RNA-binding motif protein 5 negatively regulates the activity of Wnt/β-catenin signaling in cigarette smoke-induced alveolar epithelial injury

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Abstract. Cigarette smoking is closely associated with various respiratory diseases. Oxidants and carcinogens in cigarettes are reported to induce various airway epithelial injuries. However, the underlying mechanisms remain unclear. The aims of the present study were to determine the involvement of RNA-binding motif protein 5 (RBM5) and Wnt/β-catenin signaling in cigarette smoke-induced alveolar epithelial injury, as well as the interaction between both. A549 cells were treated with cigarette smoke extract (CSE). The MTT assay was used to assess the effects of CSE on cell viability. The levels of RBM5 and Wnt/β-catenin/GSK3β were detected by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) and western blot analysis. A luciferase assay was used to assess the activity of β-catenin/T-cell factor (TCF) signaling. The results revealed that CSE inhibited A549 cell viability in both a dose- and time-dependent manner. Cytosolic and nuclear β-catenin levels were significantly increased following CSE treatment, compared with those in the control cells (P<0.05). The luciferase activity in CSE-exposed cells transfected with the TCF luciferase reporter wild-type plasmid (pGL3-OT) was significantly greater than that in cells without CSE exposure (33,167±3,085 vs. 19,978±1,916, respectively, P<0.05). Both the mRNA and protein levels of RBM5 in the CSE-treated cells were significantly reduced compared to the levels in the controls (all P<0.05). The overexpression of RBM5 inhibited Wnt/β-catenin signaling in the A549 cells, while silencing of RBM5 enhanced Wnt/β-catenin signaling. The β-catenin/TCF signaling inhibitor ICG-001 had no apparent effect on the RBM5 levels. Downregulation of RBM5 and activation of Wnt/β-catenin signaling are involved in CSE-induced alveolar epithelial injury. RBM5 acts as an upstream molecule that negatively regulates the activity of Wnt/β-catenin signaling.

Introduction

Cigarette smoking is closely associated with the development and progression of various respiratory diseases including lung cancer, chronic obstructive pulmonary disease (COPD), interstitial lung diseases and bronchial asthma (1,2). In particular, lung cancer and COPD are the main causes of death related to cigarette smoking in the world. Particles in cigarette smoke are reported to induce various lung injuries including inflammation and fibrosis (3,4).

Studies have shown that Wnt/β-catenin/T-cell factor (TCF) signaling is activated during lung injury and promotes the survival and migration of alveolar epithelial cells (5). β-catenin is a key player of canonical Wnt signaling. The activation of this signaling pathway mainly depends on the cytoplasmic accumulation and nuclear localization of β-catenin (6,7). First, a Wnt ligand binds to a seven-pass transmembrane Frizzled (Fz) receptor as well as its coreceptor LRP6 or LRP5 (8). The binding leads to depolymerization of glycogen synthase kinase-3β (GSK-3β)/APC/Axin complexes in the cytoplasm, β-catenin release and phosphorylation. It results in the accumulation and stabilization of cytosolic β-catenin, which then travels to nuclei to form complexes with members of the DNA-binding family TCF-1/lymphoid enhancer factors (LEF-1, 3 and 4). Activation of β-catenin/TCF signaling promotes the downstream target gene transcription involved in cell proliferation, migration and differentiation. These targets include cyclin D1, c-Myc and matrix metalloproteinases (MMP-2, -3, -7, -9 and -13) (9).

RNA-binding motif protein 5 (RBM5, previously referred to as g15, LUCA-15 or H37) is one of ~35 genes located in the 370-kb tumor suppressor locus on chromosome 3p21.3 (10). RBM5 is reported to induce cell cycle arrest and apoptosis by pre-mRNA alternative gene splicing (11,12). It is also suggested to act as a tumor-suppressor gene by inhibiting tumor growth and reducing metastatic potential (11,12). Both the mRNA and protein levels of RBM5 are significantly lower in non-small cell lung carcinomas (NSCLCs) than those in normal
tissues (13). The RBM5 level is negatively correlated with the smoking status in patients with lung cancer (12). In addition to lung cancer, whether the RBM5 level is also reduced in other cigarette smoke-induced lung injury has not been determined. Meanwhile, the activation of the Wnt/β-catenin signaling pathway plays an important role in diseases associated with cigarette smoking (5,14,15). Alveolar epithelial cells are the major components of the airway epithelium that are directly affected by cigarette smoking. The molecular mechanisms by which the Wnt/β-catenin signaling pathway is activated during cigarette smoke-induced lung injury remains unclear. Whether RBM5 is involved in the activation of Wnt/β-catenin signaling during lung injury also remains unknown.

