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Contraction-dependent TGF-β1 activation is required for thrombin-induced remodeling in human airway smooth muscle cells

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ABSTRACT

Aims: Thrombin is a serine proteinase that is not only involved in coagulation cascade, but also mediates a number of biological responses relevant to tissues repair, and induces bronchoconstriction. TGF-β plays a pivotal role in airway remodeling due to its effects on airway smooth muscle proliferation and extracellular matrix (ECM) deposition. Recently, bronchoconstriction itself is found to constitute a form of strain and is highly relevant to asthmatic airway remodeling. However, the underlying mechanisms remain unknown. Here, we investigated the role of contraction- dependent TGF-β activation in thrombin-induced remodeling in human airway smooth muscle (HASM) cells.

Materials and methods: Primary HASM cells were treated with or without thrombin in the absence or presence of anti-TGF-β antibody, cytochalasin D and formoterol. CFSE labeling index or CCK-8 assay were performed to test cell proliferation. RT-PCR and Western blotting were used to examined ECM mRNA level and collagen Iα1, α-actin protein expression, respectively. Immunofluorescence was also used to confirm contraction induced by thrombin in HASM cells.

Key finding: Thrombin stimulation enhanced HASM cells proliferation and activated TGF-β signaling. Thrombin induced ECM mRNA and collagen Iα1 protein expression, and these effects are mediated by TGF-β. Abrogation of TGF-β activation by contraction inhibitors cytochalasin D and formoterol prevents the thrombin-induced effects.

Significance: These findings suggest that contraction-dependent TGF-β activation could be a mechanism by which thrombin leads to the development of asthmatic airway remodeling. Blocking physical forces with bronchodilator would be an intriguing way in reducing airway remodeling in asthma.

1. Introduction

Asthma, an obstructive airway disease that affects > 300 millions people worldwide, is characterized by airway inflammation, airway obstruction and airway remodeling [1]. Airway remodeling can be referred to as alterations in structural cells and tissues of the airway in obstructive disease [2,3]. Airway remodeling is found to be associated with reduced lung function and insensitivity to glucocorticoid treatment in asthmatic patients. It is now well recognized that airway smooth muscle (ASM) remodeling play a major role in airway remodeling. ASM remodeling comprises proliferation, hypertrophy, and excessive ECM products [2,5]. On the one hand, proliferation of ASM leads to enhanced airway contractility and reduced airway caliber in fatal and nonfatal asthma [6]. On the other hand, increased ECM proteins, in turn, modulate ASM cellular behaviors including proliferation, contraction, and migration. However, the underling mechanisms of ASM remodeling are complex and not completely clear. Bronchoconstriction itself constitutes a form of strain and is relevant to the asthmatic airway remodeling. Acetylcholine, a well-known mediator in regulating bronchoconstriction, is increasingly recognized to induce airway remodeling [8]. Moreover, tiotropium bromide, an anticholinergic targeting contractile effect of acetylcholine, decreases airway

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remodeling in animal models of asthma [9,10]. Recently, it has been shown in clinical studies that compressive mechanical forces arising during methacholine-mediated bronchoconstriction may induce airway remodeling independent of inflammation [11]. Therefore, bronchoconstriction seems to play important roles in airway remodeling, and it would be clinically important to determine the mechanisms why blocking physical forces could attenuate the remodeling response.

Accumulating evidence indicates that coagulation system is activated in asthmatic airways [12–14], and uncontrolled coagulation activity contributes to the pathogenesis of asthma, in which process thrombin plays an important role [15–17]. Thrombin is the classic effector enzyme of coagulation. Beside its role in the coagulation system, thrombin has been shown to play a vital role in contributing to airway remodeling in asthma. Gabazza and coworkers [12] demonstrated that thrombin activity in induced sputum of patients with asthma was increased compared with normal control subjects, and induced sputum from these patients was able to induce proliferation of ASM cells. Similarly, increased thrombin activity in bronchoalveolar lavage fluid (BALF) is detected after segmental allergen challenge in patients with mild asthma, and BALF from allergen-provoked airways is able to promote airway fibroblast proliferation [16]. Furthermore, thrombin is sufficient to induce human ASM cells proliferation and migration [18]. Interesting, it has been found that thrombin is a potent agent triggering bronchoconstriction in guinea-pigs in vivo [19] and human bronchial rings in vitro [20]. Therefore, it would be intriguing to delineate the associations between thrombin- induced bronchoconstriction and thrombin-relevant ASM proliferation.

TGF-β1 is a particularly important mediator involved in airway remodeling in asthma [21]. TGF-β1 induces the epithelial–mesenchymal transition via a Smad3- dependent pathway in primary airway epithelial cells [22]. It also promotes the differentiation of fibroblasts into myofibroblast cells, and enhances their proliferation [23,24]. Moreover, TGF-β1 has been shown to induce human ASM cells proliferation and secretion of ECM proteins [25,26]. Over-expression of the downstream TGF-β1 signaling protein Smad2 leads to thickened ASM layer and collagen deposition in house dust mite-challenged mice [27]. TGF-β1 is increased in methacholine challenge asthma BALF [11], and it could be a modulator contributing to bronchoconstriction-mediated airway remodeling.

