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Original Article

Baicalein inhibits the invasion, migration and epithelial-mesenchymal transition of BGC-823 cells through NF-κB/Snail signaling pathway

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Abstract: Baicalin is one of the main bioactive flavone glucuronides, which is considered as a medical herb with anticancer activity. However, no detailed studies have ever been reported on its anticancer action through NF-κB/Snail signaling pathway. This study aimed to investigate the effects of baicalin on the invasion and migration of gastric cancer BGC-823 cells and epithelial-mesenchymal transition (EMT). Different concentrations of baicalein were used to treat with BGC-823 cells. MTT colorimetry was used to observe the inhibitory rate of cell proliferation. The effects of baicalein on the invasion and migration ability of gastric cancer BGC-823 cells were determined by cell invasion and migration assay. The roles of baicalein on EMT of BGC-823 cells were investigated by examining the protein levels of EMT related markers in BGC-823 cells after baicalein and/or TGF-β1 treatments by western blot. Finally, the role of baicalein in TGF-β1-mediated activation of NF-κB/Snail signaling pathway was investigated by examining the protein levels of the factors involved in NF-κB/Snail signaling pathway in BGC-823 cells after baicalein and/or TGF-β1 treatment by western blot. Our results have shown that baicalein not only inhibited the proliferation of BGC-823 cells, but also reduced the invasion and migration ability of those cells. Baicalein also inhibited EMT of BGC-823 cells and TGF-β1-mediated activation of NF-κB/Snail signaling pathway by regulating the expression of the related factors. As a result, baicalein could inhibit EMT and suppress the invasion and migration of human gastric cancer BGC-823 cells through NF-κB/Snail signaling pathway.

Keywords: Baicalein, TGF-β1, EMT, NF-κB/Snail signaling pathway, BGC-823 cells

Introduction

EMT plays a pivotal role in various cell biological processes including development, wound healing, and even tumor progression [1-3]. EMT, which is characterized by the decreased expression of epithelial markers including keratins, E-cadherin and claudin, and increased expression of mesenchymal markers such as N-cadherin and vimentin, is responsible for the invasiveness and migration of epithelial tumor cells [4, 5]. The cell polarity and cell-cell adhesion are lost in epithelial cells during EMT, combined with the function of matrix-degrading enzymes produced during this procedure, the migration and invasiveness capacity of tumor cells will be increased to facilitate the trafficking of tumor cells to surrounding areas or even long distant sites [6]. The occurrence and development of EMT is usually caused by growth factor and extracellular matrix components, within which the functional mechanism of transforming growth factor beta (TGF-β) and epidermal growth factor (EGF) have been well studied [7-9]. TGF-β has been found to play important roles in the occurrence of EMT in various cancer cells [10]. After binding to its receptors, TGF-β can active a variety of transcription factors to induce the cadherin isoform switch by down regulating E-cadherin (cell-cell adhesion molecule) and tumor suppressors [11, 12]. The NF-κB pathway has been proved to be directly involved in the development of EMT through regulating the expression of various EMT related factors including Snail, which can suppress Raf-kinase inhibitor (product of metastasis-suppressor gene) that inhibits two survival pathways implicated in EMT, including Raf-1/MEK/ERK survival pathway and NF-κB survival pathway [13-16].

Scutellaria baicalensis Georgi, which is also known as *Baikal skullcap*, is widely used in traditional Chinese medicine (TCM). The application value of the extracts of *Scutellaria baicalensis Georgi*, including baicalein, in clinical treatments has been proved in the treatment of cancer [17], inflammation [18] and viral infection [19]. Previous studies have shown that baicalein can regulate NF-κB pathway to inhibit IL-1β- and TNF-α-induced inflammatory response [20]. As NF-κB pathway makes efforts in the occurrence and development of tumor, it is reasonable to hypothesize that baicalein may also have important functions in the procedure EMT.

In this study, the effects of baicalein on the invasion and migration of gastric cancer BGC-823 cells and EMT were studied. After treatment with baicalein, the inhibitory effects on cell proliferation of gastric cancer BGC-823 cells were detected by MTT colorimetry. Cell invasion and migration assay was performed to determine the invasion and migration ability of gastric cancer BGC-823 cells before and after treatment. Western blot was used to detect the protein levels of EMT about its related markers and factors involved in NF-κB/Snail signaling pathway.

Materials and methods

Cell culture

BGC-823 cells (human gastric cancer cell line) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum in incubator at 37°C with 5% CO_2. The medium was changed once per day and 0.25% trypsin digestion was used for subculture.

