The downregulation of c-Myc and its target gene hTERT is associated with the antiproliferative effects of baicalin on HL-60 cells

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Abstract. Baicalin is a flavonoid compound isolated from Scutellaria baicalensis, a Chinese traditional medicinal herb, and is used as an anti-inflammatory, antibacterial, anxiolytic and hepatoprotective drug. Accumulating evidence has demonstrated that baicalin exhibits potent antitumor properties by suppressing cell growth, arresting cell cycle progression and inducing differentiation or apoptosis in leukemia cell lines. However, whether or not the extrinsic pathway is involved in baicalin-induced apoptosis of leukemia cells and the mechanisms underlying the antitumor activity of baicalin remain unclear. In the present study, the effect of baicalin on the expression of caspase-8, Fas cell surface death receptor (Fas) and Fas ligand in HL-60 cells was assessed, and it was demonstrated that the Fas-mediated extrinsic pathway was also involved in baicalin-triggered cell apoptosis, in addition to the intrinsic pathway. Furthermore, baicalin was able to inhibit the proliferation of HL-60 cells by arresting the cell cycle at the G0/G1 phase, and by down-regulating Myc proto-oncogene protein (c-Myc) along with its target gene, human telomerase reverse transcriptase. In summary, the results of the present study demonstrated that baicalin was able to inhibit the growth of HL-60 cells through blockade of the G0/G1 phase of the cell cycle, and significantly induce the apoptosis of cells by activating the intrinsic and extrinsic pathways. The inhibition of HL-60 cell growth was also demonstrated to be mediated by telomerase inhibition through suppression of c-Myc. The results of the present study highlight the possibility of baicalin as a promising regimen for the treatment of AML.

Introduction

Baicalin is a flavonoid compound isolated from Scutellaria baicalensis (Fig. 1A), a Chinese traditional medicinal herb used as an anti-inflammatory (1), antibacterial, anxiolytic and hepatoprotective drug (2). Accumulating evidence has demonstrated that baicalin exhibits potent antitumor properties by suppressing cell growth, arresting the cell cycle and inducing differentiation or apoptosis in leukemia cell lines (3,4), without affecting primary, or normal cells (5), which raises the possibility of using baicalin to treat patients with leukemia. Baicalin has been documented to induce apoptosis of the Jurkat T-cell acute lymphoblastic leukemia (T-ALL) cell line via the mitochondrial pathway, which involves marginal generation of intracellular reactive oxygen species (ROS), an increase in the cytosolic fractions of cytochrome c and disruption of the mitochondrial transmembrane potential (ΔΨm) (6). Another study revealed that baicalin exhibited a remarkable cytotoxic effect in T-ALL cell line CCRF-CEM, and triggered apoptosis regulator Bcl-2 (Bcl-2)-dependent, but not p53-dependent cell apoptosis (7). In addition, cyclin-dependent kinase inhibitor p27 has been suggested to serve a role in baicalin-induced apoptosis and cell cycle arrest (3). A study by Lu et al (8) confirmed that baicalin induced apoptosis in human promyelocytic leukemia HL-60 cells through the mitochondrial-involved pathway and endoplasmic reticulum-induced apoptotic cell death. While to date, whether the extrinsic pathway (death receptor pathway) is involved in apoptosis induced by baicalin in leukemia cells
has not been clarified, and the mechanisms underlying the antitumor activity of baicalin remain unclear.

The proliferation of HL-60 cells has also been demonstrated to be inhibited by baicalin (8); however, its underlying mechanism requires further elucidation. Myc proto-oncogene protein (c-Myc) is a transcription factor that participates in various cellular functions, including cell-cycle progression, proliferation, apoptosis and terminal differentiation (9). The overexpression of c-Myc mRNA and its encoded protein has been associated with neoplastic transformation in a variety of tumors (10). Therefore, perturbation of c-Myc levels is essential for influencing the growth of malignant cells, particularly those of hematopoietic origin (11). Wogonin, another major bioactive flavonoid extracted from Scutellaria baicalensis has been revealed to inhibit HL-60 cell growth via telomerase inhibition through suppression of c-Myc (12). Due to the similar molecular structure between baicalin and wogonin (Fig. 1B), it was hypothesized that baicalin may be able to inhibit the growth of HL-60 cells via the same mechanism as wogonin.

In the present study, the effect of baicalin on the expression of caspase-8, Fas cell surface death receptor (Fas), Fas ligand (FasL), death receptor (DR)4 and DR5 in HL-60 cells was assessed, and it was identified that the Fas-mediated extrinsic pathway was involved in baicalin-triggered cell apoptosis, in addition to the intrinsic pathway. Furthermore, baicalin was able to inhibit the proliferation of HL-60 cells by arresting cells at the G1/G0 phase and downregulating c-Myc along with its target gene, human telomerase reverse transcriptase (hTERT).

