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Azithromycin ameliorates airway remodeling via inhibiting airway epithelium apoptosis

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ABSTRACT

Aims: Azithromycin can benefit treating allergic airway inflammation and remodeling. In the present study, we hypothesized that azithromycin alleviated airway epithelium injury through inhibiting airway epithelium apoptosis via down regulation of caspase-3 and Bax/Bcl2 ratio in vivo and in vitro.
Main methods: Ovalbumin induced rat asthma model and TGF-β1-induced BEAS-2B cell apoptosis model were established, respectively. In vivo experiments, airway epithelium was stained with hematoxylin and eosin (HE) and periodic acid–Schiff (PAS) to histologically evaluate the airway inflammation and remodeling. Airway epithelium apoptotic index (AI) was further analyzed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), while expression of apoptosis related gene (Bax, Bcl2, Caspase-3) in lungs were measured by qRT-PCR and western blotting, respectively. In vitro experiments, apoptosis were evaluated by Flow cytometry (FCM) and TUNEL. Above apoptosis related gene were also measured by qRT-PCR and western blotting.
Key findings: Compared with the OVA group, azithromycin significantly reduced the inflammation score, peribronchial smooth muscle layer thickness, epithelial thickening and goblet cell metaplasia ($P < 0.05$), and effectively suppressed AI of airway epithelium ($P < 0.05$). Moreover, the increasing mRNA and protein expressions of Caspase-3 and Bax/Bcl-2 ratio in lung tissue were all significantly decreased in azithromycin-treated rats ($P < 0.05$). In vitro, azithromycin significantly suppressed TGF-β1-induced BEAS-2B cells apoptosis ($P < 0.05$) and reversed TGF-β1 elevated Caspase-3 mRNA level and Bax/Bcl-2 ratio ($P < 0.05$).
Significance: Azithromycin is an attractive treatment option for reducing airway epithelial cell apoptosis by improving the imbalance of Bax/Bcl-2 ratio and inhibiting Caspase-3 level in airway epithelium.

1. Introduction

Airway epithelial cell is the first physical barrier in the airway, which is also an important target of inflammatory insults [1]. Epithelial damage is now considered as a novel pathogenic mechanism of asthma, contributing to airway inflammation and remodeling [2]. Following lung injury, epithelial cells produced a big amount of transforming growth factor-β1 (TGF-β1), inflammatory cells and fibroblasts to induce persistent and severe subepithelium fibrosis [3]. Additionally, TGF-β1 played a crucial role in cell differentiation, proliferation, and apoptosis process, including apoptosis of airway epithelial cells [4,5]. Thus, it is interesting to explore novel therapeutic agents to restore epithelium integrity via inhibiting epithelial cells apoptosis process.

Long-term administration of azithromycin (AZM), the 15-member macrolide, at the dose of 250 mg daily could reduce the severity of bronchial hyperresponsiveness in patients with mild asthma [6] and in a murine model of chronic asthma, 75 mg/kg AZM ameliorates not only airway inflammation but also airway remodeling [7]. In our previous study, we also found azithromycin at the dose of 25 mg/kg possess significant protective effects against allergic airway inflammation, including significant reduction of the number of lymphocytes, eosinophils and neutrophils and inhibition of IL-2, IL-4, IL-5, IL-13, TNF-α levels in BAL fluid [8]. Importantly, azithromycin also possess significant protective effects against airway remodeling via suppressing TGF-β1 expression in rat lungs [8]. Moreover, Hodge et al. found that azithromycin could notably improve the phagocytosis of apoptotic bronchial epithelial cells by alveolar macrophages (AMs) [9]. A recent report has also demonstrated that the proportion of apoptotic bronchial epithelial cells was markedly reduced in low-dose azithromycin-treated COPD patients [10]. Conversely, corticosteroid (CS) therapy actually induced airway epithelial apoptosis in mice model [22,23]. This might be the major reason that some patients may continue to have asthma symptoms and

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a progressive decline in lung function despite their use of CS therapy. Based on these literature evidences, we hypothesized that azithromycin was likely to be a competitive drug in long-term asthma treatment by inhibiting airway epithelium apoptosis. As an inducer of apoptosis in variety epithelial cells, TGF-β has been demonstrated highly enhanced apoptosis in human bronchial epithelial cells by caspase-3 activation [11] and Bcl-2 downregulated [33].

To investigate the potential mechanisms of azithromycin in airway epithelium apoptosis, OVA-induced rat model and TGF-β1-induced BEAS-2B cells apoptosis model were used in this study. The present study showed not only peribronchial smooth muscle layer thickness, epithelial thickening and goblet cell metaplasia but also AI of airway epithelium was significantly inhibited in azithromycin-treated OVA rat model. Consistently, AZM markedly suppressed TGF-β1-induced BEAS-2B cells apoptosis. Moreover, these effects appeared to be mediated through the inhibition of level of Caspase-3 and Bax/Bcl-2 ratio in vivo and in vitro.

2. Materials and methods

2.1. Cell culture

Transformed human bronchial epithelial cells, BEAS-2B cells donated by Dr. Xiaodong Sun (Chengdu, China) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Harry Biotech, Chengdu, China) supplemented with 15% heat-inactivated FBS (Gibco, 10,099–141) and seeded on 6-well culture dishes at 1.5×10^5 cells/ml. When cells reached 60%–70% confluence, the media was changed and cells received serum-free media for 24 h. Then, cells were subjected to stimulation with 3.3, 10 and 30 μg/ml azithromycin (AZM) and recombinant human TGF-β1 (30 ng/ml, PEPROTECH, USA, 0715209) for an additional 72 h. TGF-β1 was used as a positive control in this study.

