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Research paper

Effect of Rapamycin on TGF-β_1-induced epithelial-mesenchymal transition in LoVo colonic adenocarcinoma cells ☆

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Abstract

Objective: To investigate the effect of Rapamycin on epithelial-mesenchymal transition(EMT) of LoVo colonic adenocarcinoma cells *in vitro*. **Methods:**Cultured LoVo colonic adenocarcinoma cells were divided into three groups: negative control group, EMT-inducing group(TGF-β_1) and EMT-interfering group(TGF-β_1 plus Rapamycin). E-cadherin expression in LoVo cells was detected by Western Blot, while the expression of vimentin was evaluated through immunocytochemistry. The Snail mRNA in LoVo cells was examined by RT-PCR. **Results:**TGF-β_1 induced LoVo cell switching from polygonal to spindle-shaped. TGF-β_1 enhanced the expression of vimentin, but lowered the level of E-cadherin. In contrast, Rapamycin impaired the transition induced by TGF-β_1. Rapamycin dramatically abrogated TGF-β_1-induced vimentin expression and restored E-cadherin expression in LoVo cells. Rapamycin significantly repressed the up-regulation of Snail mRNA expression induced by TGF-β_1. **Conclusion:**Rapamycin dramatically abrogated TGF-β_1 induced Snail mRNA expression in LoVo cells, hence inhibiting EMT of these cells *in vitro*.

Key words: epithelial-mesenchymal transition(EMT); Rapamycin; TGF-β_1; Snail

INTRODUCTION

Epithelial-mesenchymal transition(EMT) is a highly conserved process that has been well characterized in embryogenesis. During EMT, epithelial cells acquire fibroblast-like properties and exhibit reduced cell-cell adhesion and increased motility[1]. In pathological processes, such as oncogenesis, EMT may endow cancer cells with enhanced motility and invasiveness. Indeed, oncogenic transformation may be associated with signaling pathways promoting EMT[2].

The mechanism of EMT remains to be fully elucidated. Recently,activation of the phosphatidylinositol 3-kinase (PI3K)/AKT axis has emerged as a central feature of EMT[3]. Rapamycin, a specific inhibitor of mTOR, blocks the PI3K/Akt-mTOR pathway, and subsequently interfered with events related to that pathway[4]. Recent research showed Rapamycin could inhibit the EMT in tubular cells *in vitro* and plays a key role in preventing renal interstitial fibrosis[5]. Considering this evidence for Rapamycin in EMT, we addressed the question of whether Rapamycin could inhibit the process of EMT induced by TGF-β_1 in colorectal cancer cells.

MATERIALS AND METHODS

Cell Lines

The LoVo colonic adenocarcinoma cell line was purchased from the China center for type culture collection(Wuhan, China), and maintained in DMEM medium supplemented with 10% fetal calf serum and 1% streptomycin-penicillin(Gibco, USA), incubated at 37℃ in a humidified atmosphere containing 5% CO_2. Rapamycin(Alexis,USA) was dissolved in dimethylsulfoxide(Sigma,USA). Dimethylsulfoxide was added to the control group.

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Cell Viability

Antiproliferative effect of Rapamycin was evaluated using a modified tetrazolium salt, MTT(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay(Sigma, USA). Briefly, 1×10^4 viable cells were plated in 200 μl growth medium in 96 well plates. After 24 h, cells were treated with different concentrations of Rapamycin for 48 and 72 h. Then, 20 μl MTT was added to each well and incubated for 4 h at 37℃. The media was then removed and reduced MTT product was solubilized by adding 150 μl DMSO. The absorbance was measured at 570 nm using a microplate reader (Bio-Rad,Japan). Experiments were performed in triplicates.

RNA Isolation and RT-CR

RNA isolation was done using the Trizol reagent Kit (TaKaRa, Japan) according to the manufacturer's recommendations. Gene transcription of *GAPDH*, *Snail* was analyzed by a two-step reverse transcription-RCR. One μl(2 μg) of the cDNA was used as a template for the specific PCR reactions. Primers applied were *GAPDH*: forward 5′-GGG GAG CCA AAA GGG TCA TC-3′, reverse 5′-GCC AGT GAG CTT CCC GTT CAG-3′ (350 bp); *Snail*: forward 5′-ATC CAG TTT GGT GTC GCG GAG C-3′, reverse 5′-GAA GGG GAA GAC GCA CAG CT-3′ (763 bp). Cycling conditions of the respective PCRs were as follows: initial denaturation(4 minutes at 95℃) followed by the 30 cycles of denaturation (1 minute at 94℃), annealing (30 seconds; at 62℃) and elongation(1 minute at 72℃). Five μl of the products were run on a 2% agarose gel, stained with ethidium bromide and analyzed under UV light.

