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Celastrol inhibits TGF-β1-induced epithelial–mesenchymal transition by inhibiting Snail and regulating E-cadherin expression

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ABSTRACT

The epithelial–mesenchymal transition (EMT) is a pivotal event in the invasive and metastatic potentials of cancer progression. Celastrol inhibits the proliferation of a variety of tumor cells including leukemia, glioma, prostate, and breast cancer; however, the possible role of celastrol in the EMT is unclear. We investigated the effect of celastrol on the EMT. Transforming growth factor-beta 1 (TGF-β1) induced EMT-like morphologic changes and upregulation of Snail expression. The downregulation of E-cadherin expression and upregulation of Snail in Madin–Darby Canine Kidney (MDCK) and A549 cell lines show that TGF-β1-mediated the EMT in epithelial cells; however, celastrol markedly inhibited TGF-β1-induced morphologic changes, Snail upregulation, and E-cadherin expression. Migration and invasion assays revealed that celastrol completely inhibited TGF-β1-mediated cellular migration in both cell lines. These findings indicate that celastrol downregulates Snail expression, thereby inhibiting TGF-β1-induced EMT in MDCK and A549 cells. Thus, our findings provide new evidence that celastrol suppresses lung cancer invasion and migration by inhibiting TGF-β1-induced EMT.

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1. Introduction

The epithelial–mesenchymal transition (EMT), an important morphological event where polarized epithelial cells convert to contractile and motile mesenchymal cells, is recognized as an important process during embryonic development and tissue organization. EMT also plays a critical role in cancer invasion and metastasis. EMT induction is characterized by cell–cell junction dissolution, cytoskeletal rearrangement, increased cell motility, and synthesis of extracellular matrix [1]. One protein prominently associated with EMT is the epithelial cell adhesion molecule E-cadherin. E-cadherin is a cell–cell adhesion molecule and the loss of its expression is a hallmark of EMT. Reduction of E-cadherin increased cell mobility and promoted tumor cell invasion. Several transcriptional repressors of E-cadherin have been identified, including the zinc finger factors Snail and Slug, and the two-handed zinc factor ZEB1 [2,3]. Snail and Slug, a related Snail superfamily member, mediate E-cadherin repression and are overexpressed in epithelial cell lines during EMT [4–6]. Correlative studies have demonstrated an inverse relationship between E-cadherin and Snail expression in human samples [7].

Transforming growth factor-beta 1 (TGF-β1) is a multifunctional cytokine that regulates a wide range of cellular functions, including tissue morphogenesis, differentiation, and extracellular matrix remodeling [8]. TGF-β1-stimulated cells become spindle-shaped and undergo morphological changes, such as a decrease in cell–cell adhesion and loss of structural polarity [9]. Recent studies have revealed that TGF-β1 functions as a pro-oncogenic factor through induction of EMT; TGF-β1-induced EMT in a variety of cells is mediated by the Snail signaling pathway. Therefore, regulation of Snail expression plays a crucial role in EMT induction via TGF-β1 signaling [10].

Celastrol was identified from the traditional Chinese medicine "God of Thunder Vine" or *Tripterygium wilfordii* Hook F. almost 3 decades ago and has been used to treat cancer and other inflammatory diseases [11]. Various studies have indicated that celastrol exhibits anticancer potential and eradicates leukemia stem cells [12]. It suppresses the production of inflammatory cytokines such as interleukin-1 (IL-1), TNF-α, IL-6, and IL-8, induces the heat shock response, and disrupts heat shock protein 90 (Hsp90), possibly through its interaction with cdc37 and co-chaperone p23 [13–15]. Celastrol also inhibits NF-κB activation and arrests the cell cycle [15–18]. The molecular mechanism underlying the invasion effects of celastrol is not fully understood in TGF-β1-activated epithelial cells.

In this study, we investigated the effects of celastrol on TGF-β1-induced Snail expression in epithelial cells and explored the underlying downstream signaling mechanism. We found that celastrol

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reduced invasion of epithelial cells by inhibiting EMT through the suppression of Snail and E-cadherin expression in TGF-β1-activated MDCK and A549 cells.

2. Materials and methods

2.1. Cells and reagents

MDCK and A549 cells were maintained in DMEM supplemented with 10% heat-inactivated FBS. Lipofectamine 2000 reagent was purchased from Invitrogen (Carlsbad, CA). Anti-E-cadherin, and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Snail antibody was from Cell signaling (Beverly, MA). TGF-β1 was purchased from Calbiochem (San Diego, CA). All the chemicals not included above were from Sigma.

2.2. Cell proliferation and viability assay

All proliferation and viability assays were based on the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Cells were seeded in a 96-well plate at a density of 1×10^4 cells/well. The cells were treated with various concentration of celastrol and allowed to grow for 48 h. At the end of the experiment, the media was removed and DMSO was added with MTT solubilization solution. Absorbance was measured at 550 nm.

2.3. Western blot analysis

Cell lysates were separated on 10% SDS–polyacrylamide gels and transferred to nitrocellulose membranes. The blots were incubated with anti-E-cadherin and anti-Snail antibodies and developed with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech., Piscataway, NJ). The same blot was stripped and reprobed with anti- β-actin antibody for use as an internal control.

