The restraining effect of baicalein and U0126 on human cervical cancer cell line HeLa

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Abstract. To explore the restraining effect of baicalein and the mitogen-activation protein kinase kinase inhibitor, U0126, on human cervical cell line HeLa proliferation, apoptosis and migration. HeLa cells were treated by different concentrations of baicalein or U0126. A Cell Counting Kit (CCK)-8 assay was applied to examine cell viability. Flow cytometry was used to determine cell cycle and apoptosis. A wound healing assay was performed to detect cell migration. A terminal deoxynucleotidyl transferase dUTP nick end labeling assay was adopted to test cell apoptosis. Reverse transcription-quantitative polymerase chain reaction and western blot analysis was used to detect apoptosis gene and protein expression. CCK-8 assay demonstrated that baicalein and U0126 suppressed HeLa cell viability by dose dependence. TUNEL, Annexin V-fluorescein isothiocyanate/propidium iodide, and ratio of Bcl-2-associated X protein and B cell lymphoma 2 indicated that baicalein and U0126 induced HeLa cell apoptosis. Flow cytometry revealed that baicalein blocked the cell cycle of HeLa in G0/G1 phase. A wound healing assay demonstrated that baicalein significantly inhibited HeLa cell migration compared with control. Baicalein and U0126 markedly downregulated extracellular signal-regulated kinase 1,2, matrix metalloproteinase (MMP) 2 and MMP9 levels both in mRNA and protein. In the present study, the authors demonstrated that baicalein and U0126 may be used in cervical cancer treatment by inhibiting cell migration and inducing cell apoptosis.

Introduction

Cervical cancer is one of the most common malignant tumors in the female reproductive system (1). Cervical cancer is a complicated process involving many factors, from being a precancerous lesion, before becoming an in situ carcinoma and then an infiltrating carcinoma. Human papillomavirus (HPV) infection is one of the primary causes of cervical cancer tumorigenesis. HPV DNA can integrate into the cervical cells, and its encoded oncoprotein E6 will continue to activate the extracellular signal-regulated kinase (ERK1/2) signaling pathway, thus promoting cancerous processes and inhibiting apoptosis (2,3).

It was reported that ERK1/2 phosphorylation may be used as a marker of cervical cancer differentiation (4). The ERK1/2 signaling pathway serves a critical role in the carcinogenesis process of cervical cancer. ERK1/2 expresses in the cytoplasm in normal tissue and keeps inactivated in normal cervical epithelium proliferation. Conversely, ERK1/2 is expressed both in cytoplasm and nuclei in cervical cancer tissues, and maintains the state of activation. ERK1/2 is a tyrosine kinase involved in the signal cascade Ras/Raf/MEK/ERK. Phosphorylation is the method for activation of ERK, leading to the transmission of extracellular signals to the nucleus. It further promotes the target gene expression, such as c-Jun and c-Fos. The protein products of the later can bind with transcription factor activator proten-1 in the form of a homodimer or heterodimer, thus activating myc gene expression (5-7). Myc can activate a series of cell proliferation signals and induce cyclin DI expression, leading to cancer cells abnormal proliferation, differentiation, apoptosis and metastasis (8,9). Therefore, ERK activation can inhibit cancer cell apoptosis (10,11). Furthermore, there are numerous methods applied in clinic to treat cervical cancer by interfering in the ERK1/2 signaling pathway, such as cisplatin administration (12,13).

Baicalein belong to the group of flavonoid drugs, and is the active ingredient extracted from the root of Chinese traditional medicine \textit{Scutellaria baicalensis}. It has multiple effects, including antibacterial, antiviral, anti-inflammatory, anti-allergic, anti-oxidative and anti-tumorigenic. Recent
studies reported that baicalein may inhibit bladder cancer, prostate cancer, and liver cancer cell growth (14-16), while its role in cervical cancer cells has not been fully elucidated. The possible mechanism of baicalein includes: 1) Affecting the arachidonic acid metabolic pathway; 2) inhibiting cell proliferation; 3) promoting cell apoptosis; and 4) suppressing tumor angiogenesis formation (17,18). U0126 is the highly specific inhibitor of mitogen-activated protein kinase (MAPK) kinases, MEK1 and MEK2. It can obviously inhibit c-Fos and c-Jun mRNA and protein upregulation through blocking MAPK signal transduction, resulting in AP-1 transcriptional activity suppression (19-21).