Western blot analysis

To characterize EMT in LoVo cells the protein level of E-cadherin was detected by Western blot. Cells were lysed in buffer containing 50 mM HEPES(pH 7.6), 150 mM NaCl, 1% Triton X-100, 2 mM vanadate, 100 mM NaF, and 0.40 mg/ml phenylmethylsulfonyl fluoride. Equal amounts of protein(25 μg/lane) were subjected to 10% SDS-PAGE gel and transferred to nitrocellulose membranes. Membranes were blocked with 5% w/v nonfat dry milk in 0.05% Tween 20/phosphate-buffered saline and then incubated with rabbit anti-human E-cadherin primary antibody(1:100) (Boster, China) at 4℃ overnight. Membranes were then washed and incubated with goat anti-rabbit polyclonal antibody(Boster, China) conjugated with horseradish peroxidase. Bands were visualized by using the enhanced chemiluminescence(ECL) (Pierce, USA) Western blotting detection system.

Immunofluorescence microscopy

Cells were fixed with 4% paraformaldehyde(PFA) for 30 min, permeabilized in 2% PFA and 0.2% Triton X-100 for 10 min, and incubated in a PBS and 5% BSA blocking solution for 1 h. The slides were incubated for 2 h with mouse anti-human vimentin primary antibody(Boster, China) diluted(1:100) in PBS and 3% BSA, and then were stained for 1 h with FITC-conjugated goat anti-mouse polyclonal antibody(Boster, China)(1:500) to visualize vimentin filaments. The slides were incubated with hematoxylin for 1 min to stain nuclei. After mounting the slides, the cells were viewed at room temperature by fluorescence microscopy.

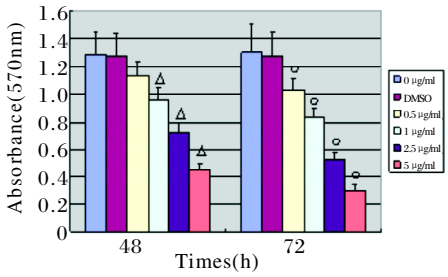
Statistical analysis

For statistical analysis, *SPSS* 12.0 was used. Means and standard deviations are given throughout and group means were compared using Student's *t* tests. *P* values < 0.05 were considered to be statistically significant.

RESULTS

Effect of Rapamycin on Lovo cell viability

The anti-proliferative effect of Rapamycin was determined at concentrations ranging from 0.5 μg/ml to 5 μg/ml(***Fig.1***). After 48 h exposure there was no significant difference in LoVo cell proliferation between untreated and vehicle control(0.1% DMSO) treated cells. Rapamycin had a concentration-dependent anti-proliferative effect, but the cell viability in the 0.5 μg/ml group was not statistically different($P > 0.05$) from the vehicle control group.



△ $P < 0.05$, *vs* control, 48 h; ○ $P < 0.05$, *vs* control, 72 h

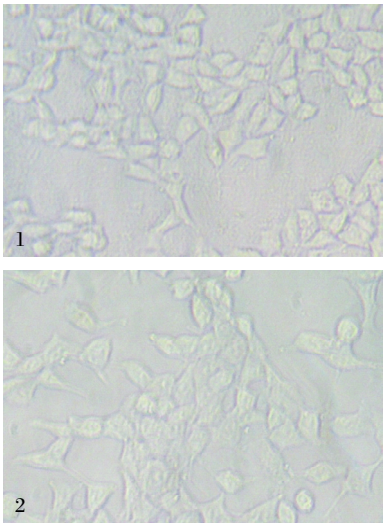
Fig. 1 Anti-proliferative activity of Rapamycin in LoVo cells

Effect of Rapamycin on TGF-β_1-induced cell morphology changes

As evident in ***Fig. 1***, treatment with Rapamycin at higher concentrations(> 0.5 μg/ml) for 48 h resulted in considerable cytotoxicity, hence concentrations of 0.5 μg/ml and under were selected for the further studies.

To characterize EMT in LoVo cells, we used microscopy to observe morphological transformation after TGF-β_1 exposure. LoVo cells undergo EMT at 48 h after 10 ng/ml TGF-β_1 treatment. As a result of EMT, the cells changed their shape from a cuboidal to a more

elongated form. ***Fig. 2*** shows that 0.5 μg/ml Rapamycin inhibited TGF-β_1-induced mesenchymal transition in LoVo cells, and the cells retained the original shape.



1.TGF-β_1(10 ng/ml); 2.TGF-β_1(10 ng/ml)+RPM(0.5 μg/ml).

