SB431542 inhibited cigarette smoke extract induced invasiveness of A549 cells via the TGF-β1/Smad2/MMP3 pathway

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Received August 27, 2017; Accepted February 19, 2018

DOI: 10.3892/ol.2018.8556

Abstract. Lung cancer has high morbidity and mortality rates. Smoking is involved in the pathogenesis of lung cancer, and tobacco smoke may increase tumor cell invasion and metastasis. The effects of cigarette smoke extract (CSE) on the carcinoma human alveolar basal epithelial A549 cell line were investigated. A549 cells were exposed to increasing concentrations of CSE for 12, 24 and 48 h, and the transforming growth factor-β1 (TGF-β1) signal pathway was inhibited by addition of SB431542, a TGF-β1 receptor antagonist. The proliferation of A549 cells was assayed by a Cell Counting kit-8, invasiveness was assayed using Transwell chambers, and TGF-β1, phosphorylated mothers against decapentaplegic homolog 2 (p-Smad2), and matrix metalloproteinase 3 (MMP3) levels were assessed by western blot analysis. The invasiveness of A549 cells and the expression of TGF-β1, pSmad2, and MMP-3 were significantly increased by CSE (P<0.05). The effects of CSE were abrogated by SB431542 (P<0.05). In conclusion, CSE increased the invasiveness of A549 cells and its effects were abrogated by SB431542 and the TGF-β1/Smad2/MMP-3 pathway may have been involved.

Introduction

Smoking is the primary cause of lung cancer. The proportion of cases attributable to smoking has reached 90% in countries with ongoing high tobacco consumption (1). Cigarette smoke contains at least 69 carcinogens, including ammonia, cadmium, nickel, nicotine (2), and nitrosamines such as 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butane (3). Tobacco smoke components not only cause cancer, but may also be involved in tumor invasiveness and metastasis. Cigarette smoking is known to increase the risk of prostate cancer metastasis (4), the metastatic ability of breast cancer cells (5), and the risk of pulmonary metastasis of breast cancer (6). Tobacco smoke may also increase the spread of lung carcinoma cells (7); however, the mechanism by which this happens is, to the best of our knowledge, unclear.

Metastasis is a well-regulated process (8) that depends on the invasion of cancer cells into surrounding tissues; it is a leading cause of cancer mortality (9), and is characteristic of lung cancer. Transforming growth factor-β (TGF-β) may be a key regulator of tumor cell invasion and metastasis. TGF-β1, TGF-β2 and TGF-β3 are members of a superfamily of secreted cytokines that regulate cellular processes, including proliferation, differentiation, migration, survival, and immunity, by ligand-receptor binding (10-12). TGF-β family members are ubiquitously expressed. The TGF-β1-induced epithelial-mesenchymal transition in lung cancer is a key first step in metastasis (13), and exposure of A549 cells to cigarette smoke extract (CSE) induces the expression, release and activation of TGF-β1 (14).

Matrix metalloproteinase (MMP) activity in tumor cell metastasis includes degrading of basement membranes and the extracellular matrix, which facilitates tumor invasion and metastasis (15,16). TGF-β1 has been reported to stimulate the expression of matrix metalloproteinase 3 (MMP3) in human corneal epithelial cells (17), but it is not known whether it has similar activity in lung cancer cells. The present study investigated the effect of CSE on the invasiveness of A549 cells and the possible involvement of TGF-β. The proliferation and invasiveness of A549 cells increased following CSE exposure. Expression of TGF-β1, mothers against decapentaplegic homolog 2 (Smad2), and MMP3 was significantly increased by CSE and partly abrogated by SB431542, a TGF-β1 receptor inhibitor. SB431542 inhibited the CSE-induced invasiveness of A549 cells via the TGF-β1/MMP3 pathway.

Materials and methods

Cell culture and reagents. The A549 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Inc.) at 37°C in a humidified 5% CO₂ atmosphere.
The Cell Counting kit-8 (CCK-8) kit was obtained from Dojindo Molecular Technologies Inc. (Kumamoto, Japan). GAPDH, TGF-β1, phosphorylated (p)-Smad2, and MMP3 primary antibodies (dilution 1:1,000) were obtained from Abcam (Cambridge, MA, USA). Solid SB431542 (cat. no. HY-I0431) was obtained from MedChemExpress (Monmouth Junction, NJ, USA). The SB431542 was dissolved in 1 ml DMEM and the concentration was adjusted to 0.1, 1.0, and 10.0% CSE as required for use in the experimental procedures. The CSE solutions were freshly prepared and used within 30 min of preparation.