Materials and methods

Reagents. Baicalin was provided by Professor Xiao Wang (Shandong Analysis and Test Center, Shandong Academy of Sciences, Jinan, China). Baicalin was dissolved in DMSO at a stock concentration of 10 mg/ml and stored at -20°C. Hoechst 33342 was obtained from Beyotime Institute of Biotechnology (Nantong, China). β-actin (cat. no. sc130065; dilution, 1:500) and c-Myc (cat. no. sc-47694; dilution, 1:400) antibodies were purchased from Santa Cruz Biotechnology (Nantong, China).

Cell culture and proliferation assay. HL-60 cells were cultured at Key Laboratory for Tumor Immunology and Chinese Medicine Immunology of Shandong Province (Institute of Basic Medicine, Shandong Academy of Medical Sciences, Jinan, China). The cells were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% newborn calf serum (Gibco; Thermo Fisher Scientific, Inc.), 100 IU/ml penicillin and 100 IU/ml streptomycin in a 5% CO2 atmosphere at 37°C. Logarithmically growing HL-60 cells were seeded on a 96-well plate at a density of 1x10^4 cells/well in 50 µl of medium in triplicate, and then different concentrations of baicalin (5, 10, 20, 40 and 80 µg/ml) in 50 µl were added. Cells treated with 0.1% (v/v) DMSO were used as the control. After incubation for 24 h, cell proliferation was detected using CCK-8, according to the manufacturer's protocol as previously described (13).

Cell cycle distribution. Following baicalin (20 µg/ml) treatment for different durations, cells (3x10^5) were washed twice with ice-cold PBS, and fixed in cold 75% ethanol at 4°C for at least 24 h. Then, the cells were rinsed with PBS, and resuspended using 0.5 mg/ml RNaseA and 20 µg/ml propidium iodide (PI) prior to incubation for 20 min at room temperature in the dark. Subsequently, the cells were analyzed using an Elite-ESP flow cytometer (Beckman Coulter, Inc., Brea, CA, USA).

Analysis of apoptosis. Hoechst 33342 staining was used to visualize the change in nuclear morphology. Following baicalin (20 µg/ml) treatment for 24 h, cells were washed twice with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. Then, washed cells were stained with 1 µg/ml Hoechst 33342 for 15 min at room temperature in the dark. Cells were observed under a fluorescence microscope and imaged. Apoptotic cells were characterized by condensed or fragmented chromatin, while normal nuclei were smooth, symmetric and oval. To quantify the cells undergoing apoptosis, Annexin V/PI (BD Pharmingen; BD Biosciences, San Jose, CA, USA) double staining was performed according to the manufacturer's protocol. Briefly, the cells were harvested by centrifugation for 10 min at 800 x g at 4°C. Subsequently, the cells were washed twice with ice-cold PBS and re-suspended in 500 µl of 1X binding buffer, stained with Annexin V and PI for 15 min at room temperature. Apoptotic cells appeared as Annexin V-positive and PI-positive.

Assays for analysis of ∆Ψm. Fluorescent dye rhodamine 123 staining and flow cytometric analysis were performed to assess the ∆Ψm. Briefly, following baicalin treatment for 24 h, the cells were incubated with 10 mg/ml Rh123 for 30 min at 37°C, and washed twice with PBS, then analyzed using fluorescence-activated cell sorting.

Reverse transcription-semi-quantitative polymerase chain reaction (RT-sqPCR) assays. Total RNA was extracted from
HL-60 cells that had been cultured with baicalin (20 µg/ml) for different durations using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RT-PCR was performed as described previously (13). The primers used in the present study were as follows: Fas (238 bp) forward, 5'-GGACCCGAGATACCAAAGTGC-3' and reverse, 5'-GCCACTGTTTCAAGGATTTAAGG-3'; hTERT (198 bp) forward, 5'-TCATCTGGATTGGAAGGAGACT-3' and reverse, 5'-TCCAGACTGCTTATTGGC-3'; c-Myc (180 bp) forward, 5'-GATTTCTGCTCTCTTGAC-3' and reverse, 5'-GACCAAGTCTGCAAAGTCA-3'; DR4 (307 bp) forward, 5'-GCACTGTTTCAAGGAGGCA-3' and reverse, 5'-TTGCTGCTCAGACGCAAAGGTC-3'; β-actin (540 bp) forward, 5'-GGGGTGCCCGCCAGGCGACGA CCA-3' and reverse, 5'-CTCCTTAATGTACGCACGAT TCC-3'. The following thermocycling conditions were maintained: 94°C for 5 min; 30 cycles of 94°C for 45 sec, 55°C for 45 sec and 72°C for 1 min; and 72°C for 10 min. The PCR products were electrophoresed on 1.5% agarose gels. The software used to quantify the bands was AlphaImager 2200 (version 0.2.1.0; Alpha Innotech, Tarzana, CA, USA).