The present study jointly used baicalein and U0126 to stimulate the cervical cancer cell line HeLa, and observe their impacts on cell apoptosis and migration.

Materials and methods

Cell line and reagents. Human cervical cancer cell line HeLa was purchased from Shanghai Bioleaf Biotechnology Co., Ltd (Shanghai, China). The cells were maintained in Dulbecco's Modified Eagle's medium containing 20% fetal calf serum and cultured at 37°C and 5% CO₂. Baicalein purchased from National Institutes for Food and Drug Control (Beijing, China) was dissolved in dimethyl sulfoxide (Beyotime Institute of Biotechnology, Jiangsu, China) at 50 mol/l. U0126 was purchased from Shanghai Haoran Biological Technology Co., Ltd. (Shanghai, China) with the excitation wavelength at 488 nm. All the experiments were repeated three times. Data were analyzed by BD Cell-Quest Pro software version 5.1 (BD Biosciences). Experiments were analyzed by BD Cell-Quest Pro software version 5.1 (BD Biosciences). Experiments were repeated three times.

Cell Counting Kit (CCK) -8 assay. HeLa cells (2×10⁵ cells/ml) were seeded in a 96-well plate in 100 µl medium. Different concentrations of baicalein (1, 2, 5, 10, 20, 50, 100, 200 and 300 µM) or U0126 (1, 2, 5, 10, 20 and 30 µM) were used to treat cells for 4 h with three replicates. Then, 10 µl CCK-8 (Beyotime Institute of Biotechnology) was added to the well for 4 h to form formazan. Finally, the plate was read at 450 nm to draw the curve.

Cell cycle detection. HeLa cells (2×10⁵ cells/ml) were seeded in a 12-well plate and treated with baicalein or U0126. The cells were collected and washed by PBS twice. Next, they were added to 1 ml 70% precooled ethanol at 4°C overnight. Then the cells were washed with PBS and added with 100 mg/l RNase (Beyotime Institute of Biotechnology) at 37°C for 30 min. Following staining with 50 mg/l propidium iodide at 4°C in the absence of light for 30 min, the cells were detected using a FACScalibur instrument (BD Biosciences, Franklin Lakes, NJ, USA) with the excitation wavelength at 488 nm. All the experiments were repeated three times. Data were analyzed using SPSS software version 19.0 (IBM SPSS, Armonk, NY, USA).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. HeLa cells were washed by PBS twice following treatment and then fixed in 4% PFA at room temperature for 10 min. The apoptotic cells were detected using a TUNEL assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. Next, the cells were treated by 3% hydrogen peroxide for 10 min to inhibit endogenous catalase. Following incubation with protease at room temperature for 30 min, the cells were washed by PBS twice. Subsequently, 50 µl TUNEL detection solution (containing terminal deoxynucleotidyl transferase and digoxigenin-labelled nucleotides) was added and incubated at room temperature for 60 min. Cells were washed with PBS three times and observed under a fluorescence microscope.

Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) assay. Following treating with 20 µM baicalein or 10 µM U0126, HeLa cells were collected and washed by PBS twice. Then the cells were resuspended in 400 µl 1X binding buffer and 5 µl Annexin V-FITC was added in the absence of light for 15 min. A total of 10 µl PI was added to the cells and incubated in darkness for 5 min. The cells were then tested using flow cytometry. The results were analyzed by BD Cell-Quest Pro software version 5.1 (BD Biosciences). Experiments were repeated three times.