**Cell Counting kit-8 (CCK-8) proliferation assay.** In brief, A549 cells were treated with CSE, then seeded into 96-well plates at a density of 5x10⁴ cells/well in 100 µl DMEM and incubated at 37°C. When the cells reached 70% confluence, the medium was replaced with an equal volume containing CSE at concentrations of 0.1, 1.0 and 10.0% and cultured for 12, 24, or 48 h before addition of 10 µl CCK-8 solution. After 1-2 h, absorbance was read at 490 nM using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

**Cell motility assay.** The invasiveness of A549 cells was assayed in BioCoat Matrigel-coated invasion chambers (BD Biosciences, Franklin Lakes, NJ, USA) with 8-μm-pore size polycarbonate membranes. Cells were grown in serum-free DMEM at 37°C for 2 h, the medium was removed and 750 µl DMEM with 10% FBS was added into the lower chamber as a chemoattractant. A549 cells were treated with CSE or SB431542, then added to each upper chamber at a density of 5x10⁴ cells/well in 2 ml DMEM with 1% FBS. After 2 h, 1.0% CSE and 100 nmol/l SB431542 were added to the upper chambers. The inserts and non-invasive cells were removed after 12 h. The invasive cells on the lower surface of the membrane were then fixed in 100% methanol for 15 min at room temperature, air dried, and stained with crystal violet for 30 min at room temperature. The numbers of cell in five random visual fields with a fluorescence microscope (Olympus Corporation, Tokyo, Japan) at a magnification of x200 were recorded.

**Western blot assays.** Cells were treated with CSE or SB431542, then separated by 1 ml 0.25% trypsin (Thermo Fisher Scientific, Inc.), then disrupted in ice-cold lysis buffer containing protease and phosphatase inhibitors (cat. no. FNN0011; Thermo Fisher Scientific, Inc.) for 30 min, and then clarified by centrifugation at 2,000 x g for 10 min at 4°C. Total protein concentration was determined using a bicinchoninic acid assay, and the sample was boiled for 5 min before loading. The cell lysate was resuspended in SDS buffer (Beyotime Institute of Biotechnology, Haimen, China), and 40 µg samples of protein were separated by 8% SDS-PAGE (Beyotime Institute of Biotechnology). The proteins were transferred to polyvinylidene difluoride membranes (EMD Millipore), blocked for 2 h with 5% bovine serum albumin (Beyotime Institute of Biotechnology) incubated with primary antibodies: Antibodies of GAPDH (cat. no. ab8245; dilution 1:1,000); TGF-β1 (cat. no. ab92486; dilution 1:1,000); phosphorylated (p)-Smad2 (cat. no. ab40855; dilution 1:1,000); and MMP3 (cat. no. ab53015; dilution 1:1,000) were obtained from Abcam. The incubation was overnight at 4°C, and then incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (cat. no. HP6023; dilution 1:1,000; Abcam) for 2 h at 20°C. Immunoreactivity was visualized by SuperEnhanced chemiluminescence kit (Millipore, Bedford, MA, USA) and the results were analyzed by Quantity One software v4.4.02 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Statistical analysis.** Experimental procedures were performed in triplicate, and the results were expressed as the mean ± standard deviation. The significance of differences between the CSE groups was assessed by one-way analysis of variance followed by Dunnett’s test. Student’s t-test was used to compare the differences in different treatment groups. Statistical analysis was performed with GraphPad Prism version 5.01 (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate statistically significant difference.

**Results**

**CSE treatment increases the invasiveness of A549 cells.** The proliferation and invasiveness of A549 cells were assayed following CSE treatment at concentrations of 0.1, 1.0, and 10.0% for 12, 24, and 48 h (Fig. 1A). At a concentration of 10.0%, CSE significantly decreased the proliferation of A549 cell; however, proliferation was not significantly affected by 1.0% CSE. Therefore, the effect of CSE on invasiveness was evaluated at a concentration of 1.0%. The results of the Transwell invasion assay (Fig. 1B) revealed that there were significantly more invasive cells on the lower membrane surface following CSE treatment compared with control cultures (P<0.05).

**CSE exposure significantly increases TGF-β1, Smad2, and MMP3 expression in A549 cells.** To determine whether CSE promoted the activation of the TGF-β1 pathway, A549 cells were treated with 1.0% CSE for 48 h, and the expression of TGF-β1 and Smad2, which mediates TGF-β1 signaling, was assessed. The expression levels of TGF-β1 and Smad2 were significantly increased following CSE exposure.
The expression of MMP3, an indicator of metastasis, was also significantly increased in A549 cells following exposure to CSE (Fig. 2C and D).

**Increased TGF-β1, Smad2, MMP3 expression and cell invasiveness in response to CSE is partly inhibited by SB431542.**

A549 cells were exposed to SB431542, a TGF-β1 receptor antagonist, to assess the involvement of TGF-β1/Smad2, and MMP3 following CSE treatment. At a density of 10 mmol/l, which was verified to have no effect on the cell proliferation in preliminary experiments (data not shown), to block the TGF-β1 receptor, SB431542 significantly decreased TGF-β1, Smad2 and MMP3 expression (Fig. 3A and B). In the Transwell invasion assay, SB431542 inhibited the effect of CSE on the invasiveness of A549 cells. The number of invasive CSE-exposed A549 cells was significantly decreased by treatment with SB431542 (Fig. 3C and D).