Western blot analysis. For immunoblotting, HL-60 cells treated with 20 µg/ml baicalin for 12 and 24 h were washed twice with cold PBS and lysed using extraction buffer (50 mM Tris-HCl pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, and 0.1% SDS) for 15 min on ice. Protein concentrations were measured using the Bradford protein assay (Beyotime Institute of Biotechnology). Protein samples (50 µg) were electrophoresed using 12% SDS-PAGE, transferred onto a polyvinylidene difluoride membrane. After blocking with Tris buffered saline-Tween-20 containing 5% non-fat dry milk for 1 h at room temperature, the membranes were incubated with primary antibodies against β-actin, cleaved caspase-3, caspase-8, caspase-9, Fas, Fasl, c-Myc, Bcl-2 and Bax overnight at 4°C. Subsequently, membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. The protein bands were visualized using an enhanced chemiluminescence detection system (EMD Millipore, Billerica, MA, USA) and imaged using a LAS-4000 mini luminescent image analyzer (Fujifilm, Tokyo, Japan). The software used to analyze the protein band density was Multi Gauge (version 3.0; Fujifilm, Tokyo, Japan).

Statistical analysis. Results are expressed as the mean ± standard error mean. The data were analyzed using one-way analysis of variance and Tukey's honest significant test post hoc analysis using SPSS (version 13.0; SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Baicalin inhibits the proliferation of HL-60 cells, and c-Myc together with hTERT participates in its growth arrest. The effect of baicalin on the growth of HL-60 cells was detected using a CCK-8 assay. As presented in Fig. 2A, baicalin inhibited the proliferation of HL-60 cells in a dose-dependent manner with an IC_{50} value of 21.8 µg/ml at 24 h. Therefore, a baicalin concentration of 20 µg/ml was selected to perform the majority of the following experiments. At this concentration, baicalin arrested cell growth in a time-dependent manner (Fig. 2B).

c-Myc possesses an essential function in controlling cell growth. To investigate the roles of c-Myc in growth inhibition, the mRNA and protein levels of c-Myc gene expression were detected in HL-60 cells exposed to 20 µg/ml baicalin for different durations. As presented in Fig. 2C-F, baicalin resulted in a significant downregulation of c-Myc mRNA and protein levels compared with untreated cells. In line with the aforementioned results, it was demonstrated that the mRNA expression of hTERT, the catalytic subunit of the telomerase holoenzyme complex, a direct transcriptional target of c-Myc (14), consequently decreased (Fig. 2C and D).

Baicalin causes G_1/G_s arrest in HL-60 cells. To investigate the mechanisms underlying growth inhibition by baicalin in HL-60 cells, cell cycle analysis was then performed using flow cytometry. As presented in Fig. 3, the time-course study revealed that exposure of HL-60 cells to baicalin significantly increased the proportion of cells in G_1/G_s phase from (29.2±0.5%) in the vehicle treated cells to (37.6±3.2%; P<0.05), accompanied by a reduction in the percentages of HL-60 cells in the G_2/M and S phases. In addition, cells in the sub-G_s phase rapidly increased at 24 h and the sub-G_s hypodiploid population reached 20.1±2.9% (data not shown). The data indicated that inhibition in cell cycle progression and induction of apoptosis may be associated with the inhibitory effect of baicalin treatment on the proliferation of HL-60 cells.

Baicalin induces apoptosis in HL-60 cells. To further characterize apoptosis induced by baicalin, Hoechst 33342 staining and Annexin V/PI analysis was performed. The nuclei of untreated cells were round and large in size, exhibiting homogeneous blue fluorescence. In contrast, a number of the cells treated with baicalin for 24 h showed condensed or fragmented nuclei, which are characteristics of cell apoptosis (Fig. 3A). In addition, the induction of apoptosis was time-dependent (Fig. 4B and C). Baicalin induced apoptosis in 12.6±4.3% of HL-60 cells after 12 h treatment, while it induced 32.3±5.1% of cells after 24 h treatment.

