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Piperlongumine inhibits TGF-β-induced epithelial-to-mesenchymal transition by modulating the expression of E-cadherin, Snail1, and Twist1

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ABSTRACT

Cancer is a life-threatening disease, and the occurrence of metastasis, which increases the lethality of primary tumors, is increasing. The epithelial-to-mesenchymal transition (EMT) is a biological process by which epithelial cells lose cell-cell adhesion properties and acquire mesenchymal properties, including motility and invasiveness. EMT is considered an early stage of metastasis; therefore, inhibiting EMT may be an effective anticancer therapy. In the present study, the antimetastatic effect of piperlongumine (PL) was assessed in human cancer cells. PL is a single component isolated from long pepper (*Piper longum*) and it has been studied for its antibacterial, anti-angiogenic, and antidiabetic activities. Migration assays (wound healing assay) and transwell invasion assays showed that PL inhibited the migration and invasion of cancer cells. Western blotting and immunofluorescence imaging showed that TGF-β upregulated the transcription factors Snail1 and Twist1 and downregulated E-cadherin, a marker of epithelial cells, inducing EMT. PL might inhibit TGF-β-induced EMT by downregulating Snail1 and Twist1 and upregulating E-cadherin in cancer cells. In summary, PL might inhibit TGF-β-induced EMT, suggesting that it is a promising anticancer agent.

1. Introduction

Cancer is a life-threatening disease that is prevalent worldwide, and it is the second leading cause of death (Siegel et al., 2015). A large number of studies have been performed to elucidate the mechanisms underlying cancer (Hanahan and Weinberg, 2011). Anticancer agent development was initially based on killing cancer cells using their characteristics and environment, which are different from those of normal cells. However, in 90% of cancer patients, the cause of death is metastasis (Sleeman et al., 2011; Steeg, 2006). Accordingly, the target of antitumor therapy is changing, with a trend toward inhibiting metastasis (Klein, 2009; Steeg, 2006). The epithelial-to-mesenchymal transition (EMT), which is a key process during metastasis, is a morphological event in which polarized epithelial cells are converted to mesenchymal cells characterized by contractility and motility (Kong et al., 2011; Lamouille et al., 2014; Thiery et al., 2009).

The idea that EMT is a process that occurs before tumor metastasis has existed for a long time. The activation of certain transcription factors induces EMT, which led many researchers to use EMT to investigate anticancer therapy. Various EMT signaling pathways and EMT-related genes have been identified, and recent research has shown that the invasiveness and metastasis of cancer cells can be suppressed by inhibiting EMT (Davis et al., 2014; Derynck et al., 2014; Ginnebaugh et al., 2014).

In the present study, we show that piperlongumine (PL), an alkaloid compound isolated from long pepper (*Piper longum*), has an inhibitory effect on TGF-β induced EMT. PL has been studied for its cytotoxic, antibacterial, antiangiogenic, antidiabetic, and antiplatelet aggregation properties (Adams et al., 2012; Aodah et al., 2016; Bezerra et al., 2013; Zheng et al., 2016). Despite extensive research on the anticancer effects of PL, there are few reports on the antimetastatic effect of PL (Chen et al., 2015; Song et al., 2016). The present study hypothesized that PL is an effective inhibitor of EMT during cancer progression, and may therefore be used an agent for epithelial tumors. Moreover, we investigate the antimetastatic effect of PL by assessing the inhibition of migration and invasion in epithelial cancer cells like MCF7 and A549, the anti-EMT properties, and the role of PL in the regulation of E-cadherin and the Snail1/Twist1 axis.

2. Material and methods

2.1. Cell culture and reagents

MCF7 and A549 cells were maintained in Dulbecco's modified

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Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/stereptomycin antibiotics. PL was purchased from Sigma-Aldrich (St. Louis, MO). The antibody β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), E-cadherin were purchased from BD Biosciences (San Jose, CA, USA), Snail1 was purchased from Cell Signaling Technology (Beverly, MA, USA), and Twist1 was purchased from Abcam (Cambridge, MA, USA).

2.2. Cell proliferation assay

All proliferation assays were based on the 3-[4,5-dimethythiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) method and 5-ethynyl-2′-deoxyuridine (EdU) assay. For MTT method, cells were seeded in a 96-well plate, 1×10^4 cells per well. After overnight culture, PL was added to the cells and further cultured for 24 h. The media was removed and DMSO was added at MTT solubilization solution. Absorbance was measured at 550 nm. The Click-iT EdU imaging Kit purchased from ThermoFisher (Waltham, MA, USA) were used to observe the proliferation of MCF7 and A549 cells. Both cells were grown in 4-chamber slides and were treated with PL (0, 0.5, 5.0 and 10.0 μM) for 24 h. Before fixation, permeabilization, and EdU staining, both cells were incubated 50 μM EdU. The cell nuclei were stained with DAPI and the cells were examined using a Leica fluorescence microscope. For colony forming assay, single-cell suspensions of 5×10^3 cells were seeded into 6-well plate and allowed to attach for 24 h at 37 ˚C in culture medium. Cells were then treated with various concentrations, 0, 0.5, 1.0, 2.0, 5.0, and 10.0 μM, of PL. After 15 days, colonies were fixed with 100% methanol for 10 min at room temperature and stained with 0.1% crystal violet. Plates were washed with PBS and were photographed.

