used to test cell apoptosis according to the manufacturer’s description. Briefly, harvested cells were washed three times with pre-cooled PBS and re-suspended in 500 μL binding buffer containing 5 μL of Annexin V-FITC and 5 μL PI for 30 min. Apoptotic rate was analyzed by FACS Calibur (BD, USA). The early apoptotic cells (Annexin-V⁺/PI⁻) were distributed in the lower right quadrant. The late apoptotic cells (Annexin-V⁺/PI⁺) were distributed in the upper right quadrant. The lower left quadrant indicated the normal cells.

Transmission electron microscopy analysis

HeLa cells were harvested after incubation with 40 μM of BG for 48 h and processed as described elsewhere [11]. A JEM 1410 transmission electron microscopy (JEOL, Inc., USA) was used to analyze samples at 80 kV.

Optical microscope

HeLa cells were incubated with various concentrations of baicalin or BG for 24 or 48 h. Phase-contrast images of cells were observed and photographed by an inverted microscope.
BG induced apoptosis in HeLa cells

Statistical analysis

GraphPad Prism 5 was used to perform statistical analysis. All the experimental data were shown as means ± standard deviations (SD). One-way ANOVAs or Student’s t test (2-tailed) was used to compare multiple sets of data. P-value < 0.05 was considered to be statistically significant.

Results

BG induces growth inhibition in different cancer cells

The structures of baicalin and BG were shown in Figure 1A and 1B. Cancer cell lines HepG2, HeLa, KB and AGS were challenged with different concentrations of baicalin or its transformed product BG. Cell viabilities were quantified by CCK-8 assay. As shown in Figure 1C-E, these two drugs showed a similar inhibition ratio in HepG2, KB, AGS cells at the same concentration. However, BG showed a higher inhibition ratio than baicalin in human cervical cancer HeLa cells (Figure 1F).

The cytotoxicity of BG in human cervical carcinoma HeLa cells

To further confirm whether BG and baicalin could influence the viability of HeLa cells, a CCK-8 assay was used to detect the inhibition
BG induced apoptosis in HeLa cells

In brief, cells were treated with baicalin and BG (0-200 μM) for given times. As shown in Figure 2A, BG reduced cell viability in a dose- and time-dependent manner. Moreover, BG showed higher inhibition rate than baicalin at the same concentrations and treatment times (Figure 2B-D). Similar results were further confirmed by morphology analysis with an optical microscope. As demonstrated in Figure 2E, cells exhibited morphological changes, including a decrease in the number of cells, a change in shape and reduced volume after incubation with increasing concentrations of baicalin or BG. In addition, compared with baicalin, a higher number of atrophic cells treated with BG were observed at the same concentration (Figure 2E). These findings demonstrated that BG results in growth inhibition of HeLa cells, indicating its cytotoxic effect on cervical cancer cells.

Figure 2. The cytotoxicity of BG in human cervical carcinoma HeLa cells. (A) HeLa cells were incubated in the presence of absence of varying concentrations of BG for 24, 48, or 72 h. The inhibition rate was determined by CCK-8 assay. HeLa cells were incubated with different concentrations of BG or baicalin for 24 h (B), 48 h (C), or 72 h (D), and the inhibition rates were determined by CCK-8 assay. (E) Morphological and numerical changes of HeLa cells were analyzed by microscope after challenged with BG or baicalin at different concentrations. Data above are representative of three representative experiments.
BG induced apoptosis of HeLa cells in a concentration- and time-dependent manner

