Original Article

Baicalin inhibits TGF-β1-induced epithelial-to-mesenchymal transition and suppresses pancreatic cancer cell migration and invasion

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Abstract: The epithelial-mesenchymal transition (EMT) is an important cellular process during which epithelial polarized cells become motile mesenchymal-appeared cells, which, in turn, induces the metastasis of cancer. Baicalin is one of the main bioactive flavone glucuronides derived as a medical herb from the dried roots of Scutellaria baicalensis Georgi, and has been shown to possess anticancer activity. However, no detailed studies have so far been reported on its action on human pancreatic cancer. The aim of this study was to investigate the potential functions and molecular mechanisms of baicalin as an inhibitor of the transforming growth factor-β1 (TGF-β1)-induced EMT in human pancreatic cancer cells. In this study, we demonstrated that baicalin inhibited TGF-β1-induced EMT, as well as the expression of Snail and Slug in PANC-1 cells. Baicalin also inhibits cell migration and invasion during inhibition of TGF-β1-induced EMT. Moreover, baicalin inhibits phosphorylation of Smad2 and Smad3 during inhibition of TGF-β1-induced EMT. Taken together, our results showed that baicalin inhibited the TGF-β1-induced EMT and suppressed pancreatic cancer cell migration and invasion through inhibiting Smad signal pathway. Thus, baicalin may have potential as therapeutic or supplementary agents for the treatment of pancreatic cancer.

Keywords: Baicalin, pancreatic cancer, epithelial-to-mesenchymal transition (EMT), invasion

Introduction

Pancreatic cancer is the fourth most common cause of cancer-related deaths worldwide. Despite improvements in surgical and chemotherapeutic approaches over the past decades, the average overall 5-year survival rate is less than 5% [1]. One contributor to the poor prognosis is the limited understanding of the pathogenesis of pancreatic cancer. Therefore, it is important to elucidate the molecular mechanisms associated with the occurrence, development and metastasis of pancreatic cancer.

The epithelial-mesenchymal transition (EMT) refers to the transdifferentiation of epithelial cells into mesenchymal cells under certain physiological and pathological conditions. EMT occurs in a variety of processes, such as embryonic development, wound healing, fibrosis, and early stage tumor metastasis [2-4]. It is well known that transforming growth factor-β (TGF-β) is a well-known inducer of EMT in various cancer cells [5, 6]. During TGF-β1-induced EMT, the morphological transition of epithelial cells into fibroblastoid-like or mesenchymal-like cells occurs and this results in the loss of epithelial markers such as E-cadherin and γ-catenin and the gain of mesenchymal markers such as N-cadherin and vimentin [7]. Thus, inhibiting TGF-β1-induced EMT of pancreatic cancer cells may be a therapeutic method for human pancreatic cancer.

Baicalin is one of the main bioactive flavone glucuronides derived as a medical herb from the dried roots of Scutellaria baicalensis Georgi. Baicalin has been shown to possess anti-bacterial, and anti-inflammatory and anti-oxidant properties [8-10]. It has also been shown to exert a potential for anticancer activity against various human cancers [11-13]. Shu et al. reported that baicalin significantly inhibited cell growth and colony-formation, induced cell apoptosis in human gallbladder carcinoma cells in vitro [14]. Baicalin may reduce the TGF-β1-mediated EMT, anchorage-independent growth and cell migration of human breast cancer cells.
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[15]. However, no detailed studies have so far been reported on its action on human pancreatic cancer. The aim of this study was to investigate the potential functions and molecular mechanisms of baicalin as an inhibitor of the TGF-β1-induced EMT in human pancreatic cancer cells.

Materials and methods

Cell culture

Human pancreatic cancer cell line PANC-1 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Sijiqing biochemical, Hangzhou, China) and maintained at 37°C in a 5% CO₂ atmosphere.

Cell proliferation assay

Cells were washed with serum-free RPMI-1640 medium and incubated overnight under a serum-free condition. Cells (1 × 10⁴ cells per well) were seeded in triplicate on a 96-well plate and incubated overnight before treating with baicalin (5, 10 and 20 μM) (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. After incubation, 100 ml of medium containing 0.5 mg/ml MTT (Sigma-Aldrich, St. Louis, MO, USA) was added to each well for 4 h. The medium was then removed and the formazan crystals were solubilized in dimethylsulfoxide. The mean absorbance at 570 nm in each set of samples was measured using a 96-well plate reader (Dy-natech, Chantilly, VA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from PANC-1 cells using the RNeasy Kit (Qiagen, Hilden, Germany) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and first-strand cDNA was synthesized from 10 μg total RNA using random primers and Superscript II reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA). The quantitative PCR analysis was done using an ABI PRISM 7700 Sequence Detector System and the SYBR Green PCR Master Mix kit (Applied Biosystems, Wellesley, MA), according to the manufacturer’s suggestions. The primer sequences used in this study are as follows: Snail Forward: 5’-TCGGAGCCTAAGCTAGCCTA-3’; Reverse: 5’-AGATGAGCATTGCCAGCGAG-3’; Slug Forward: 5’-CGAAGCACTAACCAACATACGTTG-3’; Reverse: 5’-CTGAGGATCTGTTGTTGATG-3’. GAPDH Forward, 5’-AGAAAGCTGGGCTCGATT-3’; Reverse: 5’-AGGGGCACTCCCAGCGCTCTC-3’. Quantitative real-time PCR data were calculated by the 2^ΔΔCT method.