2.4. RNA extraction and semi-quantitative RT-PCR

Total RNA was extracted from cells with Trizol (Invitrogen) according to the manufacturer's protocol. Approximately 1 μg of total RNA was used to prepare cDNA using the Superscript First Strand cDNA synthesis Kit (Bioneer, Daejeon, South Korea). The following primers were used in this study: 5′-AACATCCTCAGCCAA-GATCC-3′ and 5′-GCACCTGACCCTTGTACGTG-3′ for E-cadherin; 5′-TCTAGGCCCTGGCTGCTACAA-3′ and 5′-ACATCTGAGTGGGTCTGG AGGTG-3′ for Snail; 5′-CCATCACCATCTTCCAGGAG-3′ and 5′-CCTGCTTCACCACGTTCTTG-3′ for GAPDH. PCR was performed with Platinum Taq polymerase (Invitrogen) under the following conditions: 30 cycles of 96 °C for 40 s, 55 °C (E-cadherin and Snail) or 60 °C (GAPDH) for 40 s, and 72 °C for 1 min followed by 10 min at 72 °C. All the PCR reactions were repeated at least three times. GAPDH was amplified as an internal control. The intensity of each band amplified by RT-PCR was analyzed using MultiImageTM Light Cabinet (version 5.5, Alpha Innotech Corp., San Leandro, CA).

2.5. Immunofluorescent staining

MDCK or A549 cells were treated with or without 1 μM celastrol for 30 min and then incubated with TGF for 72 h. Cells were fixed with 4% paraformaldehyde in PBS at room temperature for 10 min. Nonspecific sites were blocked by incubating with 200 μl of 1% BSA in PBS at 37 °C for 15 min. A rabbit polyclonal antibody against E-cadherin was diluted 1:200 in PBS containing 1% BSA and incubated with the coverslips at 37 °C for 1 h. Cells were then washed with 1% BSA/PBS for 10 min at room temperature before incubating with a 1:200 dilution of FITC-labeled goat anti-rabbit IgG antibody at room temperature for 45 min, and then the coverslips were rinsed with a 1% BSA/PBS solution for 10 min. Then the cells were stained with 4,6-diamidino-2-phenylindole (DAPI) for another 1 min at room temperature. The coverslips containing the cells were then mounted with AquaMount (Lerner Laboratories, New Haven, CT) containing 0.01% 1,4-diazobicyclo(2,2,2)octane.

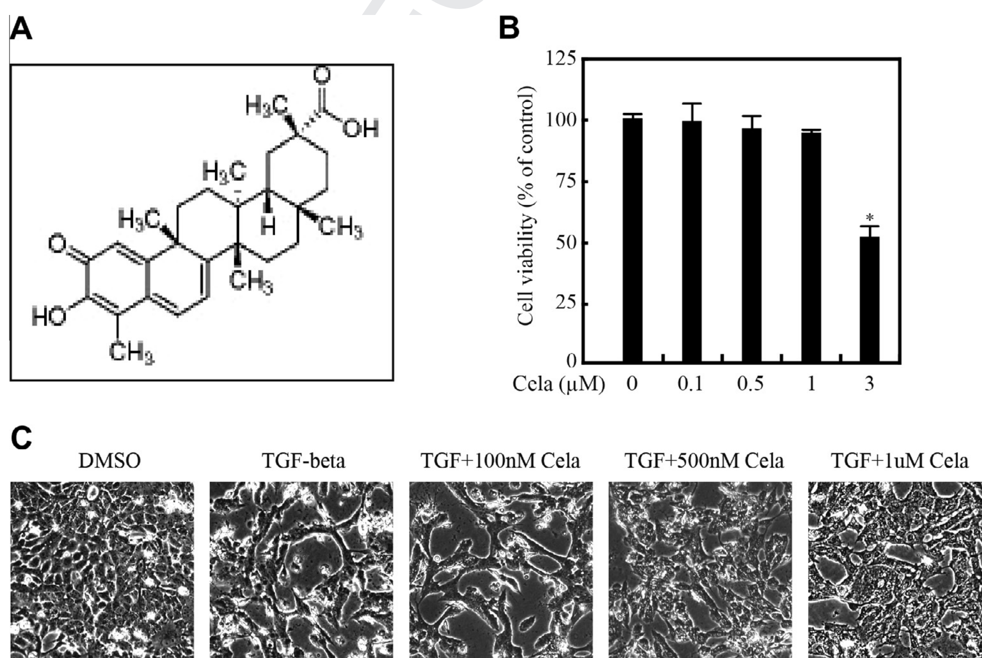

Fig. 1. Celastrol inhibits TGF-β1-induced morphological changes in MDCK cells. (A) Chemical structure of celastrol. (B) MDCK cells were treated with 0–3 μM celastrol for 48 h and cell viability was measured by MMT assay; *$P < 0.05$ versus vehicle. (C) MDCK cells were pretreated with the indicated concentration of celastrol for 30 min and then stimulated with 5 ng/ml TGF-β1 for 72 h. TGF-β1 treatment induces cell elongation and increases scattering, while celastrol inhibits the activation of these processes in a dose-dependent manner. Scale bar 20 μm, magnification 40×.

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