Effects of Retinoic Acid on the β-catenin/TCF Pathway in Cultured Porcine Tracheobronchial Epithelial Cells*

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Summary: The effects of retinoic acid on the β-catenin/TCF pathway in cultured porcine tracheobronchial epithelial cells (TBEC) were investigated. After TBEC were treated with retinoic acid at various concentrations, mRNA and protein changes of β-catenin in cytoplasm, nucleus and whole cell of the TBEC were observed by immunocytochemical stain, RT-PCR and Western blotting. And the changes of the target gene cyclinD1 of β-catenin/TCF pathway were also observed. It was found that there was no significant difference in β-cat mRNA level after retinoic acid treatment. However, the expression of β-catenin in the whole cell and cytoplasm was elevated with the increase of retinoic acid concentration ($P<0.01$). The nuclear protein β-catenin and target gene cyclinD1 of β-catenin/TCF pathway was decreased ($P<0.05$). It was indicated that retinoic acid could increase β-catenin level of the whole cell protein and decrease nuclear β-catenin, downregulating β-cat/TCF signaling activity and reducing target gene cyclinD1 protein level. As a result, retinoic acid can downregulate β-catenin/TCF pathway in porcine tracheobronchial epithelial cell, suggesting that retinoic acid can inhibit the proliferation and accelerate differentiation of tracheobronchial epithelial cells.
Key words: β-catenin; retinoic acid; cyclinD1; tracheobronchial epithelial cells

Retinoic acids (RA) and their derivatives play an important role in the process of embryogenesis and repair of the lung. RA is the ligand of the RA receptor (RAR) and the retinoid X receptor (RXR) and can respectively bind with them as transcriptional factors to regulate cell differentiation. β-catenin (β-cat) can bind with RAR to regulate downstream gene expression. β-cat is a key mediator in wnt signaling. When wnt signaling is activated, the phosphorylation of β-cat can be inhibited, then β-cat is increased in cytoplasm, leading to β-cat entering the nucleus binding with TCF activating target gene cylinD1, which can regulate cell cycle and promote cell proliferation. Therefore, β-cat is coactive protein within wnt signaling system and retinoid receptor signaling system influencing embryogenesis, cell proliferation and carcinogenesis and so on. In this study, we investigated the expression of β-cat protein and mRNA level in trancheobronchial epithelial cells (TBEC) after RA treatment by immunocytochemistry, Western blotting and reverse transcription-polymerase chain reaction (RT-PCR) in order to study the effects and regulative mechanisms of RA on β-cat/TCF pathway in cell proliferation and differentiation.

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1 MATERIALS AND METHODS

1.1 Main Reagents

DMEM/F-12 culture medium (1 : 1), Transferrin and TRIZOL were from GIBCO (USA). Insulin, epidermal growth factor (EGF), hydrocortisol, RA and bovine serum albumin were from Sigma (USA). Anti-β-catenin monoclonal antibody and Anti-cyclinD1 monoclonal antibody were from Santa Cruse (USA).

1.2 Isolation and Culture of Porcine Airway Epithelial Cells

Trachea and bronchus were obtained from the freshly slaughtered pig. A modified method of Wu *et al* [12] for isolating cells has been described previously. About $10^6/cm^2$ were plated in culture flasks coated with rat tail collagen. The culture medium was DMEM/F-12 supplemented with 5 μg/ml insulin, 10 μg/ml transferrin, 0.5 μg/ml hydrocortisol, 10 ng/ml epidermal growth factor (EGF), and antibiotics (100 IU/ml penicillin, 100 μg /ml streptomycin). The primary cells were round, after 2—3 days, cell confluent, showing conspicuously tilt appearance. The cells at the first passage were used in experiment. The epithelial cells were identified by anti-vimentin and anti-cytokeratin protein.

1.3 Experiment Treatment and Grouping

The experiment was divided into three groups: control group and two experimental groups with addition of 1 μg/μl and 2 μg/μl RA into culture medium respectively. After RA treatment for 12 h and 24 h, the cells were isolated.

1.4 Immunocytochemistry

In each group, the slides were fixed with cold

acetone and incubated with a monocolonal anti-β-catenin (1 : 500) overnight at 4 ℃ and a secondary antibody, then detected with DAB kit for colour development. The nuclei were counterstained with Heamtoxylin. In negative controls, PBS was used to replace primary antibody.