The aims of the present study were to determine the level of RBM5 in cigarette smoke-injured lung epithelium and the involvement of RBM5 in the activation of Wnt/β-catenin signaling during lung injury. A549 cells were treated with cigarette smoke extract (CSE) at a series of concentrations and for various times. The present study demonstrated that the level of RBM5 in cigarette smoke-injured lung epithelium was significantly reduced and that RBM5 acts as an upstream molecular regulator of Wnt/β-catenin signaling activity during lung injury.

Materials and methods

Preparation of CSE. CSE was prepared using a popular type of cigarette in China (Hong Shuang Xi, 12 mg of tar, 1.1 mg of nicotine), as previously described (16). Briefly, a syringe-driven apparatus device was designed and operated to allow a stream of smoke to flow into a tube-shaped trap, which was maintained at room temperature. The smoke then entered a 1.5-liter flask submerged in liquid nitrogen. The amount of smoke obtained was determined by the increase in the weight inside the flask. The collected smoke particles were dissolved in dimethyl sulfoxide (DMSO) at 40 mg/ml, and the solution was sterile-filtered through a 0.22-µm syringe filter (Millipore, Watford, UK). The CSE solution was prepared by dissolving the condensate in DMSO, which was then stored in small vials at -80°C.

Cell culture. The human alveolar epithelial cell line A549 was obtained from Jilin University. The cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS; Gibco Waltham, MA, USA) and antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin) at 37°C in 5% CO2.

MTT assay. The cells (5x10^3/well) were plated in 96-well microtiter plates. After 24 h of culture in regular medium, the cells were then cultured in serum-free medium either supplemented with CSE (5, 10, 15, 20, 40, 60, 80 and 160 µg/ml, respectively) or DMSO for 6, 12, 24, 48 and 72 h, respectively. Next, 10 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA) and 100 µg/ml of the appropriate primer pair. The resulting amplified DNA fragments were separated by electrophoresis through a 1.5% agarose gel, and the resulting bands were visualized and scanned using a white ultraviolet transilluminator (Ultra-Violet Products Ltd., Cambridge, UK) and quantified by densitometry.

Immunofluorescence staining. The cells were fixed with cold 100% methanol at -20°C for 5 min and permeabilized with PBS-0.5% Triton X-100 for 5 min. After blocking with 5% bovine serum albumin for 1 h at 37°C, the cultures were incubated with rabbit polyclonal anti-β-catenin antibody (1:150 dilution) overnight at 4°C. After washing, the cells were incubated with Cy3-conjugated goat anti-rabbit secondary antibody (1:200 dilution) for 1 h at room temperature. Finally, DAPI (Sigma-Aldrich) was used to stain the nuclei. Fluorescence images were captured using a fluorescence microscope (Nikon Eclipse E600; Nikon, Tokyo, Japan).

Western blot analysis. The cells were plated in culture dishes for 24 h in RPMI-1640 and 10% FBS before exposure to CSE. The supernatant of the cultured cells was discarded, and the attached cells were washed twice with PBS. The cells were then lysed at 4°C for 60 min. The cell lysates were then centrifuged for 30 min at 12,000 x g and 4°C. For cell fractionation into the cytoplasmic and nuclear extracts, the Cytoplasmic Extraction kit (CWBAO, Beijing, China) was used according to the manufacturer's instructions. The protein concentrations were determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of lysates (40 µg) were separated using 12% SDS-PAGE and transferred onto nitro-
cellulose membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% non-fat milk diluted in buffer (10 mM Tris-HCl, 100 mM NaCl and 0.1% Tween-20) for 1 h at room temperature. The membranes were then probed with antibodies including anti-RBM5 (ab85504), anti-β-catenin (E247, ab32572), anti-His-H3 (acetyl K27, ab4729), anti-p-β-catenin (phospho-Y654, ab24925) and anti-β-actin [ACTN05 (C4), ab3280] from Abcam (Cambridge, MA, USA), anti-Wnt-2 (11160-1-AP), anti-Wnt-7a (10605-1-AP), anti-FRZB (12884-1-AP), anti-p-GSK-3β (phospho-Y216, ab75745) and anti-GSK-3β from Proteintech Group, Inc. (Chicago, IL, USA; 22104-1-AP), overnight at 4˚C. Horseradish peroxidase-conjugated secondary anti-