MTT assay

Cells were collected at exponential phase and diluted to a density of 5×10^4/mL. Then cells were plated onto 96-well cell culture plates with 100 μL per well. Cells were divided into two groups: group 1 was subjected to baicalein treatment with a serial concentration of 0, 5, 10 and 15 μg/mL and group 2 was used as a blank control. Cells were collected at 4, 24 and 36 hours after treatment and 20 μL of tetrazolium blue solution was added into each well 4 hours before terminating the experiment. The supernatant was discarded and 150 μL of DMSO was added to each well. After shaking for 10 minutes on a shaker, absorbance at 490 nm was checked by a microplate reader. Three repeats were involved in each experiment and the inhibition rate was calculated with the following formula: inhibition rate = (mean value of control group-mean value of baicalein treatment group)/mean value of control group × 100%.

Cell invasion assay

The bottom of the transwell membrane was coated with matrigel and hydrated. Cells were cultured in 6-well plates and divided into baicalein treatment group and control group. Cells in baicalein treatment were subjected to baicalein treatment with a serial concentration of 0, 5, 10 and 15 μg/mL. After incubation for 24 h, the cells were digested and resuspended in serum-free RPMI 1640 medium to make a final concentration of 5×10^5 cells/mL. Then 200 μL single cell suspension was added to the transwell upper chamber and 500 μL RPMI 1640 medium with 10% FBS was added to lower chamber. Five repeats were used for each experiment. After incubation for another 24 h, transwell chamber was gently washed twice with PBS. A cotton swab was used to wipe cells from the surface of the membrane. After removal of non-invasive cells, invaded cells were incubated with methanol for 30 min. Cells were then stained with 0.1% crystal violet at room temperature for 20 min and crystal violet was removed by three times' washing with PBS. The numbers of the invaded cells in lower chamber were calculated under a microscope and five visual fields from both the margin and center areas were used to calculate the mean value.

Cell migration assay

The experimental procedure of cell migration assay was the same as that of the cell invasion experiment. The difference was that the surface of polycarbonate porous membrane was not covered with matrigel. The remaining steps and methods were the same. Five visual fields were randomly selected under 200-fold microscopic to count the cell numbers and the average numbers were used to represent the tumor cell migration capacity.

Establishment of epithelial-mesenchymal transition (EMT) model and baicalein treatment

BGC-823 cells were treated with different concentrations of TGF-β1 (2.5, 5 and 10 ng/mL) and the effects of different concentrations of

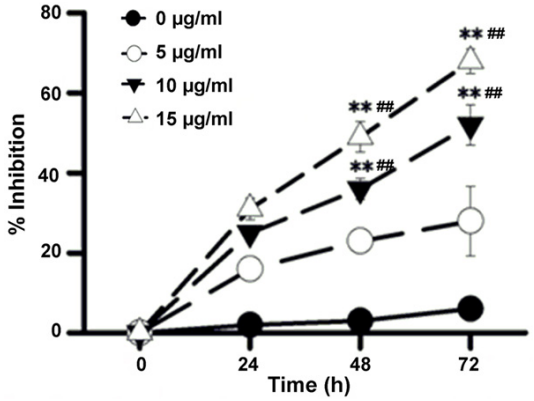

Figure 1. Inhibitory effect of baicalein on proliferation of gastric cancer BGC-823 cells. All experiments were repeated three times. Data are means ± SD. **P<0.01, compared with control group. ##P<0.01, compared with group 1.

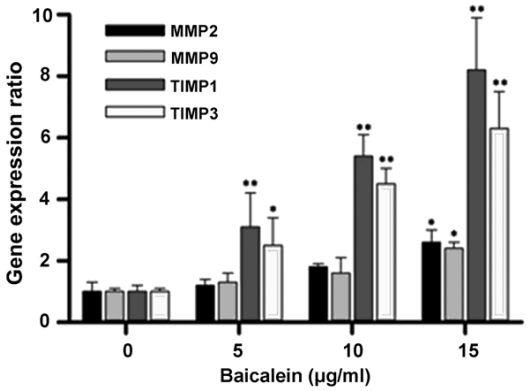

Figure 2. Inhibitory effects of baicalein on the of invasion and migration ability of gastric cancer BGC-823 cells. All experiments were repeated three times. Data are means ± SD. *: compared with control group, *P*<0.05; **: compared with control group, *P*<0.01.

