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Azithromycin ameliorates OVA-induced airway remodeling in Balb/c mice via suppression of epithelial-to-mesenchymal transition

Yue Pu[a], Yuanqi Liu[a], Shiping Liao[b], Shikun Miao[a], Liming Zhou[a], Lihong Wan[a,*]

[a] Department of Pharmacology, West China School of Basic Medical Sciences & Forensic Medicine, Sichuan University, Chengdu, Sichuan 610041, PR China
[b] Functional Laboratory, West China School of Basic Medical Sciences & Forensic Medicine, Sichuan University, Chengdu, Sichuan 610041, PR China

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ABSTRACT

Azithromycin is a potent agent that prevents airway remodeling. In this study, we hypothesized that azithromycin (35 mg/kg orally) alleviated airway remodeling through suppression of epithelial-to-mesenchymal transition (EMT) via downregulation of transforming growth factor-beta 1 (TGF-β1)/receptor for activated C-kinase1 (RACK1)/snail in mice. An ovalbumin (OVA)-induced Balb/c mice airway allergic inflammatory model was used. Airway inflammation and remodeling were evaluated with hematoxylin and eosin (HE), periodic acid–Schiff (PAS), and Masson staining. E-cadherin and N-cadherin (molecular markers of EMT) were analyzed by immunofluorescence, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), and western blotting; α-smooth muscle actin (α-SMA) was evaluated using immunohistochemistry (IHC), qRT-PCR, and western blotting; and expression of TGF-β1/RACK1/Snail in lungs was measured by qRT-PCR and western blotting. Our data showed that azithromycin significantly reduced inflammation score, peribronchial smooth muscle layer thickness, goblet cell metaplasia, and deposition of collage fibers ($P < 0.05$), and effectively suppressed airway EMT (upregulated E-cadherin level, and downregulated N-cadherin and α-SMA levels) compared with the OVA group ($P < 0.05$). Moreover, the increasing mRNA and protein expressions of TGF-β1 and RACK1 and mRNA level of Snail in lung tissue were all significantly decreased in azithromycin-treated mice ($P < 0.05$). Taken together, our results suggest that azithromycin has the greatest effects on reducing airway remodeling by inhibiting TGF-β1/RACK1/Snail signal and improving the EMT in airway epithelium.

1. Introduction

Azithromycin, a macrolide antibiotic, exhibits promising effects on exacerbations of asthma [1]. Our previous studies have indicated that azithromycin (25 mg/kg) has significant protective effects against allergic airway inflammation and airway remodeling by inhibiting transforming growth factor-beta 1 (TGF-β1) expression [2] and inhibiting epithelial cell apoptosis in rat lungs [3]. Interestingly, azithromycin has been reported to inhibit TGFβ1-induced airway epithelial-mesenchymal transition (EMT) by suppressing Smad3 production in airway epithelial cells [4], implying that azithromycin might be used to treat asthma through ameliorating airway EMT. However, the exact cellular mechanism of azithromycin on airway EMT in asthma has never been investigated.

Airway remodeling is the key pathologic feature of asthma and is associated with the persistence of asthma symptoms and poor clinical outcomes [5]. The pathogenesis of asthmatic airway remodeling is generally believed to be associated with epithelial-mesenchymal transition [6]. Epithelial-mesenchymal transition is a dynamic process in which epithelial cells gradually lose their epithelial characteristics and acquire mesenchymal features. Currently, increasing evidence demonstrated that TGF-β1 is the initiation of EMT, and the development of EMT is generally accompanied with the loss of epithelial markers such as E-cadherin and the expression of mesenchymal markers such as N-cadherin and α-smooth muscle actin (α-SMA) [7]. In recent years, a number of studies have highlighted a critical role of snail on E-cadherin regulation [8] via binding E-cadherin promoters (E-boxes) [9], especially in asthma [10]. Moreover, previous studies have demonstrated that Snail was directly activated by the TGF-β1/Smad signaling pathway [11,12] or indirectly mediated by receptor for activated C-kinase1 (RACK1) [13], which is the downstream target gene of TGF-β1 in primary hepatic stellate cell (HSCs) [14]. Notably, azithromycin was also documented to recover the reduction of E-cadherin level induced by TNF-α in gingival epithelial cells [15].

Based on these literature evidences, we designed our study to investigate whether azithromycin possessed its antifibrotic effect through

* Corresponding author at: Department of Pharmacology, 3-17 Renmin South Road, West China School of Basic Medical Sciences & Forensic Medicine, Sichuan University, Chengdu, Sichuan 610041, PR China.
E-mail address: wanlihong1976@sina.com (L. Wan).

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suppressing EMT by blocking activation of the TGF-β1 pathway in OVA-induced Balb/c mice.

2. Materials and methods

2.1. Animals and ethics statement

Twenty-four male Balb/c mice, aged 5 to 6 weeks and weighing 20 ± 2 g, were obtained from Da-Shuo Biological Technology Co., Ltd. (Chengdu, China). The mice were acclimated for 1 week under standard conditions of ambient temperature (22 °C ± 1 °C), humidity (60% ± 10%), and with a 12-h light/dark cycle (lights on at 8:00 am). All experimental procedures were conducted in accordance with the guidelines of the Experimental Research Institute of Sichuan University in agreement with the guidelines of the Canadian Council on Animal Care (Permit Number: 2003-149). Animal ethics approval has been obtained from West China School of Basic Medical Sciences and Forensic Medicine.

2.2. Experimental groups

All mice were randomly divided into four groups (n = 6) including the control group (saline challenge + saline treatment), the OVA group (OVA challenge + saline treatment), the dexamethasone (DEX) group (OVA challenge + 0.7 mg/kg DEX treatment), and the azithromycin (AZM) group (OVA challenge + 35 mg/kg AZM treatment, corresponding to human clinical equivalent dosage of 250 mg/d).