Luciferase assay. The cells were plated onto a 12-well plate 1 day prior to transfection. Following confirmation of 70-80% confluency, the cells were transfected with 0.4 µg of TCF luciferase reporter plasmids (wild-type, pGL3-OT; mutant, pGL3-OF, respectively) for 24 h. Meanwhile, the cells in each group were cotransfected with 0.1 µg of β-galactosidase expression vector (Santa Cruz Biotechnology) at a dilution of 1:500 were then added to the solution and allowed to incubate for 1 h at room temperature. The protein bands were then detected using an Enhanced Chemiluminescence kit (Pierce Biotechnology Ltd., Rockford, IL, USA). The protein levels were quantified by densitometry using Quantity One software (Bio-Rad Laboratories).

Silencing of RBM5 in the cells. A short hairpin RNA (shRNA) sequence targeting human RBM5 and a non-target sequence were obtained from Shanghai Genechem. The RBM5 siRNA sequences used were forward, 5'-CCACAAAAGAUGGCAUUGATT-3' and reverse, 5'-UCAAUGCCAUCUUUGGUGGTT-3'; and those of the non-target shRNA (Scramble) were forward, 5'-UUCUCCGAACGUGUCAGUTT-3' and reverse, 5'-ACGUGACACGUUCGGAGAATT-3'. The transfections with siRNA were performed using Lipofectamine 2000 according to the manufacturer’s protocol. Briefly, the A549 cells were seeded into 6-well plates and transfected the next day with 100 pmol of shRBM5 or shScramble using 10 µl of Lipofectamine 2000. The cells were harvested 48 h after transfection.

Treatment of the cells with the β-catenin/TCF inhibitor. The novel small-molecule ICG-001 (S2662; Selleckchem, Houston, TX, USA) which selectively inhibits β-catenin/TCF-mediated gene transcription, was previously described (23,25). A549 cells were seeded onto 6-well plates and treated with 12.5 µl of ICG-001 or DMSO on the second day. After treatment for 24 h, the cell culture medium was replaced with medium containing the same dose of ICG-001 or DMSO on the second day. After treatment for 24 h, the cell culture medium was replaced with medium containing the same dose of ICG-001 or DMSO. The cells were continuously incubated for another 24 h. Then, the protein was extracted from the cells and subjected to western blot analysis.

Results

Cytotoxicity of CSE on cell viability and growth. The effects of CSE on cell growth in vitro were evaluated using an MTT assay. A549 cells were treated with various concentrations of CSE for 6, 12, 24, 48 or 72 h, respectively. The results showed that...
CSE exposure inhibited cell growth and viability (Fig. 1A). Compared with the DMSO-treated control cells, which were closely adherent, the CSE-exposed cells showed delayed confluence and larger interspaces between the cells under light microscopy. The shrinkage and death of cells were more apparent after exposure to 80 µg/ml CSE for 48 h (Fig. 1B).