Table 1. The inhibitory effects of baicalein on the of invasion and migration ability of gastric cancer BGC-823 cells

Groups	Concentration/ $\mu g \cdot mL^{-1}$	Invasion/ cells	Migration/ cells
Control	0	228.4±9.1	174.2±11.6
1	5	122.2±11.2*	98.4±13.2*
2	10	86.5±2.1*	67.3±7.4*
3	15	56.4±6.8*	42.7±8.2*

All experiments were repeated three times. Data are means ± SD. *p<0.1.

TGF-β1 on EMT were detected by Western blot. Based on the result of northern blot, 10 ng/mL TGF-β1 was used to treat BGC-823 cells to establish EMT model. To investigate the effects of baicalein on EMT, four groups of cell with different treatments were included. The serum-free medium was added into group 1 cells to sever as a negative control; cells in group 2 (TGF-β1) were treated with serum-free medium containing 10 ng/mL TGF-β1; cells in group 3 (baicalein group) and positive control group were firstly treated with a serial different concentrations of baicalein (0, 5, 10 and 15 μg/mL) followed by treatment with 10 ng/mL TGF-β1.

Western blot

The total protein was extracted by conventional method and BCA method was used to check the concentration. After denaturation at 95°C for 5 min, 40 μg protein was used for 12% SDS-PAGE gel running. After that, proteins were transferred to PVDF membrane. Membrane was blocked for 2 h with 5% skim milk. The membrane was then incubated with the primary antibody overnight at 4°C followed by incubation with the secondary antibody for 2 h at 37°C. After adding ECL solution, exposure step was performed in dark room.

Statistical analysis

Each experiment was repeated for three times. Differences of the quantity values between groups were analyzed with the one-way analysis of variation (ANOVA) with Student's Newman-Keuls by Bandscan 5.0 software. *P* value of <0.05 meant statistically significant; *p* value of <0.01 meant significantly different.

Results

Inhibitory effect of baicalein on proliferation of gastric cancer BGC-823 cells

After treatment with different dose of baicalein and different length of treatment period, MTT colorimetry was used to observe the inhibitory rate of cell proliferation. The data have shown that inhibitory effect was increased with the increase of baicalein concentration. Significant differences were found between groups treated with different concentrations of baicalein (*P*<0.01) (**Figure 1**).

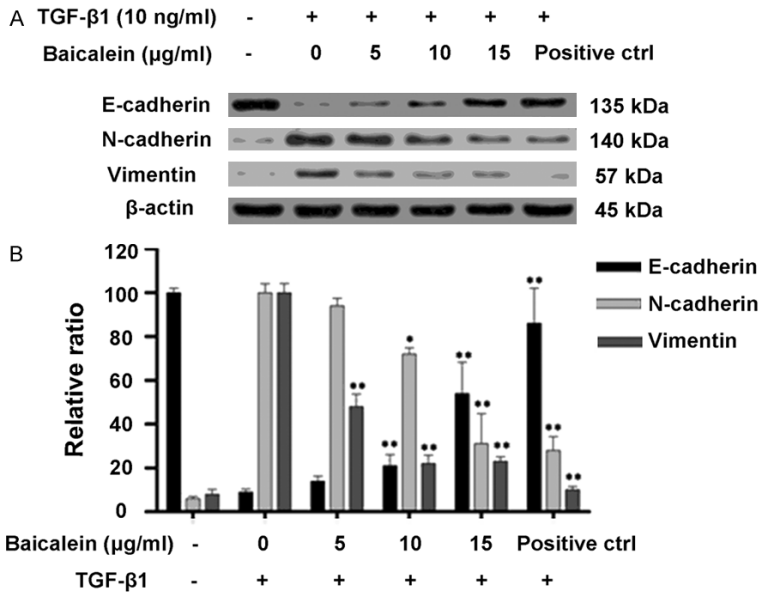

Figure 3. The effect of different doses of TGF-β1 and baicalein on the expression of EMT related markers. A. The protein expression levels of E-cadherin, N-cadherin, vimentin and β-actin were determined by Western blot. B. Quantitative analysis of protein expression levels of E-cadherin, N-cadherin, vimentin and β-actin using Image-Pro Plus 6.0 software and normalized to β-actin. All experiments were repeated three times. Data are means ± SD. ***P*<0.01 compared with control group.

Inhibitory effects of baicalein on the of invasion and migration ability of gastric cancer BGC-823 cells

The invasion and migration assay have shown that invasion and migration rates of the cells treated with different doses of baicalein were all significantly lower than those of the control group (*P*<0.01) (**Table 1**). With increased baicalein concentration, increased inhibitory effect was observed, indicating that baicalein can inhibit the invasion and chemotaxis of BGC-823 cells (**Figure 1**; **Table 1**).