Wound healing assay. A total of 6×10⁶ HeLa cells were seeded in a 24-well plate for 24 h to form a monolayer. Then the cells were scratched using a sterile toothpick to form a straight line. Following washing with PBS, the cells were treated with baicalein (20 µM). Finally, the plate was observed under a light microscope at different time points. The wound areas were imaged and automatically calculated using TScratch software version: 1.0 (Computational Science and Engineering Laboratory, ETH Zurich, Zurich, Switzerland) (22).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using TRIzol (Beyotime Institute of Biotechnology) and reverse transcribed to cDNA using the RevertAid First Strand cDNA Synthesis kit (cat no. K1622; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The primers used were designed by Primer 6.0 Premier (Premier Biosoft International, CA, USA). RT-qPCR was applied to test target gene expression. Reaction conditions: 55°C for 1 min, followed by 35 cycles of 92°C for 30 sec, 58°C for 45 sec and 72°C for 35 sec. GAPDH was used as internal reference. 2−ΔΔCt was applied to calculate relative expression level (23). The primers sequences were as follows: ERK1/2 forward, 5'−AATCACACGGTACACTGAAATGC-3' and reverse, 5'-CATCACATCCATACATAATG-3'; GATA2-associated X protein (Bax) forward, 5'-CATATACCCCGTCTCAAGCGC-3' and reverse, 5'-CCAGGCCCAGATGGGTCTCTGAT-3'; B cell lymphoma 2 (Bcl-2) forward, 5'-GGTGGGCTATGTGTTGC-3' and reverse, 5'-GGTGGTATGGCTACATCC-3'; Cyclophilin forward, 5'-GCTGGAAAGTTGAACATC-3' and reverse, 5'-CTCCTCCTTCGCACACATTG-3'; MMP9 forward, 5'-TACAGGATATGGCTCAGACC-3' and reverse, 5'-GTCAGTGAGGCCGAGACCTGAG-3'; Bax forward, 5'-GGGGTCCTCGGCTC-3'; and reverse, 5'-GACGATCATCGTACCTGGAT-3'.

Western blot analysis. HeLa cells were incubated on ice for 20 min and protease inhibitor and radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology) was added to extract protein. Following centrifugation at 9,000 g for 10 min at 4°C, the supernatant was moved to a new Eppendorf
Protein concentration was determined using a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology) according to the manufacturer’s protocol. The protein was separated using 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane. Following blocking with 5% skim milk for 1.5 h, the membrane was incubated with primary antibodies, cyclin D1 (catalog no. ab137875), ERK1/2 (catalog no. ab151279), MMP2 (catalog no. ab37150), MMP9 (catalog no. ab137867), bax (catalog no. ab182733), bcl-2 (catalog no. ab32124), and GAPDH (catalog no. ab8245) (dilution 1:1,500; Abcam, Cambridge, UK) at 4˚C for 8 h. Then the membrane was incubated with secondary antibody conjugated to horse radish peroxidase (catalog no. ab131368, dilution 1:3,000; Abcam) at 37˚C for 30 min and washed with PBS containing 20% Tween 20. Finally, the membrane was visualized using 3,3’-Diaminobenzidine substrate and images were captured. Protein bands were quantified using Quantity One 1-D Analysis software version 4.6.9 (Bio-Rad, Hercules, CA, USA). Experiments were repeated for three times.

Statistical analysis. Data was presented as mean ± standard deviation and analyzed using SPSS software version 19.0 (IBM SPSS, Armonk, NY, USA). Student’s t-test or one-way analysis of variance followed by the Bonferroni post hoc test was applied for data comparison. P<0.05 was considered to indicate a statistically significant difference.