Baicalin induces the activation of caspases. To determine the mechanism of apoptosis induction by baicalin treatment, the activities of caspases, which have been demonstrated to be essential mediators in the apoptosis pathway (15), were analyzed using western blotting. Treatment of cells with baicalin resulted in marked time-dependent caspase-3, caspase-8 and caspase-9 activation, and cleavage, which indicated that the extrinsic and intrinsic pathways were associated with apoptosis induced by baicalin (Fig. 5).

Baicalin-induced apoptosis is mediated through intrinsic pathway. The decline in ΔΨm has been considered to be an important event in intrinsic pathway, which leads to matrix condensation and rapid cytochrome c release (16),...
Figure 2. Inhibitory effect of baicalin on the growth of HL-60 cells. HL-60 cells were treated with (A) various concentration of baicalin for 24 h; and were treated with (B) 20 µg/ml baicalin for the indicated time (0-48 h). The cell viability was determined by using a Cell Counting Kit-8 assay, the cell growth inhibition rate was calculated as described in the Materials and methods. Values represent the mean ± standard deviation of experiments performed in triplicate. (C) Baicalin downregulated the expression of c-Myc and hTERT mRNA. (D) The semi-quantitative analysis of c-myc and hTERT mRNA was calculated and is presented. (E) Baicalin downregulated the expression of c-Myc protein. (F) Quantification of c-Myc protein expression in HL-60 cells. *P<0.01 vs. control. Con, control, hTERT, human telomerase reverse transcriptase; c-Myc, Myc proto-oncogene protein.

Figure 3. Baicalin induces G<sub>0</sub>/G<sub>1</sub> cell cycle arrest in HL-60 cells. (A) Following exposure to baicalin (20 µg/ml) for the indicated times (0-48 h), HL-60 cells were stained with propidium iodide, then the DNA content was analyzed through flow cytometry. (B) Quantitative data from A. Values represent the mean ± standard deviation (n=3). *P<0.05 vs. control.
followed by caspase-9 activation. Flow cytometry analysis of Rh123-stained HL-60 cells was performed to investigate the intrinsic pathway. As presented in Fig. 6A, treatment of HL-60 cells with baicalin for 24 h led to a marked decrease in Rh123 fluorescence compared with the control group, presenting dissipation of ΔΨm.

The Bcl-2 family of proteins is a key factor in the regulation of cytochrome c release from mitochondria (17). To further confirm the involvement of the intrinsic pathway, anti-apoptotic factor Bcl-2 and pro-apoptotic factor Bax were detected using western blotting. The protein expression of Bax was increased notably, but that of Bcl-2 decreased following exposure of HL-60 cells to baicalin for 12 and 24 h (Fig. 6B). As a result, the ratio of Bcl-2/Bax declined significantly (Fig. 6C). These results demonstrated that the intrinsic signaling pathway was involved in baicalin-treated apoptosis, in accordance with the previous studies (6,8).

Baicalin induces apoptosis through the Fas-mediated extrinsic pathway. To clarify which receptor mediated the extrinsic pathway, the death receptors, including DR4, DR5 and Fas, were detected using RT-PCR. The results revealed that exposure of HL-60 cells to baicalin for 24 h significantly
triggered Fas expression, but no significant effects of baicalin on the mRNA levels of DR4 or DR5 were noted (Fig. 7A and B). Western blot assays were then performed, which further confirmed that baicalin induced Fasl and Fas expression throughout the time period examined (Fig. 7C and D). The data presented in the present study suggest that activation of the extrinsic Fas-associated pathway partially contributed to baicalin-induced apoptosis.
Discussion

As previously reported (8), the present study confirmed that baicalin exhibits high dose- and time-dependent anticancer activity. To determine the molecular mechanisms of the inhibitory effect of baicalin in HL-60 cells, cell cycle analysis was performed using flow cytometry. The results demonstrated that baicalin induced significant G0/G1 arrest 48 h later; however, it was reported by Ikezoe et al (3) that baicalin arrested HL-60 cells at the G2/M phase. Consistent with the G0/G1 arrest, the results of our previous study demonstrated the baicalin upregulated the expression of cyclin-dependent kinase inhibitors, including p21 and p27 (15), which are involved in the G1 to S transition (18). It was concluded that baicalin reduced cell viability partially through arresting cells at the G0/G1 phase. However, the appearance of the hypodiploid peak in the cell cycle distribution demonstrated that apoptosis also contributed to cell proliferation inhibition by baicalin. Furthermore, the cell morphological changes observed and Annexin V/PI staining results further confirmed this, and are concordant with preceding reports (3,4,6,8).