Effects of baicalein on mRNA levels of MMP2, MMP9, TIMP1 and TIMP3

After treatment with different concentrations of baicalein (0, 5, 10 and 15 μg/mL) for 24 h, the mRNA levels of MMP2 and MMP9 in human gastric cancer BGC-823 cells were increased slightly compared with those in control group at the concentrations lower than 15 ug/ml. The mRNA levels of TIMP1 and TIMP3 after treatment were significantly higher than those in control group (*P*<0.05, *P*<0.01), and mRNA levels were further increased with the increased concentration of baicalein (**Figure 2**).

Effect of baicalein on EMT of BGC-823 cells

Western blot analysis showed that the protein level of E-cadherin in BGC-823 cells was significantly decreased with the increased TGF-β1 concentration (2.5, 5 and 10 ng/mL) (*P*<0.01), while protein level of interstitial markers N-cadherin and vimentin were significantly increased with the decreased TGF-β1 concentration (2.5, 5 and 10 ng/mL) (*P*<0.01) (**Figure 3A**).

In order to investigate the effects of baicalein on EMT, four groups of cell with different treatments were included. The serum-free medium was added into group 1 cells to sever as a negative control; cells in group 2 (TGF-β1) were treated with serum-free medium containing 10 ng/mL TGF-β1; cells in group 3 (baicalein group) and positive control group were firstly treated with a serial concentrations of baicalein (0, 5, 10 and 15 μg/mL) followed by treatment with 10 ng/mL TGF-β1. After that, the protein levels of EMT related markers E-cadherin, N-cadherin and vimentin were detected by Western blot. The data have shown that protein levels of E-cadherin in cells of baicalein group were significantly higher than that in TGF-β1, and increased E-cadherin levels were observed with the increased baicalein concentration (*P*<0.01). While the protein levels of N-cadherin and vimentin in cells of baicalein group were significantly lower than that in TGF-β1, and decreased E-cadherin levels were observed with the increased baicalein concentration (*P*<0.01) (**Figure 3B**).

Baicalein inhibits TGF-β1-mediated activation of NF-κB/Snail signaling pathway

The effect of baicalein on TGF-β1-mediated activation of NF-κB/Snail signaling pathway were determined by detecting the protein levels of NF-κB/Snail signaling pathway related factors in BGC-823 cells after TGF-β1 and/or baicalein. Four groups of BGC-823 cells includ-

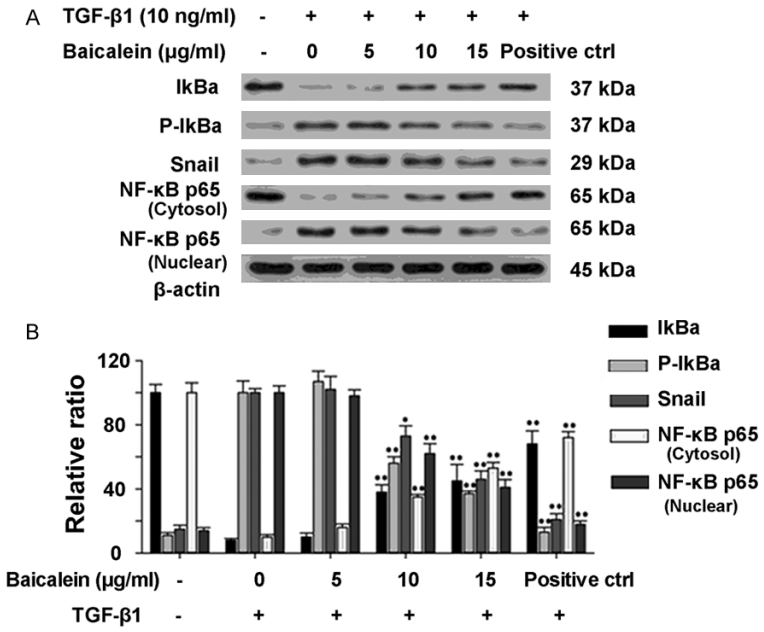

Figure 4. Baicalein inhibits TGF-β1-mediated activation of NF-κB/Snail signaling pathway. A. The protein expression levels of IkBa, p-IkBa, Snail, NF-κB p65 (cytosol) and NF-κB p65 (nuclear) were determined by Western blot. B. Quantitative analysis of protein expression levels of IkBa, p-IkBa, Snail, NF-κB p65 (cytosol) and NF-κB p65 (nuclear) using Image-Pro Plus 6.0 software and normalized to β-actin. All experiments were repeated three times. Data are means ± SD. $^{**}P<0.01$ compared with control group.