Increased cytoplasmic accumulation and nuclear translocation of β-catenin in the A549 cells following CSE treatments. To determine the effects of CSE on the β-catenin levels in A549 cells, the β-catenin protein levels were analyzed by western blot analysis. The results showed that the total, cytosolic and nuclear β-catenin levels were increased upon exposure to CSE, compared to those in the control cells. The increases in nuclear β-catenin were more significant at concentrations of 10-80 µg/ml, and total β-catenin and cytosolic β-catenin at concentrations of 40-80 µg/ml (all P<0.05). In addition, the increases were dose-dependent (Fig. 2). The levels of β-catenin in the A549 cells exposed to CSE were also examined by immunofluorescence staining. The results showed that strongly positive β-catenin cells were detected in both the cytoplasm and nuclei of the CSE-treated cells and that the expression was more significant in the nuclei of cells exposed to 40 µg/ml CSE for 48 h, while the β-catenin expression was only slightly positive in the control cells (Fig. 3A). To further study the mechanisms, other molecules involved in the Wnt/β-catenin/TCF pathway were examined. Compared to the untreated control groups, western blot
analysis showed that the levels of phosphorylated β-catenin and total GSK-3β were decreased, while phosphorylated GSK-3β, FRB, Wnt2 and Wnt7a were increased following treatment with 40 µg/ml CSE (Fig. 3B).

Activation of β-catenin/TCF signaling in the A549 cells following CSE treatments. The luciferase activity assay showed that the luciferase activity in the CSE-exposed cells transfected with pGL3-OT (wild-type) was significantly greater than that in the cells without CSE exposure (33,167±3,085 vs. 19,978±1,916, respectively; P<0.05). Meanwhile, the luciferase activity in the CSE-exposed cells transfected with the TCF luciferase reporter mutant plasmid (pGL3-OF) was similar to that in the cells without CSE exposure (35,657±2,301 vs. 32,908±2,350, respectively, P>0.05). These results suggest that the β-catenin/TCF signaling pathway was activated in cells following CSE treatment (Fig. 4).

Downregulation of RBM5 in the A549 cells following CSE treatments. To determine the effect of CSE on the RBM5 level in cells, we examined both the mRNA and protein levels of RBM5 in the A549 cells following treatment with various concentrations of CSE at different time-points. The real-time PCR results showed that CSE inhibited the RBM5 mRNA levels in both a dose- and time-dependent manner. The fold-change in gene expression was significantly reduced at 40 and 80 µg/ml at 24 h; at 10, 20, 40 and 80 µg/ml at 48 h; and at 0, 20, 40 and 80 µg/ml at 72 h (all P<0.05) (Fig. 5A and B). In addition, the western blot analysis showed that the RBM5 protein levels were reduced in the cells treated with CSE for 48 h and that the levels were significantly reduced at 20 and 80 µg/ml. The RBM5 expression level after treatment with 40 µg/ml CSE was slightly greater than that after treatment with 20 µg/ml CSE. These results suggest that CSE inhibited RBM5 protein expression (Fig. 5C and D).

RBM5 regulates Wnt/β-catenin signaling in the A549 cells. To determine the effects of RBM5 on Wnt/β-catenin signaling in the A549 cells, we either overexpressed wild-type RBM5 or knocked down RBM5 using RBM5 shRNA in the cells. As shown in Fig. 6, compared with the control group,
overexpression of RBM5 significantly inhibited β-catenin expression in the A549 cells (P<0.05), increased phosphorylation of β-catenin and GSK-3β, and increased total GSK-3β expression. In contrast, silencing of RBM5 significantly enhanced the β-catenin level in the A549 cells (P<0.05), reduced phosphorylation of β-catenin and GSK-3β and reduced total GSK-3β expression. Furthermore, the cells were treated with the β-catenin/TCF signaling inhibitor ICG-001. The data showed that ICG-001 had no apparent effect on the RBM5 expression level in the cells. These studies suggest that RBM5 is an upstream molecule that regulates Wnt/β-catenin signaling and negatively regulates this pathway in A549 cells (Fig. 6).

Discussion

The primary purpose of the present study was to determine whether the RBM5 level is reduced and whether it regulates Wnt/β-catenin signaling in cigarette smoke-induced lung injury. The A549 cell line has been considered to be a preferred cellular model for studies related to type II alveolar epithelial cells, which secrete specific type II cell markers including surfactant proteins and some other markers (17,18). In the present study, A549 cells were exposed to various concentrations of CSE for different periods of time. We observed that CSE is cytotoxic to alveolar epithelial cells as it inhibited the viability of cells in a dose- and time-dependent manner. Our study is consistent with previous reports (19,20). Cigarette smoke is a complex mixture of harmful chemicals, some of which are known carcinogens. Recent research has shown that A549 cells are more vulnerable to harmful chemicals in CSE than other types of cells (21).