Results

Baicalein and U0126 suppressed HeLa induced cell apoptosis. To explore the inhibitory effect of baicalein and U0126 on HeLa cells, different concentrations of baicalein (1, 2, 5, 10, 20, 50, 100, 200 and 300 µM) or U0126 (1, 2, 5, 10, 20 and 30 µM) were used to treat HeLa cells for 24 h. CCK-8 results demonstrated that HeLa cell viability significantly decreased by administration of baicalein or U0126 with dose dependence (P<0.05; Fig. 1A). To investigate baicalein and U0126 impact on HeLa cell apoptosis. HeLa cells were treated with 20 µM baicalein and 10 µM U0126. A TUNEL assay revealed that both baicalein and U0126 induced HeLa cell apoptosis synergistically (Fig. 1B). To further examine which phase of apoptosis was baicalein and U0126 mainly act on. Annexin V/PI staining indicated that baicalein and U0126 increased both early and late phases of apoptosis with synergistic effect (Fig. 1C).

Baicalein and U0126 restrained the MCF-7 cell cycle. As ERK can regulate cell cycle, the authors intended to clarify the impact of baicalein and U0126 on the HeLa cell cycle.
Baicalein (20 µM) or U0126 (10 µM) was added to HeLa cells for 24 h and flow cytometry demonstrated that, compared with control group, cell content increased in G0/G1 phase, reduced in S phase, and had no significant change in G2/M phase in the baicalein group (Fig. 2A). U0126 addition markedly declined cell content in S phase, when compared with the single baicalein group (Fig. 2A). In addition, cyclin D1 expression was investigated in HeLa cells treated by baicalein or U0126. Both RT-qPCR and western blot analysis demonstrated that cyclin D1 mRNA and protein levels reduced following 20 µM baicalein treatment for 24 h. U0126 further declined cyclin D1 expression (Figs. 2B and C).

**Baicalein inhibited HeLa migration.** To study the effect of baicalein on HeLa cell migration, the authors treated HeLa cells with 20 µM baicalein for 1, 3 and 6 h. A wound healing assay demonstrated that the scratch width in the baicalein group was significantly larger than that in control, indicating that baicalein can restrain HeLa cell migration (Fig. 3).

**Baicalein and U0126 affected ERK signaling pathway and apoptosis related molecule expression in HeLa cells.** As a previous study reported that baicalein may regulate the ERK signaling pathway (24), the present study tested the effect of baicalein on the ERK signaling pathway and related proteins in HeLa cells. RT-qPCR results demonstrated that baicalein obviously declined ERK1/2, MMP2 and MMP9 levels in HeLa cells. Moreover, it significantly elevated levels of pro-apoptotic factor Bax mRNA expression and downregulated apoptosis suppression gene Bcl-2 mRNA level (Fig. 4A). Furthermore, the expression of proteins of the ERK signaling pathway was analyzed. Western blotting revealed that ERK1/2 phosphorylation, MMP2 and MMP9 levels were markedly declined in HeLa cells treated by baicalein.

![Figure 2. Baicalein and U0126 restrained the HeLa cell cycle. (A) The HeLa cell cycle was examined using flow cytometry. (B) cyclin D1 mRNA expression determined by reverse transcription-quantitative polymerase chain reaction. (C) Cyclin D1 protein expression detected by western blot analysis. Lanes: 1, control; 2, control + DMSO; 3, baicalein (20 µM); 4, U0126 (10 µM); 5, baicalein (20 µM) + U0126 (10 µM). DMSO, dimethyl sulfoxide.](image)

![Figure 3. Baicalein inhibited MCF-7 migration. HeLa cell migration ability was determined using a wound-healing assay. Magnification, x100. DMSO, dimethyl sulfoxide.](image)
In addition, Bax and Bcl-2 proteins presented a similar trend with mRNA expression. Baicalein, together with U0126, more obviously suppressed ERK signaling pathway related proteins expression, when compared with single baicalein treatment (Fig. 4B).

Discussion

Cervical cancer is a common malignant tumor in females (1). At present, radiotherapy, surgery and chemotherapy are the main treatment methods. All of these methods improve patient’s survival through suppressing cancer cell proliferation and metastasis, and promoting cell apoptosis. However, there are numerous adverse reactions of current chemotherapy drugs (25,26). Thus, searching for effective drugs for cervical cancer treatment is of great significance.