2.2. Apoptosis assay

2.2.1. Flow cytometry (FCM)

Apoptosis was measured by using an annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Annexin V-FITC/PI, Vazyme Biotech, A211-02). In brief, cells were plated at a density of 4×10^5 cells per well into 6-well plate and cultured in DMEM medium supplemented with 15% heat-inactivated fetal bovine serum and antibiotics at 37 °C in 5%CO_2 (v) in a humidified incubator for 24 h and treated with different concentrations of azithromycin. Untreated cells were used as negative control. After 72 h, the cells were harvested by trypsinization (Solarbio, T1350-100), washed twice with cold PBS (2000 rpm, 5 min), and resuspended in 100 μl of binding buffer. Annexin V-FITC (5 μl) and propidium iodide (PI, 5 μl) were added to each sample, and the mixture was incubated in the dark for 10 min at room temperature. Cells were immediately subjected to FACS analysis (BD Accuri C6, USA) within 1 h. Ex = 488 nm and Em = 530 nm. Both PI and Annexin V negative cells were considered as normal, PI negative and Annexin V positive cells were considered as early apoptotic, cells that were both PI and Annexin V positive were considered as late necrotic, and cells that were PI positive and Annexin V negative were considered as mechanically injured during the experiment. All the experiments were conducted in triplicates. Apoptosis rate (%) = number of apoptotic cells/total number of nucleated cells × 100%.

2.2.2. TUNEL staining

TUNEL staining for apoptotic cells was carried out using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay according to the manufacturer's instructions. Briefly, after 72 h treatment with different concentrations of azithromycin, the cells were fixed by adding 4%paraformaldehyde (Kelong Chemical Reagent Factory, China) and incubating for 30 min. The fixed cells were washed in PBS, permeabilized with 0.1% Triton X-100 for 10 min on ice, washed in PBS twice, and then incubated with 50 μl of terminal deoxynucleotidyl transferase end-labeling solution for 60 min at 37 °C in a humidified chamber in dark. Cells containing green granules in the nucleus were regarded as positive for TUNEL. Nuclei were stained by DAPI. Fluorescence signals were detected with a fluorescence microscope system (IX73, OLYMPUS, Tokyo, Japan). DAPI and apoptosis positive cells were enumerated using the National Institutes of Health ImageJ software.

2.2.3. Animals and ethics statement

Forty male Sprague-Dawley (SD) rats, aged 6–7 weeks and weighing 190–210 g, were obtained from Da-Shuo Biological Technology Co., Ltd. (Chengdu, China), and used after 1 week of acclimation. 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 our institution. The holding room was under standard conditions of ambient temperature (20 ± 1 °C), humidity (60 ± 10%), and with a 12-h light/dark cycle (lights on at 8:00 AM) throughout the whole study.

2.3. Experimental groups

All rats were divided into four groups randomly ($n = 10$): the control group (saline challenge), the OVA group (OVA challenge + saline treatment), the dexamethasone (DEX) group as a positive control (OVA challenge + 0.5 mg/kg DEX treatment), and the azithromycin (AZM) group (OVA challenge + 25 mg/kg AZM treatment, corresponding to human clinical equivalent dosage 250 mg/daily) [6,10].

2.4. OVA-induced rat asthma model and treatment

A schematic diagram of the treatment schedule is shown in Fig. 1. Rats were immunized by a subcutaneous injection of 1 mg of OVA (Sigma-Aldrich, USA) in 1 ml saline and 200 mg aluminum hydroxide (Aldrich, USA) in 1 ml of saline on day 1 and 6. From day 7 to 13, after initial sensitization, rats were given aerosol challenges with 1%OVA for 30 min using an ultrasonic nebulizer (NE-U12; Omron Co., Tokyo, Japan) once daily. The control rats received aerosol challenges with saline in a similar manner. From day 7 to 13, OVA-sensitized rats were intragastrically with 0.2 ml of saline containing DEX (0.5 mg/kg) or AZM (25 mg/kg) 1 h prior to OVA administration, while the control group was treated in the same way with normal saline. On day 14, all rats were sacrificed by decapitation. The inferior lobes of right lungs from 10 animals in each group were rapidly removed, dissected and stored at −80 °C.

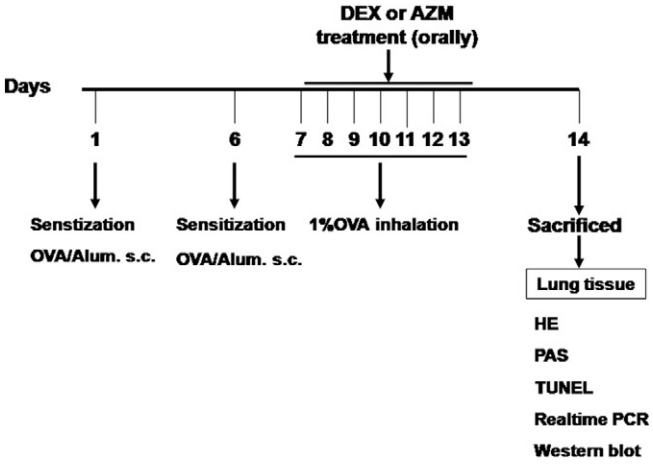

Fig. 1. Rat model of airway inflammation and treatment with azithromycin.