In the present study, we observed that both cytosolic and nuclear β-catenin levels in the CSE-treated cells were significantly elevated following CSE exposure for 48 h, compared with those in the control cells. According to the western blot results, there were increased nuclear β-catenin levels in the cytoplasm as well as increased cytosolic β-catenin and total β-catenin levels in the nuclei. These results were also confirmed by an increased number of β-catenin-positive cells in both the cytoplasm and nuclei as examined by immunofluorescence staining. The changes were dose-dependent. Unfortunately, proteins were only collected from cells treated for 48 h. Thus, we were unable to determine whether the changes were also time-dependent. These findings suggest that CSE upregulates β-catenin levels and promotes β-catenin cytosolic accumulation and nuclear translocation. In addition, the luciferase activity assay showed that the luciferase activity in the CSE-exposed cells transfected with the TCF luciferase reporter wild-type plasmid (pGL3-OT) was significantly greater than that in cells without CSE exposure. These results suggest that Wnt/β-catenin signaling was activated in the CSE-exposed lung epithelial cells. Previous studies have shown that Wnt/β-catenin signaling is activated in lung injury (5,14,15,22). Moreover, aberrant activation of WNT signaling also has been reported in the airway of cigarette smoke-associated COPD (23). Thus, the present study is in line with these reports, confirming that aberrant activation of Wnt/β-catenin signaling exists in cigarette smoke-associated airway injury and is responsible for cell proliferation and tissue remodeling at the late stage of injury in response to CSE stimuli. However, one report has contradicted these studies, suggesting that smoking downregulates the Wnt/β-catenin pathway in the human airway epithelium and contributes to the dysregulation of airway epithelium differentiation observed in smoking-related airway disorders (24).

Previous studies suggest that frequently reduced RBM5 levels exist in different types of tumors including breast cancer (25), schwannoma (26), and ~75% of primary lung cancers (27). We previously reported that the protein levels of RBM5 are lower in NSCLC compared with non-tumorous tissues (13). In addition, we studied the possible involvement of RBM5 in drug resistance during chemotherapy using a cisplatin-sensitive and a cisplatin-resistant NSCLC cell
line (10). These studies suggest that RBM5 plays a role in suppressing tumor development and progression. Additionally, the RBM5 level has been shown to be higher in the adult thymus compared with that in the fetal thymus, which suggests that RBM5 may also be involved in normal development (28). Furthermore, the inhibitory effects of RBM5 on tumor growth both in vitro and in vivo have been suggested due to its induction of G1 cell cycle arrest and apoptosis (25). In the present study, for the first time, we observed that both the mRNA and protein levels of RBM5 in the CSE-injured lung epithelium also were significantly reduced compared to the controls. The reductions in human alveolar epithelial cells were both time- and dose-dependent. Similarly, a study from another group showed that a decreased RBM5 level was more frequently observed in the alveolar epithelium of smokers than non-smokers (13). Together, these data suggest that loss of RBM5 contributes to cell proliferation and tumor transformation. The possible molecular mechanisms may involve cell cycle arrest and induction of apoptosis.

Given that RBM5 was downregulated and Wnt/β-catenin signaling was activated in the CSE-exposed alveolar epithelial cells, we postulated that RBM5 may be involved in the activation of Wnt/β-catenin signaling in CSE-induced lung injury. To further study whether RBM5 regulates β-catenin signaling, we examined the protein level of β-catenin in the A549 cells following the overexpression of wild-type RBM5 or the silencing of RBM5 using RBM5 shRNA in these cells. The present study revealed that overexpression of RBM5 significantly inhibited Wnt/β-catenin signaling in A549 cells, while silencing of RBM5 significantly enhanced Wnt/β-catenin signaling in the A549 cells. However, the β-catenin/TCF signaling inhibitor ICG-001 had no apparent effect on the β-catenin signaling in A549 cells, while silencing of RBM5 significantly enhanced Wnt/β-catenin signaling: arrows point the way. Development 131: 1663-1677, 2004.