1.5 RT-PCR Analysis

Total RNA was isolated by using TRIZOL reagent. Reverse transcription was performed with M-MLV RT according to the manufacturer's instructions. PCR primers for β-catenin were picked up based on the published sequence and devised by Shanghai Shenyou Company. The forward primer was 5'-TCC GTA GTA AAG GCG AAC-3', and backward primer 5'-TGG ACC ACA AGC CGA GTA-3', with a 480bp fragment. The amplification procedures were 40 cycles of 94 ℃ for 3min, 94 ℃ for 30 s, 55 ℃ for 30 s, 72 ℃ for 30 s, 72 ℃ for 3 min. β-actin cDNA was amplified as internal control using following primers: forward: 5'-GGC TAC AGC TTC ACC ACC AC-3', and backward: 5'-TAC TCC TGC TTG CTG ATC CAC-3', with a 498bp fragment product. The amplification procedures were 20 cycles of 94 °C for 30 s; 55 °C for 30 s; 72 °C for 40 s.

1.6 Western blotting Analysis

Cells were lysed in lysis buffer (50 mmol/L Tris-HCl, pH 8.0, 100 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT, 1 % Triton X-100, 0.1 % SDS, 50 mmol/L NaF, 1 mmol/L $NaVO_3$) plus protein inhibitors (10 μg/ml each of aprotinin, leupeptin, pepstatin and 1 mmol/L PMSF) to obtain whole cell protein. And cells were lysed according to the method of NE-PER™ Nuclear and Cytoplasmic Extraction Reagents to obtain nuclear and cytoplasmic protein. Lysate was cleared by centralization and protein concentration determined by BCA kit. Equal amount of protein (50—75 μg) from each sample were subjected to SDS-PAGE (7.5 % and 12 % gel), transferred to nitrocellulose membrane. The membrane was incubated with anti-beta-catenin and cyclinD1 antibody (1 : 1000; 1 : 200) overnight, then incubated with horseradish perosidase-conjugated secondary antibody (1 : 10 000) for 1 h, then developed with ECL kit.

1.7 Statistical Analysis

Data were expressed as $\bar{x} \pm s$. Difference was considered significant when $P<0.05$ as determined by Student's t-test (SPSS).

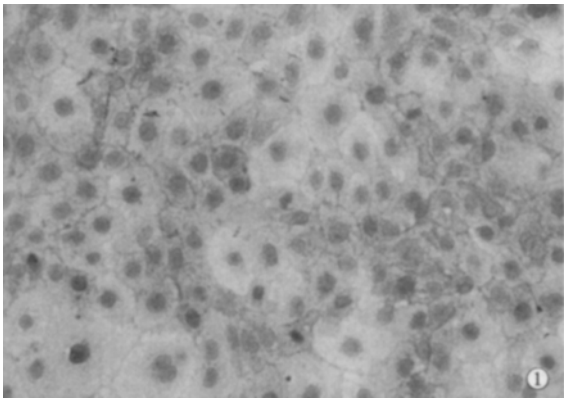
2 Results

2.1 Morphologic changes of TBEC

Under phase contrast microscopy, TBEC fused and connected tightly, showing conspicuously tilt appearance. TBEC was diffusely distributed throughout the cytoplasm for cytokeratin protein and was completely negative for vimentin protein. It was concluded that 99% cultured cells were airway epithelial cells. After TBEC were treated with 1 μg/μl and 2 μg/μl retinoic acid, vacuoles in the cytoplasm were increased, and confluence was delayed, but no detachment was observed.

2.2 Immunocytochemistry results

In control group, TBEC had β-catenin stained granular brown-yellow color at cellular membrane and cytoplasm (fig. 1). After 2μg/μl RA treatment, TBEC express higher levels of β-catenin at the membrane and cytoplasm, especially at the membrane (fig. 2).



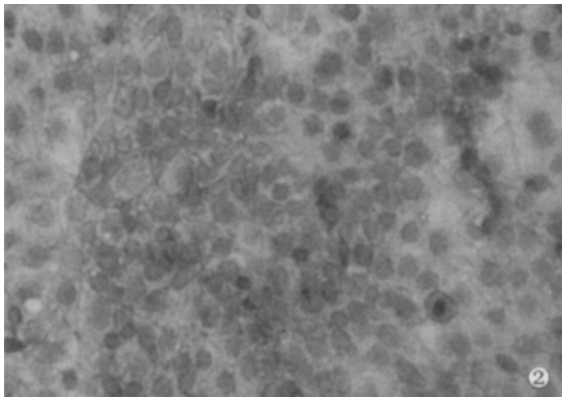


Fig. 1 In control group, TBEC showed linear brown-yellow color at the membrane and granule brown-yellow color in the cytoplasm (SABC×200)

Fig. 2 After RA treatment, the expression of β-catenin at the membrane and cytoplasm increased significantly, especially at the membrane (SABC×200)

2.3 RT-PCR results

Total RNA were respectively extracted after TBEC were treated with RA at various concentrations for 12 h and 24 h (repeat for many times). The PCR products were analysed by Gel analystic system. The ratio of the average absorbency (A) between β-actin and β-catenin was analyzed by variance. The results indicated that the expression of β-catenin mRNA level had no difference after RA treatment ($P>0.05$, fig. 3).