Baicalein has been demonstrated to exhibit antitumor effects in numerous types of cancer (27-29). However, the antitumor effect and the associated mechanisms in breast cancer cells remain unclear. The present study revealed that baicalein significantly inhibited the proliferative and metastatic ability of breast cancer cell line, HeLa, by regulating the expression levels of MMP2 and MMP9 via inhibition of the ERK signaling pathway. To the best of the authors’ knowledge, the present study is the first to investigate the antitumor effect of baicalein on breast cancer cell line, HeLa.

The ERK signaling pathway is important for tumor cell proliferation through regulating the cell cycle (30). ERK can facilitate cancer cell growth by promoting cell movement from the G1 phase to the S phase (31). The current results exhibited that baicalein blocked HeLa cells in G0/G1 phase, and U0126 also decreased cell content in S phase. In addition, cyclin D1 level declined following baicalein and U0126 treatment, indicating that both baicalein and U0126 may restrain breast cancer proliferation through blocking the cell cycle.

Abnormal apoptosis is an important reason for carcinogenesis. Apoptosis is a specific process with featured biochemical processes and cell morphology changes, including the death receptor and mitochondrial pathways. They can activate caspases through a series of signal transduction, thus leading to the degradation of the nucleus and various substrates in the cytoplasm (32,33). Caspase-3 is the most common executor of the apoptosis pathway (34,35). The Bcl-2 family serves a critical role in regulating apoptosis, while ERK1/2 phosphorylation can achieve anti-apoptosis and promote proliferation through phosphorylating Bcl-2, activating transcriptional factors, and interfering TNF related apoptosis inducing ligand (36,37). Baicalein and Bcl-2 are important members in Bcl-2
family, and the ratio of Bax and Bcl-2 determines whether cell apoptosis occurs or not (38,39). These results indicated that baicalein and U0126 treatment both restrained HeLa cell viability and induced cell apoptosis. Furthermore, they significantly declined Bcl-2 expression and enhanced Bax level. All of these results suggested that baicalein and ERK signaling pathway inhibitor may suppress cervical cancer cell proliferation and induce apoptosis.

Tumor invasive metastasis is a complicated pathological process affected by multiple factors, genes and processes. Extracellular matrix (ECM) degradation and basement membrane integrity damage are the prerequisites of tumor cell metastasis (40,41). The role of MMPs in tumor metastasis mainly includes: 1) Destructing local tissue structure and promoting tumor growth; 2) destructing the basement membrane barrier, which facilitates tumor metastasis; 3) promoting tumor neovascularization by improving the ECM. The degradation of ECM mainly depends on proteolytic enzymes; MMPs are an extremely important type of proteolytic enzyme (42,43). MMPs are Zn²⁺- and Ca²⁺-dependent proteases that may be classified by four types according to specific substrates: 1) Collagenase (MMP-1, -8 and -13); 2) gelatinase (MMP-2 and -9); 3) stromelysin (MMP-3, -7, -10 and -11); 4) Membrane type metal protein enzymes (MMP-14, -15, -16 and -17) (44,45). MMP2 and MMP9 can specifically degrade the main component of basement membrane, collagen type IV (46,47). MMP2 and MMP9 serve an important role in cervical cancer invasion and metastasis. It was reported that MMP2 and MMP9 is significantly overexpressed in cervical cancer tissue, thus leading to ECM and vascular basement membrane acceleration of degradation. Therefore, it facilitates cancer cells emigrating from the primary lesion and enhances tumor cell metastatic ability (48). Moreover, MMP2 is an early marker of tumor, and its high expression is one of the important characteristics of tumor metastasis (49). These results revealed that baicalein and U0126 markedly decreased MMP2 and MMP9 levels in HeLa cells, and their combination further declined MMP2 and MMP9 mRNA and protein expression.

Taken together, baicalein and U0126 can induce HeLa cell apoptosis and restrain migration through the ERK signaling pathway. Their combination can further suppress breast cancer cell proliferation and metastasis, providing great significance for breast cancer treatment.

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References


liposomal formulation of cisplatin, lipoplatin, to treat cisplatin-resistant cervical cancer.

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