Western blot

Cells were harvested using RIPA lysis buffer (Solarbio, Beijing, China) supplemented with PMSF protease inhibitor (Solarbio, Beijing, China). The protein concentrations of the samples were determined using a protein quantitation kit (Invitrogen, Carlsbad, CA, USA). In total, 30 μg of the cell lysates were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Pall). Membranes were blocked for 2 h in 5% nonfat dried milk. Membranes were incubated with specific antibodies at 4°C overnight. The following primary antibodies were used in this study: anti-N-cadherin, anti-E-cadherin, anti-vimentin, anti-Snail, anti-Slug, anti-p-Smad2, anti-Smad3, anti-Smad2 and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were then incubated with the corresponding fluorescent secondary antibodies (Odyssey). Reactive bands were visualized with enhanced chemiluminescent reagents (GE Healthcare, Piscataway, NJ).

Transwell assay

Transwell chambers (Corning, USA) with an 8-μm pore were used to assess migration in 24-well plates. After treatment with baicalin, cells at a density of 1 × 10⁵/ml in FBS-free DMEM, was seeded into the upper chambers. The lower chambers were filled with DMEM containing 10% FBS as a stimulatory factor. The chambers were incubated in a humidified tissue culture incubator at 37°C, 5% CO₂ atmosphere. After 48 h, cells on the upper side of the membrane were wiped off, the membranes were fixed with 95% ethanol for 20 min, stained by crystal violet for 30 min, and counted using a microscope with a 40 × objective.

To assess cell invasion, BioCoat Matrigel (Sigma, St. Louis, MO, USA) (300 μg/ml, 100 μl per chamber) was applied to the upper insert chambers 3 h before following the procedure for the migration assay described above.
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Statistical analysis

All experiments were carried out in triplicate and repeated independently at least three times. Data are presented as means ± SD (standard deviation). Statistical significance was assessed by the one-way analysis of variance (ANOVA). P<0.05 was considered to indicate statistical significance.

Results

Effect of baicalin on the viability of PANC-1 cells

We measured the effect of baicalin on PANC1-1 cell viability. As shown in Figure 1, we observed that concentrations of baicalin below 10 μM had no influence on cell proliferation, however, while 20 μM baicalin remarkably inhibited PANC-1 cell proliferation. Therefore, the cells were treated with selected doses (5 μM and 10 μM baicalin) in subsequent experiments.

Baicalin suppresses TGF-β1-induced EMT in PANC-1 cells

A growing body of evidence indicates that TGF-β1 significantly decreases E-cadherin expression and concomitantly increases N-cadherin and vimentin expression. So, in order to investigate the role of baicalin during the TGF-β1-induced EMT, we examined the effect of baicalin on the TGF-β1-induced EMT of PANC-1 cells. As indicated in Figure 2, TGF-β1 obviously reduced the expression of E-cadherin, and increased the expression of N-cadherin and vimentin, as compared with the control group. Whereas, baicalin significantly reversed this effect. These results suggest that baicalin suppresses TGF-β1-induced EMT in PANC-1 cells.

Baicalin suppresses TGF-β1-induced snail and slug in PANC-1 cells

It has been reported that many transcription factors, including Snail (also called Snail1), Slug (also called Snail2) and ZEB1/2 could suppress the expression of E-cadherin. Therefore, we asked whether these transcription factors could be involved in the effects of baicalin on the TGF-β1-induced EMT of PANC-1 cells. As shown in Figure 3A, treatment of PANC-1 cells with baicalin decreased the mRNA expression levels of Snail and Slug.
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Baicalin inhibits the TGF-β1-induced EMT in pancreatic cancer cells by inhibiting the expression of Snail and Slug in a concentration-dependent manner. Moreover, consistent with the results of RT-qPCR, baicalin also reduced the protein expression of Snail and Slug in PANC-1 cells (Figure 3B).

Baicalin inhibits cell migration and invasion during inhibition of TGF-β1-induced EMT

To examine the effect of baicalin on PANC-1 cell migration, we performed a Transwell assay. As shown in Figure 4A, baicalin significantly inhibited cell migration induced by TGF-β1 in a concentration-dependent manner. In addition, PANC-1 cells treated with baicalin exhibited a decrease in the number of cells migrating through the Matrigel-coated membrane in Boyden chambers, compared with untreated PANC-1 cells (Figure 4B).