Fig. 2 Effect of Rapamycin on LoVo cell morphology(×100)

Effect of Rapamycin on the TGF-β_1-induced EMT phenotype

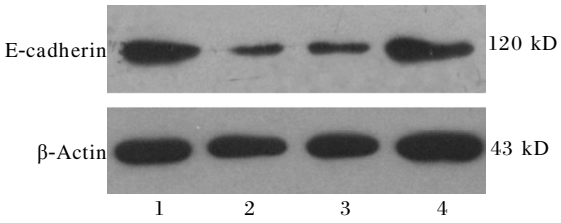
The morphological changes characteristic of cells undergoing EMT were accompanied by a shift in gene expression from an epithelial to a mesenchymal repertoire. To determine whether EMT-associated molecular alterations had occurred in LoVo cells, we detected the expression of epithelial and mesenchymal markers.

In comparison with the control group, TGF-β_1 repressed the expression of E-cadherin as assessed by Western blot analysis(***Fig. 3***). Rapamycin counteracted the repression of E-cadherin induced by TGF-β_1. The relative expression level of E-cadherin in 0.5 μg/ml Rapamycin was 4.7 fold compared with TGF-β_1 group ($P<0.05$). Next, we evaluated the expression of vimentin, a mesenchymal marker, by immunofluorescence. Differential vimentin expressions in LoVo cells were observed upon treatment with TGF-β_1 in the presence or absence of Rapamycin(***Fig. 4***). The untreated cell did not produce vimentin, whereas TGF-β_1 induced the LoVo cells to produce vimentin filaments. Rapamycin impaired the ability of TGF-β_1 to induce vimentin expression.

Taken together, these data indicated that Rapamycin reversed TGF-β_1-induced EMT, in association with repression of vimentin, and regained expression of endogenous E-cadherin.

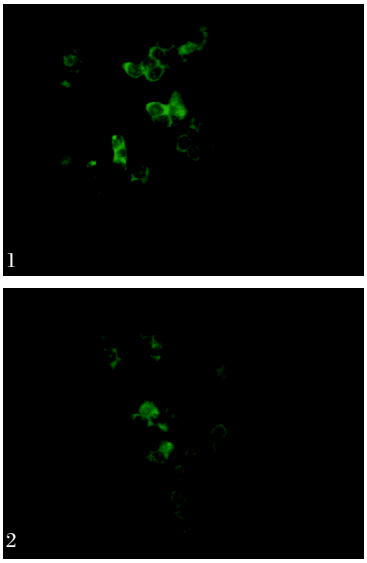
Effect of Rapamycin on the TGF-β_1-induced Snail mRNA experession

The induction of snail gene is an important mechanism



1.Control group;2.TGF-β_1(10 ng/ml);3.TGF-β_1(10 ng/ml)+RPM (0.1 μg/ml); 4.TGF-β_1(10 ng/ml)+RPM(0.5 μg/ml).

Fig. 3 Effect of Rapamycin on the expression of E-cadherin induced by TGF-β_1

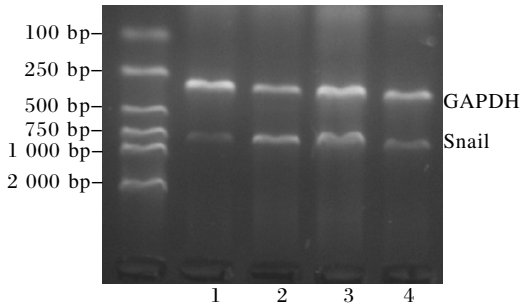


1.TGF-β_1(10 ng/ml); 2.TGF-β_1(10 ng/ml)+RPM(0.5 μg/ml).

Fig. 4 Effect of Rapamycin on the expression of vimentin induced by TGF-β_1

for inducing EMT. To determine whether Rapamycin could have an effect on the expression of snail induced by TGF-β_1, we analyzed the expression of snail mRNA in LoVo cells.

As shown in ***Fig. 5***, TGF-β_1 up-regulates Snail mRNA in LoVo cells. Rapamycin treatment partially reversed the TGF-β_1-induced upregulation of Snail mRNA, exposure to 0.5 μg/ml Rapamycin cut the relative expression level of Snail mRNA by 44.7%($P<0.05$).



1.Control group; 2.TGF-β_1(10 ng/ml); 3.TGF-β_1(10 ng/ml) +RPM(0.1 μg/ml); 4.TGF-β_1(10 ng/ml)+RPM(0.5 μg/ml).

Fig. 5 Effect of Rapamycin on the expression of Snail mRNA induced by TGF-β_1

DISCUSSION

Cancer metastases, rather than primary tumors, are responsible for most cancer deaths[6]. One important insight into metastases is that cancer cells increase their motility and invasiveness in a manner reminiscent of the epithelial-mesenchymal transition(EMT) that occurs during embryonic development and oncogenesis. In the process of EMT, epithelial cells undergo a phenotypic switch to form mesenchymal cells that are similar in appearance to fibroblasts. The change in cell type results in the loss of polarity and also the loss of tight intercellular adhesions maintained by epithelial cells *via* adherens junctions. This is thought to allow dynamic cellular migration and increase embryogenic diversity[7].