Therefore, using HASM cells as a bronchoconstriction model, in vitro, we aimed to elucidate that thrombin-mediated human ASM remodeling required contraction- dependent TGF-β activation.

2. Materials and methods

2.1. Reagents and antibodies

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin were purchased from GIBCO BRL (Gaithersburg, MD, USA). The stimulants used were thrombin (Sigma, St. Louis, MO), and active human recombinant TGF-β1 (Peprotech, Rocky Hill, NJ, USA). Inhibitors used were the pharmacologic inhibitor of actin reorganization cytochalasin D (abcam), the β2-adrenoreceptor agonist formoterol (Sigma, St. Louis, MO), and the pan-TGF-β blocking antibody, clone 1D11 (R&D Systems, Minneapolis, USA). The antibodies used were anti-phosphorylated Smad2, anti-total Smad2 (Cell Signaling Technology, Danvers, MA); anti-collagen Iα1, anti-GAPDH (Beijing Biosynthesis biotechnology, Beijing, China), and α-actin (Beyotime Biotech, Shanghai, China).

2.2. Isolation and culture of HASM cells

HASM cells were dissected from the lobar or main bronchus obtained from the patients undergoing resection for lung carcinoma. Six patients were sequentially sampled and each isolate was treated as separate sample. This study was approved by Ethics Committee of Nanfang Hospital, Southern Medical University (NFEC-201109-K1) and the patients gave their written, informed consent. Pure ASM bundles were dissected free from surrounding tissue. The cells were maintained as primary culture in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in humidified air containing 5% CO_2. After 20 to 25 d, HASM cells in primary culture grew to confluence. The cells showed a typical hill-and-valley appearance and revealed a positive expression of smooth muscle α-actin by immunofluorescence techniques, with a purity of ≥95%. All experiments were performed in cells from passages 3 to 6 of subculture.

2.3. CCK-8 assay

HASM cells were harvested and seeded in flat-bottomed 96-well culture plates (8×10^3 cells/well) and maintained at 37 °C in a humidified incubator overnight. The cells were then treated with thrombin (1 U/ml) for 12, 24, 48, and 72 h using three replicates. Then, 10 μl CCK-8 solution (Dojindo, Tabaru, Japan) was added to each well in a 100 μl volume. After incubation for 2 h, the optical density (OD) of each sample was measured at 450 nm using microplate reader (Spectra Max MD5, Molecular Devices, USA). Culture medium (DMEM) without cells was used as blank control.

2.4. Flow cytometry detection of CFSE-labeled HASM cells

Carboxyfluorescein diacetate, succinimidyl ester (CFDASE) is a cell-tracking dye used to examine HASM cells proliferative activity. It diffuses into the cytoplasm where its acetate groups are cleaved to yield a highly fluorescent derivative (CFSE) retained in the cells. In mitosis, daughter cells exhibit half the fluorescence intensity of their mother cell, allowing the visualization of the number of rounds of cell division. In the study, cells were harvested and washed twice in PBS containing 1% bovine serum albumin. The cells were then labeled with 5 μM CFSE (Invitrogen, Eugene, Oregon, USA) at 37 °C for 10 min in the dark. After incubation, the labeling was quenched with 2 volumes of ice-cold 10% FBS for 5 min. After being washed twice, the CFSE-labeled cells were seeded at 1×10^6 cells/ml in 35-mm culture plates. At least 24 h after seeding, drugs were added into each culture plates, and the cells were further incubated for 48 h. After incubation, cells were harvested, washed twice, re-suspended in PBS, and analyzed immediately using a flow cytometer. The data was analyzed using ModFit LT 3.2 program (Verity Software House, USA). Proliferation index was calculated as the sum of cells in all generations divided by the number of original parent cells.

2.5. Western blot analysis

Cultured cells were lysed on ice for 20 min using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotech, China), containing a protease inhibitor cocktail (Sigma, St. Louis, MO) or phosphatase inhibitors (Sigma, St. Louis, MO). Spin at 14,000 rpm (16,000*g*) in an Eppendorf microfuge for 10 min. Transfer the supernatant to a new tube and discard the pellet. Cell protein concentrations were determined using the Enhanced BCA Protein Assay Kit (Beyotime Biotech, China). For immunoblotting, equal amounts of proteins (25 μg/lane) were subjected to electrophoresis on a 10% SDS-polyacrylamide gel and electroblotted to PVDF membrane (Bio-Rad, Hercules, CA, USA). The membranes were blocked for 1 h with 5% bovine serum albumin in TBST buffer (20 mM Tris, 500 mM NaCl and 0.1% Tween-20). After incubation with specific primary antibodies diluted in blocking buffer (1:1000 for anti-phosphorylated Smad2, anti-total Smad2 and anti-collagen Iα1) overnight at 4 °C, membranes were washed and incubated for 1 h at room temperature with secondary antibody. The target proteins on the membrane were then immunodetected by the ECL-detecting reagents according to the manufacturer's instruction. The resulting bands were densitometrically

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