2.3. Cell migration assay

Migration was assessed by a wound healing assay. Cells were seeded at 2×10^4 MCF-7 and A549 cells/well in 6-well plate and were cultured for 24 h. After scraping the cell monolayer with a sterile micropipette tip, the wells were washed with PBS, and treated with TGF-β (10 ng/ml) or co-treated with TGF-β (10 ng/ml) and various concentrations of PL (0.5, 1.0, 2.0, and 5.0 μM). The first image of each scratch was acquired at time zero. At 24 h, each scratch was examined and captured at the same location and the healed area was measured.

2.4. Transwell invasion assay

The invasion of tumor cells was assessed in Transwell chambers equipped with 8 μm pore size, 6.5 mm diameter polyvinylpyrrolidone-free polycarbonated membranes (Corning Costar Inc., Corning, NY, USA) that were coated with 1 mg/ml fibronectin. The cells were seed onto the upper wells at a concentration 1×10^5 MCF-7 and A549 cells/well were cultured for 24 h following treatment with TGF-β (10 ng/ml) or co-treated with TGF-β (10 ng/ml) and various concentrations of PL (0.5, 1.0, 2.0, and 5.0 μM). The bottom chambers of the Transwell were filled with conditioned medium (DMEM supplemented with 1% FBS and 1% penicillin/stereptomycin antibiotics). After incubation for 24 h, cells were fixed with 100% methanol for 10 min at room temperature, stained with 0.1% crystal violet and counted under a light microscope.

2.5. Western blotting

MCF-7 and A549 cells were treated with TGF-β (10 ng/ml) or co-treated with TGF-β (10 ng/ml) and various concentrations of PL (0.5, 1.0, 2.0, and 5.0 μM) for 24 h. After lysing cells with RIPA buffer, proteins were resolved by SDS-PAGE and immunoblotted using primary antibodies such as anti-E-cadherin (Abcam; cat. no., ab184633; dilution 1:1000), anti-Snail1 (Cell Signaling Technology, Inc.; cat. no., 3895; dilution, 1:1000), anti-Twist1 (Abcam, cat. no.; ab175430; dilution, 1:1000) and anti-β-actin (Santa Cruz Biotechnology, Inc.; cat. no., sc47778; dilution, 1:1000) for 2 h at room temperature. Subsequent to treatment with the appropriate secondary antibodies, goat anti-mouse IgG (Santa Cruz Biotechnology, Inc.; cat. no., sc2005; dilution, 1:2000) for 2 h at room temperature, the immunoreactive bands were visualized by standard ECL method.

2.6. Immunofluorescence staining

MCF7 and A549 cells were grown in 4-chamber slides in serum free media, and were treated with TGF-β (10 ng/ml) or co-treatment with TGF-β (10 ng/ml) and various concentrations of PL (0.5, 1.0, 2.0, and 5.0 μM). After 24 h incubation, cells were fixed with 4% paraformaldehyde at 4 ˚C. Cells were washed with PBS containing 0.1% BSA and incubated with anti-E-cadherin antibody for 1 h followed by 1 h incubation with fluorescence-tagged secondary antibody, then counterstained with DAPI for 5 min. Cell images were captured at 400× magnification on a Leica fluorescence microscope.

2.7. Statistical analysis

The results are presented as mean ± SE, and statistical comparisons between groups were carried out using one-way ANOVA followed by the Student's *t*-test.

3. Results

3.1. PL inhibits the growth of human cancer cell in vitro

The effect of PL on the proliferation of the human cancer cell lines MCF7 and A549 was examined first. To determine the drug concentration that causes 50% growth inhibition (IC_{50}), cells were treated with different concentrations of PL for 24 h, and cell viability was measured using the MTT assay. The IC_{50} values for both cell types were < 10.0 μM – actually, 4.68 μM for MCF7 and 7.84 μM for A549 (Fig. 1A). The long-term effects of PL were determined by culturing MCF7 and A549 cells with or without PL for 10 days and performing colony formation assays. PL inhibited colony formation in cancer cells in a dose-dependent manner. At a concentration of 5.0 μM, PL almost completely inhibited colony formation in both human cancer cell lines (Fig. 1B). In addition, we observed cell proliferation by EdU detection. As concentration of PL increased, EdU positive cells reduced, which is consistent with MTT results (Fig. 1C). According to these findings, doses of 0.5, 1.0, 2.0, and 5.0 μM PL were used for subsequent experiments.

3.2. Effect of PL on the TGF-β-induced migration of human cancer cells

TGF-β acts as a pro-oncogenic factor through the induction of EMT (Araki et al., 2011; Zavadil and Bottinger, 2005). Because EMT is associated with tumor progression, the effect of PL on TGF-β-induced EMT was assessed to determine the role of PL in cell migration. Cancer cell lines were treated with TGF-β or co-treated with TGF-β and predetermined PL concentrations (0.5, 1.0, 2.0, and 5.0 μM), and wound-healing assays were performed. TGF-β-treated cancer cells showed a ≥1.5-fold increase in migration, whereas treatment with PL inhibited TGF-β-induced migration in a dose-dependent manner. At a concentration of 5.0 μM, PL reduced TGF-β-induced migration to 13% of the TGF-b control in MCF7 cells as a fold ratio decreased from 1.52 to 0.20. In A549 cells, PL reduced TGF-β-induced migration to 29% of the TGF-b control as a fold ratio decreased from 1.57 to 0.45 (Fig. 2A and B). These data indicate that PL inhibits the migration of cancer cells during TGF-β-induced EMT.

3.3. Effect of PL on the TGF-β-induced invasion of human cancer cells

We examined whether PL inhibits the TGF-β-induced invasiveness of cancer cells. Treatment with TGF-β alone considerably increased the