ing negative control group, TGF-β1 group, baicalein group and positive control group were used with the same treatment as described previously. We found that significantly higher protein levels of IkBa and NF-κB p65 (cytosol) were found in baicalein group compared with those in TGF-β1 (except the cells treated with 5 μg/mL baicalein, P<0.01), and increased IkBa and NF-κB p65 (cytosol) levels were observed with the increased baicalein doses (**Figure 4**). In contrast, protein levels of p-IkBa, Snail and NF-κB p65 (nuclear) were significantly lower in baicalein group than those in TGF-β1 group, and decreased p-IkBa, Snail and NF-κB p65 (nuclear) levels were observed with the increased baicalein doses (**Figure 4**).

Discussion

EMT is a series of cell biological events that can alter the interaction between cells, and cells and matrix, and eventually lead to the release of epithelial cells to surrounding tissue or distant sites [21]. Previous studies have shown that the deregulation of EMT is the main cause for many tumors of epithelial tissues [2]. Baicalein, which is one of the extracts from an herb widely used in traditional Chinese medicine, has been proved to play pivotal roles in the clinical treatment of cancer and other diseases [17-19]. In our study, baicalein was used to treat gastric cancer BGC-823 cells to investigate its roles in cancer development. Our data have shown that the proliferation, invasion and migration rates of the BGC-823 cells with baicalein treatment were significantly lower than those of the cells without baicalein treatment. In addition, the inhibitor effects of baicalein on cell proliferation, invasion and migration of BGC-823 cells were increased with the increased doses of baicalein, indicating that baicalein can inhibit the development of tumor at the stage of the proliferation, invasion and migration of tumor cells.

The occurrence and development of EMT can be triggered and regulated by a variety of differentiation and growth factors, within which TGF-β plays its roles through tyrosine kinases [10, 22]. TGF-β can bind to its receptor to active a variety of transcription factors to induce the cadherin isoform switch by down regulating E-cadherin (cell-cell adhesion molecule) and tumor suppressor [11, 12]. Consistent results were found in our study. After TGF-β1 treatment, decreased protein levels were found in E-cadherin and increased protein levels were found in vimentin and N-cadherin (mesenchymal markers) (**Figure 3**). In addition, the changes of the expression of EMT related factor became more significant with increased concentration of TGF-β1 (**Figure 3A**). However, the changes of TGF-β1 brought to the expression of E-cadherin, vimentin and N-cadherin were compensated by adding baicalein (**Figure 3B**), and increased rates of compensation were observed with increased baicalein concentration, indicating that baicalein may have the ability to interact with TGF-β pathway to suppress the occurrence and development of EMT.

With the ability of regulating various EMT related factors, NF-κB pathway has been shown to play pivotal roles in the occurrence and development of EMT. Among all the NF-κB regulated factor, Snail has been well studies to show its function to suppress Raf-kinase inhibitor (product of metastasis-suppressor gene) that inhibit two survival pathways implicated in EMT including Raf-1/MEK/ERK survival pathway and NF-κB survival pathway [13-16]. Previous studies have shown that TGF-β can interact with NF-κB survival pathway [23]. Consistent results were found in our study. TGF-β1 decreased the protein level of IkBa (NF-κB inhibitor) and increased the content of its inactivated form p-IkBa (**Figure 4**). In addition, TGF-β1 also increased the protein level of Snail and NF-κB p65 in nuclear and reduce the content of NF-κB p65 in cytosol (**Figure 4**), indicating that TGF-β1 can active NF-κB signaling pathway. However, the effects of TGF-β1 brought to the factors involved in NF-κB signaling pathway were reversed by adding baicalein (**Figure 4**), and the reverse efficiency became more significant with the increased baicalein concentration (**Figure 4**), indicating that baicalein can inhibit the TGF-β mediated activation of NF-κB/Snail signaling pathway. Therefore, further studies are still needed to further elucidate the mechanism.

Conclusion

In this study, EMT in human gastric cancer BGC-823 cells was induced by TGF-β1 treatment. Our data have shown that baicalein not only can inhibit the proliferation of BGC-823 cells but also can reduce their invasion and migration ability. Baicalein can inhibit EMT of BGC-823 cells and TGF-β1-mediated activation of NF-κB/Snail signaling pathway by regulating the expression of the related factors. Our data have shown that baicalein could inhibit EMT and suppress the invasion and migration of human gastric cancer BGC-823 cells possibly through NF-κB/Snail signaling pathway.

Disclosure of conflict of interest

None.

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