Apoptosis is an important mechanism which can lead to the cell death [12]. Therefore, we used multiple techniques including transmission electron microscopy (TEM), TUNEL and FCM to explore whether BG induced HeLa cell death was due to apoptosis. After incubation with BG for 48 h, cells were observed under TEM. Compared with the control group, cells treated with drugs showed irregular morphology including chromatic agglutination, karyopyknosis, and nuclear fragmentation (Figure 3A). To further demonstrate whether BG could induce apoptosis in HeLa cells, a TUNEL assay was used to observe the DNA cleavage of apoptotic cells. As shown in Figure 3B, we used DAPI to locate the nuclei of the cells. TUNEL-positive cells indicated apoptotic cells. We also assessed cell apoptosis by flow cytometry after double staining with Annexin-V and PI. As shown in Figure 3C and 3D, the percentage of early apoptotic cells (Annexin-V⁺/PI⁻) was elevated in a dose-and time-dependent manner after exposure of HeLa cells to 0-80 μM of BG for specific times. Taken together, these observations suggested that BG induced HeLa cell apoptosis.

BG induces caspase-3 dependent apoptosis in HeLa cells

It has been reported that Bcl-2 family proteins, in particular the Bcl-2/Bax ratio, is a key factor in apoptotic process [13]. In this study, the expression of Bcl-2 was down-regulated while...
BG induced apoptosis in HeLa cells

Bax was up-regulated in a concentration-dependent manner in HeLa cells with the treatment of BG for 48 h (Figure 4A). We further confirmed the expression of caspase 3, caspase 9, and PARP proteins, which are key factors in the mitochondrial apoptosis pathway [14]. As shown in Figure 4B, the level of full-length forms of caspase 3, caspase 9 and PARP gradually decreased while the corresponding cleaved proteins significantly increased after dose-dependently exposure to the drug. These results indicated that BG triggered the apoptosis process in HeLa cells in a dose-dependent manner.

Discussion

Baicalin is a widely used herb in traditional Chinese medicine formulated with other herbs due to its multiple pharmacological effects, such as anti-tumor [15], anti-viral [16], anti-proliferation [17] and anti-allergy [18] properties. In recent years, baicalin has been extensively studied in order to obtain novel compounds with a higher affinity [9]. Many natural products have been reported as precursor drugs to produce double or multiple target drugs with significant anti-tumor activities [19]. For example, evodiamine, a quinazolinocarbo-line alkaloid with diverse biological activities has been isolated from the fruits of the Chinese herb Evodiae fructus. Sheng and others designed and synthesized a number of evodiamine derivatives. One in particular, amino-10-hydroxyevodiamine, demonstrated excellent antitumor activity against multiple cancers both in vivo and in vitro [20, 21].

In this study, we demonstrated for the first time that BG induced a higher growth inhibition ratio of HeLa cells than baicalin. Furthermore, we also observed BG could trigger apoptotic response in HeLa cells, as evidenced by the formation of apoptotic bodies using TEM, DNA fragmentation by TUNEL assay and caspase-dependent apoptotic effectors and mechanisms by Western blot analysis. Apoptosis is an essential homeostatic process and help prevent cancer and autoimmune disease, amongst others [22] by maintaining the balance between the processes of cellular proliferation and death [23].

Intrinsic and extrinsic are the two main pathways that lead to apoptosis. The extrinsic pathway is activated by triggering cell-surface-expressed death receptors or tumor necrosis factor receptor. The intrinsic apoptotic pathway is usually initiated by cellular stress and is regulated primarily by the Bcl-2 protein families [24]. Bcl-2 family-related proteins are required to initiate cell death [25]. Caspases, which belong to the cysteine protease group, are actively involved in the process of inflammatory and apoptosis. Caspase 3 and caspase 9 are two key proteins to initiate the process of apoptosis [23]. Based on our experimental results, we hypothesized that Bcl-2 and the caspase family proteins play important roles in BG-induced apoptosis in HeLa cells. Therefore, we further validated the decreased expression of PARP, caspase 3, caspase 9 and Bcl-2, while increased expression of Bax and cleaved-PARP, -caspase 3 and -caspase 9.

In conclusion, we demonstrated that BG could induce a higher rate of apoptosis in HeLa cells than baicalin, mediated by a caspase-dependent pathway. This compound may act as a

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Disclosure of conflict of interest

None.

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BG induced apoptosis in HeLa cells