**Discussion**

CSE exposure increased the proliferation and invasiveness of A549 cells; it also increased MMP3 production and TGF-β1/Smad2 pathway activity, which were inhibited by SB431542, a known TGF-β1 receptor antagonist.

The smoke generated from the tobacco in cigarettes exposes the smoker to upwards of 4,000 different xenobiotic chemicals (19,20), and exposure to cigarette smoke increases the risk of lung cancer (21). Cigarette smoke has also been associated with pancreatic cancer metastasis (22) and with the increased metastatic ability of breast cancer cells via promotion of the epithelial-mesenchymal transition (5). CSE has also been reported to enhance the metastatic ability and invasiveness of lung cancer cells (7). In the present study CSE increased the invasiveness of A549 cells (Fig. 1B and C).

Metastasis is a complex multistep process and a leading cause of cancer-associated mortality (9). MMP3 is a proteolytic enzyme that is active in metastasis, capable of degrading structural components of the extracellular matrix (23) and disrupting intercellular and cell-extracellular matrix adhesions (24). MMP3 activity contributes to tumor invasion and metastasis, and is indicative of a poor survival prognosis (25). CSE exposure increased the expression of MMP3 in A549 cells (Fig. 2C and D) and may have increased the invasiveness of lung cancer cells by upregulating the MMP3 expression, which is in line with previous reports of MMP3 activity in lung cancer metastasis (26). As there are several other MMPs involved in the cancer metastasis, their roles should be studied in future experiments.

TGF-β is a mediator of cancer invasion and metastasis (27). TGF-β signals are transferred to the nucleus via TGF-β type I or type II receptors that phosphorylate canonical Smad2/3 downstream effectors (28). In the present study, CSE increased TGF-β1 and Smad2 activity (Fig. 2A and B). In vitro, treatment with an anti-MMP3 antibody was found to result in a dose-dependent decrease in active TGF-β1 (27). Activated TGF-β can regulate the secretion, expression, and activation of MMP3, resulting in a bidirectional regulatory loop (29).
SB431542 is a TGF-β1 receptor kinase inhibitor that interrupts the activation of downstream signaling pathways (30). SB431542 has previously been reported by Tanaka et al (31) to induce an in vivo antitumor immune response associated with TGF-β activity. Matsuyama et al (32) reported that SB431542 exerted antitumor activity by inhibiting the proliferation of osteosarcoma cells. Xi et al (33) revealed that SB431542 inhibited the invasiveness of RPMI 8226 cells by decreasing the expression of MMP3. In the present study, SB431542 significantly inhibited the activity

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Figure 2. Levels of TGF-β1, p-Smad2, and MMP3 in A549 cells was assayed by western blotting following CSE exposure for 48 h. (A) Western blot analysis reveals that levels of TGF-β1 and p-Smad2 were increased following exposure to 1.0% CSE exposure, with (B) densitometry analysis confirming this. (C) MMP3 expression in A549 cells was significantly increased following exposure to 1.0% CSE, with (D) densitometry analysis confirming this. ***P<0.001 vs. control; #P<0.05, CSE+SB431542 vs. CSE only. CSE, cigarette smoke extract; TGF-β1, transforming growth factor-β1; p-Smad2, phosphorylated mothers against decapentaplegic homolog 2; MMP3, matrix metalloproteinase 3.

Figure 3. Levels of TGF-β1, p-Smad2, and MMP3 increased by CSE treatment in A549 cells was significantly inhibited by SB431542. (A) A549 cells were treated with 1.0% CSE with or without 10.0 mmol/l SB431542 for 48 h, the levels of TGF-β1, p-Smad2, and MMP3 was assayed by western blot analysis, with (B) densitometry analysis confirming this. (C) The invasiveness of A549 cells was determined by Transwell assays and (D) quantified. ***P<0.001 vs. control; #P<0.05, CSE+SB431542 vs. CSE only. CSE, cigarette smoke extract; TGF-β1, transforming growth factor-β1; p-Smad2, phosphorylated mothers against decapentaplegic homolog 2; MMP3, matrix metalloproteinase 3.
of the TGFβ1/Smad2 pathway and decreased MMP3 expression in A549 cells exposed to CSE (Fig. 3A and B), and reduced the invasiveness of CSE-treated A549 cells (Fig. 3C and D).

In the current study, the promotion of the invasiveness of lung cancer cells by CSE was associated with the activation of the TGFβ1/Smad2 pathway and regulation of MMP3 expression. The effects of CSE were partially reversed by SB431542, a TGFβ1 receptor antagonist that may have therapeutic potential in cancer, which could be proven in other in vitro models, such as HAC-84 and GLC-15 cells, or in vivo experiments in the future.

Acknowledgements
Not applicable.

Funding
No funding was received.

Availability of data and materials
All the data and materials are available upon reasonable request.

Authors' contributions
KL contributed to the study design and contributed to data analysis, CY contributed to performing experiments and KH contributed to data analysis.

Ethics approval and consent to publish
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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