The factor most capable of inducing and completing EMT is transforming growth factor(TGF) β[8,9]. The mechanism of EMT remains to be fully elucidated. Grille[10] and Bakin[11] indicated that an activation of the phosphatidylinositol 3-kinase(PI3K)/AKT axis was emerging as a central feature of EMT. They reported that squamous cell carcinoma lines engineered to constitutively express active Akt underwent EMT, characterized by down-regulation of the epithelial markers desmoplakin and E-cadherin, and up-regulation of the mesenchymal marker vimentin. The cells lost epithelial cell morphology and acquired fibroblast-like properties. They constitutively expressed active Akt, exhibited reduced cell-cell adhesion, increased motility on fibronectin-coated surfaces and increased invasiveness in animals. The authors believed that the AKT-driven EMT may confer the motility required for tissue invasion and metastasis. Future therapies based on AKT inhibition may complement conventional treatments by controlling tumor cell invasion and metastasis.

The mammalian target of Rapamycin, mTOR, is a serine/threonine protein kinase that belongs to the phosphoinositide 3-kinase(PI3K)-related kinase family. mTOR presents an attractive therapeutic target in the PI3K/Akt pathway because it acts downstream of broader functional groups of upstream proteins. Rapamycin binds the 12-kDa immunophilin FK506-binding protein (FKBP12) and forms a complex that inhibits mTOR[12,13]. Copeland reported that Rapamycin could inhibit EMT in tubular cells *in vitro* and plays a significant role in preventing renal interstitial fibrosis[5]. Considering this evidence for Rapamycin in EMT, the question of whether Rapamycin could inhibit the process of EMT induced by TGF-β_1 in colorectal cancer cells is worth further discussion. In the present research, we showed that LoVo cells underwent changes in cell shape, switching from polygonal to spindle-shaped forms after treatment with TGF-β_1(10 ng/ml) for 48 h. In contrast, 0.5 μg/ml Rapamycin inhibited the TGF-β_1-induced mesenchymal transition in LoVo cells, and the cells retained their epithelial morphology.

The morphological changes characteristic of cells undergoing EMT was accompanied by a shift in gene expression from an epithelial to a mesenchymal repertoire. To determine whether Rapamycin affected such a shift, we examined the expression of EMT-related molecular markers, which include E-cadherin, an epithelial marker, and vimentin, a mesenchymal marker. A critical molecular feature of EMT is the downregulation of E-cadherin, a cell adhesion molecule present in the plasma membrane of most normal epithelial cells. E-cadherin is required for the formation of stable adherens junctions and thus the maintenance of an epithelial phenotype. Previous research that silenced the expression of E-cadherin not only showed a morphological shift from an epithelial to a fibroblastoid phenotype, characteristic of EMT, but also a concomitant increase in invasive cell behaviour[14]. Reduced expression of E-cadherin has been reported in colorectal cancers, being associated with tumor progression and metastasis[15-17]. In our research, cells treated with 10 ng/ml TGF-β_1 showed a lower level E-cadherin, Rapamycin restored the expression of E-cadherin down-regulated by TGF-β_1 in LoVo cells. LoVo cells did not produce vimentin, whereas, TGF-β_1 induced this cell line to produce vimentin filaments, and Rapamycin dramatically abrogated the vimentin expression.

Finally, we determined whether Rapamycin affected the expression of Snail mRNA induced by TGF-β_1. The transcriptional factor Snail is a zinc-finger protein originally shown to be involved in mesoderm formation. Snail expression is inversely correlated with E-cadherin transcription. Snail has been described to be a direct repressor of E-cadherin in vitro and in vivo through an interaction of their COOH-terminal region with a 5-CACCTG-3 sequence in the E-cadherin promoter. Both have been suggested to be involved in the acquisition of resistance to apoptosis, thereby promoting tumor survival[18,19]. Analysis of Snail in human CRC has shown that 78% of the tumor samples examined overexpressed this protein[20]. Our experiments showed that Rapamycin dramatically abrogated TGF-β_1 induced Snail mRNA expression in LoVo cells, hence inhibiting EMT of these cells *in vitro*.

In conclusion, our studies demonstrated that the Rapamycin reversed TGF-β_1-induced EMT, in association with repression of vimentin, and a regained expression of endogenous E-cadherin, and subsequently inhibited the up-regulation of the invasive ability induced by TGF-β_1. It is suggested that Rapamycin is a valuable agent in the prevention and cure of tumor

metastases.

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