Baicalin inhibits phosphorylation of Smad2 and Smad3 during inhibition of TGF-β1-induced EMT

The TGF-β1-induced EMT is mediated through Smad signaling pathways. After TGF-β1 stimulation, Smad2 and Smad3 are phosphorylated and formed Smad2/3 complex, and then induce the EMT. Therefore, we examined the effect of baicalin on the phosphorylated levels of two primary signal proteins in TGF-β1/Smad pathway-Smad2 and Smad3. As shown in Figure 5, TGF-β1 significantly promoted the phosphorylation of Smad2 and Smad3, however, baicalin suppresses TGF-β1-induced the phosphorylation of Smad2 and Smad3 in PANC-1 cells.

Discussion

In this study, we demonstrated that baicalin inhibited TGF-β1-induced EMT, as well as the expression of Snail and Slug in PANC-1 cells. Baicalin also inhibits cell migration and invasion during inhibition of TGF-β1-induced EMT. Moreover, baicalin inhibits phosphorylation of Smad2 and Smad3 during inhibition of TGF-β1-induced EMT.
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It is well known that EMT plays a critical role in tumor invasion and metastasis from in vitro and in vivo pancreatic cancer studies [16]. Several lines of evidences showed that stimulation of TGF-β1 could induce EMT in pancreatic cancer [17-19]. Upon activation of the TGF-β1 pathway, pancreatic cancer cells that exhibit more overt and irreversible EMT lead to more aggressive and metastatic tumors. Therefore, inhibition of TGF-β1-mediated EMT might be a rational strategy to prevent metastasis. It has also been reported that the forced expression of E-cadherin suppresses cancer metastasis, and an E-cadherin mutation in a tumor decreases cellular adhesion and increases cellular motility, invasion and metastasis [20, 21]. In this study, we observed that baicalin effectively upregulates the expression of EMT-associated protein E-cadherin, but downregulates the expression of N-cadherin and vimentin, all in a dose-dependent manner in PANC-1 cells. Consistent with the above-described results, baicalin also inhibits cell migration and invasion during inhibition of TGF-β1-induced EMT in PANC-1 cells, suggesting that the baicalin-mediated inhibition of EMT may inhibit the migration and invasion of pancreatic cancer cells.

Zinc finger protein snail and slug are proteins encoded by the snail gene. Snail is a family of transcription factors that promote the repression of the adhesion molecule E-cadherin to regulate EMT in the development of tumor [22]. Multiple lines of evidence show that TGF-β1 induced Snail expression during EMT development [23-25]. In this study, we observed that the EMT-related transcriptional repressors, Snail and Slug, were differentially reduced when cells were treated with baicalin. These results suggest that these transcriptional repressors are the upstream regulators of E-cadherin in the baicalin-mediated EMT.

Smad signaling pathway is activated as a function of progression in TGF-β-induced EMT, making it an attractive therapeutic target to prevent TGF-β-induced EMT and the subsequent enhance of tumor invasion [20, 26]. In terms of its activation mechanism, TGF-β1 interacts with TGF-βRII, which in turn activates TGF-βRI. Subsequently, an active TGFRI directly phosphorylates Smads, namely Smad2 and Smad3. These activated R-Smads associate with Smad4, and the complex then translocates into the nucleus. After this translocation event, the complex interacts with DNA-binding factors, including the EMT-inducing factors such as Snail, Slug, Twist and so on in order to regulate responsive genes [27-29]. Previous studies showed that the TGF-β1-mediating signaling molecule Smad2 potentiates the EMT and EMT-related genes in enhancing skin cancer aggressiveness [30]. In addition, it has been reported that genetic inactivation of individual Smads protects pancreatic cancer cells from the acquisition of a migrating mesenchymal phenotype.

Figure 5. Baicalin inhibits phosphorylation of Smad2 and Smad3 during inhibition of TGF-β1-induced EMT. PANC-1 cells were pretreated with the indicated concentration of baicalin for 2 h and then stimulated with TGF-β1 (5 ng/ml) for 24 h. A. The protein expression levels of p-Smad2 and p-Smad3 were determined by Western blot. B and C. Quantitative analysis of protein expression levels of p-Smad2 and p-Smad3 using Image-Pro Plus 6.0 software and normalized to GAPDH. All experiments were repeated at least three times. Data are means ± SD. *P<0.05 compared with control group, #P<0.05 compared with TGF-β1 group.
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[17]. In line with these results, in the present study, we observed that baicalin inhibits phosphorylation of Smad2 and Smad3 during inhibition of TGF-β1-induced EMT. These data indicate that baicalin probably prevents EMT and invasion in the TGF-β1-induced signaling pathway by inhibiting the activation of Smad2 and Smad3.

In summary, we demonstrated that baicalin inhibited the TGF-β1-induced EMT and suppressed pancreatic cancer cell migration and invasion through inhibiting Smad signal pathway. Thus, baicalin could have potential as therapeutic or supplementary agents for the treatment of pancreatic cancer.

Disclosure of conflict of interest

None.

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