2.4 Western blotting results

β-catein protein levels in whole cell, cytoplasm

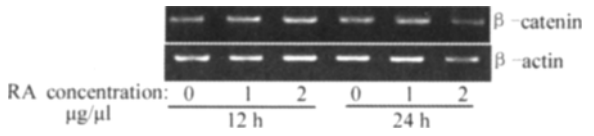


Fig. 3 After RA treatment, β-catein mRNA level had no difference among various groups ($P > 0.05$)

and nucleus were respectively observed after TBEC were treated with RA at various concentrations for 12 h and 24 h. The increased β-catenin protein levels in the whole cell and cytoplasm were observed with the increased retinoic acid concentration, whereas β-catenin and cyclinD1 protein levels in the nucleus were markedly decreased after RA treatment (fig. 4-7).

Fig. 4 The whole cell β-catenin protein increased with the increased RA concentration

Fig. 5 The cytoplasmic β-catenin protein increased with the increased RA concentration

Fig. 6 The nuclear β-catenin protein decreased with the increased RA concentration

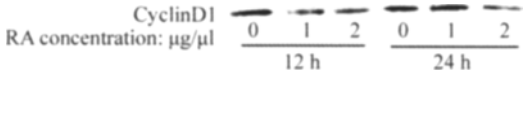


Fig. 7 CyclinD1 protein level decreased after RA treatment

3 Discussion

The β-catenin protein is an important component of β- cat/E-cd complex to maintain strong cell-cell adhesion and tissue integrity in epithelium. The cytoplasmic β-catenin protein level is eliminated by adenomatous polyposis coli(APC)-dependent proteasomal degradation patheways regulated phosphorylately by glycogen synthase kinase 3β (GSK3β). And the β-catenin protein is also a component in wnt signaling, when wnt activating, the phosphorylation of β-catenin could be inhibited. Then the cytoplasmic β-catenin protein will translocate to the nucleus, where it interacts with TCF/LEF to activate target genes involved in embryogenesis and oncogenesis. Several genes have been demonstrated to be activated by the β-cat/TCF complex are c-myc, cyclinD1 and PPARδ, which take part in cell cycle and cell proliferation. RA and their derivatives play an important role in the process of embryogenesis and repair of the lung. RA is the ligand of the RA receptor (RAR) and the retinoid X receptor (RXR) and can respectively bind with them as transcriptional factors to regulate cell differentiation.

In this study, there was no significant difference in the β-cat mRNA level after RA treatment between experimental groups and control groups, but β-catenin protein level in the whole cell and cytoplasm was increased with the increased RA concentration. It could be concluded that the increased β-catenin protein level that occurred after RA treatment could be the result of decreased degradation, not increased protein synthesis. There are several factors affecting β-catenin degradation[2]. We know RA could respectively interact with RAR and RXR and act as a kind of transcriptional factor regulating cell differentiation. Recently, many results have proved RXR played an important role in regulating the degradation of β-catenin[3]. It was shown in this study that high concentration RA had no effect on the mRNA level, but could increase the protein level, we infer that RA inducing the increasing of β-catenin protein level is due to β-catenin degradation complex suppressed by RXR.

β-catenin nuclear translocation could transmit signal to the nucleus, which is very important in wnt signal. Therefore, we investigate the location and level change of β-catenin and cyclinD1 in TBEC after RA treatment, and the effect of retinoic acid on β-catenin/TCF pathway in TBEC. In cultured TBEC, it was found that the cellular β-catenin level was increased significantly after RA treatment, whereas the nuclear β-catenin level decreased. The results illustrate that RA failed to stimulate β-catenin nuclear translocation in TBEC. It has been reported previously that the β-cat protein level in the whole cell was increased, but the nuclear β-catenin and cyclinD1 protein level had no change [3]. CyclinD1 is cell cycle proteins that regulate G1 -S cell cycle. cyclinD1 high expression had relations with the development and prognosis of the tumor. The data suggest that a kind of molecular carcinogenesis mechanism of cyclinD1 is gene amplification and transcriptional upregulation. Many pathways take part in the transcriptional activation of cyclinD1 such as Ras gene family, Dbl superfamily, $PP60^{v\text{-}src}$, β-cat/TCF signalling[1]. In this study, it was revealed that RA could markedly repress cyclinD1 and nuclear β-cat expression after tracheobronchial epithelial cells were treated with RA. It had been reported that RA decreased cyclinD1 protein expression paralleling of human bronchial epithelial

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