It is well known that caspases, a conserved family of cysteine proteases, serve a central role in the execution of cell apoptosis (19). They are generally divided into two classes: The initiator caspases, which include caspase-2, -8, -9 and -10; and the effector caspases, which include caspases-3, -6 and -7 (15). Classical apoptosis occurs through the extrinsic and intrinsic pathway in all mammalian cells. Both pathways converge on activation of initiator and effector caspases (15). The former depends on death receptors, including Fas or TNF-related apoptosis-inducing ligand (TRAIL) receptors, DR4 and DR5 (20). When bound by their specific death ligand, these receptors are activated, resulting in the formation of a death-inducing signaling complex and activation of the initiator caspase-8, which further directly or indirectly activates effector caspase-3 (20). The latter depends on mitochondria. Once the apoptotic stimuli are received, including heat shock, oxidative stress, DNA damage or cytotoxic drugs, the permeability of the inner mitochondrial membrane increases, and the mitochondrial membrane potential is disrupted (21). Subsequently, cytochrome c is released, triggering caspase-9, and downstream effectors caspase-3 and/or -7 activation (21).

To elucidate the mechanism of apoptosis elicited by baicalin in HL-60 cells, the protein expression of caspase-3, -8, and -9 was detected using western blotting. Consistent with previous reports (6,8), the results of the present study demonstrated that caspase-3 and -9 were cleaved, and activated following baicalin treatment, demonstrating a role for mitochondria in the process of ongoing apoptosis in HL-60 cells. Though caspase-8 activation had been confirmed in cervical cancer HeLa cells (22) and in human colorectal carcinoma SW620 cells (23), the roles of caspase-8 in baicalin-induced apoptosis in human leukemia HL-60 cells remained to be clarified. In the present study, increased expression of cleaved caspase-8 was detected following exposure to baicalin for 12 h, and more notably after 24 h. Thus, it was postulated that, in addition to the intrinsic signaling pathway, the extrinsic pathway may also contribute to apoptosis induced by baicalin in HL-60 cells.

To further confirm the convergence in apoptosis signaling, molecular markers associated with the extrinsic and intrinsic pathways were analyzed. Similar to the results acquired from hepatoma cell lines (24) and the leukemia-derived T cell line (6), it was demonstrated that baicalin caused A1V1m disruption of HL-60 cells and increased the expression of Bax, while it decreased expression of Bcl-2, confirming that the intrinsic pathway participated in apoptosis induced by baicalin.

It is well established that caspase-8 activation is initiated by the activation of death receptors. Currently, six distinct death receptors are known, including Fas (APO-1/CD95), the TRAIL receptor (R)-1 (also named DR4), TRAIL-R2 (also named DR5), tumor necrosis factor-R1, DR3 and DR6. Of these receptors, Fas and the TRAIL receptors have been extensively investigated (25). A previous study validated that modulation of Fas expression on the surface of tumor cells may potentiate the induction of apoptosis in tumor cells in response to chemo- and immuno-therapeutic agents (26). In addition, baicalin has been reported to trigger Fas and FasL expression during HeLa cell apoptosis (21). However, in the T-cell acute lymphoblastic leukemia cell line CCRF-CEM, it appeared that baicalin treatment exhibited no significant effect on the expression of Fas (7). In the present study, the upregulation of Fas and its ligand FasL was detected at the mRNA and protein level. However, there was no change in DR4 and DR5 mRNA expression. This discrepancy is likely attributed to the specific cell type investigated. The data of the present study implied that the Fas receptor, but not DR4 and DR5, was involved in baicalin-induced caspase-8 activation.

An investigation regarding the involvement of transcription factors in cell proliferation may be useful to perform a more in-depth analysis of the exact mechanism by which baicalin exhibits antitumor activity. c-Myc, as a transcription factor, serves an important role in cell proliferation and is overexpressed in numerous human tumors (27). Evidence has demonstrated that c-Myc binds to thousands of promoters of its target genes, including hTERT (28). It has been confirmed that c-Myc is involved in the control of telomerase activity, which is associated with cell proliferation in normal cells and tumors (29), through its ability to induce the transcriptional activation of hTERT. In the present study, the downregulation of c-Myc was detected at the mRNA and protein level, in addition, transcriptional activation of hTERT was also decreased, in line with the inhibition of HL-60 cells.

In conclusion, the results of the current study demonstrated that baicalin is able inhibit the proliferation of the AML HL60 cell line through G0/G1 phase arrest and significant apoptosis induction via the intrinsic, and extrinsic pathways. In addition, the inhibition of HL-60 cell growth was also mediated by telomerase inhibition through suppression of c-Myc. These results raise the possibility that baicalin may be a promising regimen for treatment of AML.

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