2.3. OVA-induced mice airway allergic inflammatory model and treatment

Balb/c mice were sensitized by an intraperitoneal injection of 20 μg of OVA (Sigma-Aldrich, USA) in 0.2 mL saline and 20 mg aluminum hydroxide (Aldrich, USA) in 0.2 mL of saline on days 0 and 14. From day 21, mice were given aerosol challenges with 1%OVA for 60 min using an ultrasonic nebulizer (NE-U12; Omron Co., Tokyo, Japan) once daily. The control mice were challenged with saline instead of 1%OVA aerosol in a similar manner. From day 21, OVA-sensitized mice were administrated with 0.2 mL of saline containing DEX (0.7 mg/kg) or AZM (35 mg/kg) orally 1 h prior to the 1%OVA challenge, whereas the control mice were treated in the same way with normal saline. On day 28, all mice were euthanized and decapitated. The inferior lobes of the right lungs from 6 animals in each group were rapidly removed, dissected, and stored at −80 °C. The left lung lobes were immediately fixed in 10% (v/v) neutral-buffered formalin for 24 h at 4 °C and embedded in paraffin. The schematic diagram of the treatment schedule is shown in Fig. 1.

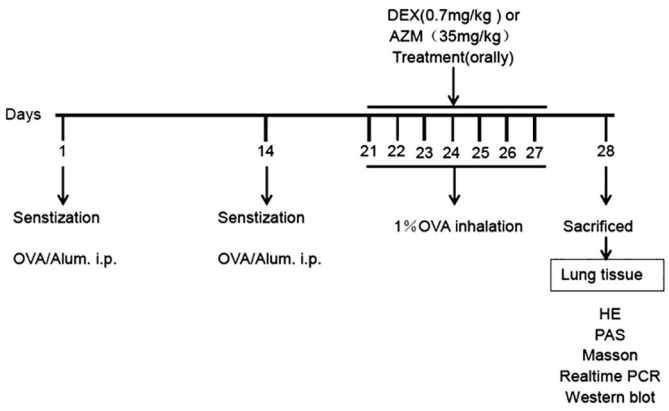

Fig. 1. Mouse model of airway allergic inflammatory and treatment with azithromycin.

2.4. Lung tissue histopathology

The tissues were sectioned at 4 μm thickness and stained with hematoxylin and eosin (HE) (Sigma, St. Louis, MO), periodic acid–Schiff (PAS) solution (IMEB, San Marcos, CA), and Masson trichrome staining to assess the inflammation, epithelial injury, and degree of collagen deposition, respectively. The airway wall thickness (Wat) and the bronchial smooth muscle thickness (Wam) were quantified by Image-Pro® Plus 6.0 software (Media Cybernetics, Bethesda, MD) by adjusting to basement membrane length (μm), and the inflammatory degree was scored by two independent observers blinded to the experiment. The detail methods refer to our previous publication [3]. An increment of 0.5 was given if the inflammation scores were in between two grades, and the total inflammation score was calculated by the addition of both peribronchial and perivascular inflammation scores (n = 10 airways from 3 to 5 animals). The thickness of the epithelial layer and mucus accumulation in the epithelial layer were quantified using PAS-stained sections with Image-Pro® Plus 6.0 software by dividing the epithelial area (μm^2) or the PAS^+ area in the epithelium (μm^2) by the length of the basement membrane (μm) around a bronchus. The bronchi (0.4–0.6 mm in diameter) in each animal were randomly chosen to quantify the fibrosis score. The degree of fibrosis was graded from 0 to 3 using Masson-stained sections, and the detail methods refer to our previous publication [16].

2.5. Immunohistochemistry (IHC)

Paraffin-embedded sections of lung tissue were assessed with IHC. After quenching with 3% H_2O_2, the sections were incubated with anti-α-SMA antibody (1:2000, G13044, Servicebio, China) overnight at 4 °C, followed by incubation with a secondary antibody (G1211, Servicebio, China) at room temperature for 50 min. Immunoreactivity was visualized with diaminobenzidine (DAB) (G1211, Servicebio, China). Brown staining was considered positive. Images were captured using an Olympus microscope (IX51; Olympus Corporation, Tokyo, Japan).The sections were evaluated by Image-Pro Plus 6.0 software, and the average of optical density (AOD) was quantified at 200× magnification.

2.6. Immunofluorescence

Co-staining was performed on lung tissue sections. Antigen retrieval was performed in a pressure cooker using ethylenediamine tetraacetic acid (EDTA; pH 8.0) for 20 min in boiling water. Then, sections were incubated overnight at 4 °C with primary antibodies raised against E-cadherin (GB11082; 1:200; Guge, China) and N-cadherin (GB11135; 1:400; Guge, China). Subsequently, CY3 conjugated goat anti-rabbit IgG antibody (GB25303; 1:300; Guge, China) was used as a secondary antibody and incubated with the sections for 50 min at room temperature. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, 1:200) for 10 min at room temperature. Images were captured using an Olympus microscope (IX51; Olympus Corporation, Tokyo, Japan).

2.7. mRNA and protein preparation

The total RNA and protein of lung were extracted by the Eastep®Super Total RNA Extraction Kit (Promega, China). Protein concentrations were determined using the BCA protein assay kit (Beyotime Institute of Biotechnology, China).

2.8. qRT-PCR

Total RNA (1 μg) was reverse-transcribed with a Revert Aid First Strand cDNA Synthesis Kit (Thermo, USA). The CFX96™ Real-Time PCR Detection System (Bio-Rad, USA) was applied for the mRNA amplification of related genes using the following forward and reverse primers