Dexamethasone suppresses bleomycin-induced pulmonary fibrosis via down-regulation of jagged1/notch1 signaling pathway

Yan Zhou¹, Shiping Liao², Bo Wang², Zhongwei Zhang¹

¹Department of Intensive Care Unit, West China Hospital, Sichuan University, Chengdu 610041, Sichuan, P. R. China; ²Functional Laboratory, West China School of Preclinical and Forensic Medicine, Sichuan University, Chengdu 610041, Sichuan, P. R. China

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Abstract: Dexamethasone (DEX) plays an important role in attenuating bleomycin (BLM)-induced lung fibrosis in mice. Recently, inhibition of Notch signaling is a potential therapeutic strategy for pulmonary fibrosis. To investigate whether dexamethasone exerts protective effect on BLM-induced pulmonary fibrosis via inhibiting the Notch signaling, BLM-induced rat model was used. All SD rats received daily intraperitoneally administration of DEX (3 mg/kg) or saline 1 day after BLM instillation daily for 28 days. Histological changes in the lung were evaluated by hematoxylin and eosin and Masson’s trichrome staining. The expression of α-smooth muscle protein (α-SMA) was measured by immunohistochemistry (IHC). The mRNA and protein level of Jagged1, Notch1 and transforming growth factor-β1 (TGF-β1) was analyzed by qPCR and Western blot, respectively. Our data showed that BLM induced severe alveolitis and pulmonary fibrosis; together with significant elevation of α-SMA, TGF-β1, Jagged1 and Notch1. DEX administration notably attenuated the degree of alveolitis and lung fibrosis with the decreasing of α-SMA, TGF-β1, Jagged1 and Notch1 in lungs. These results demonstrated that DEX (3 mg/kg) may suppress BLM-induced pulmonary fibrosis in rat via down-regulation of Jagged1/Notch1 signaling pathway.

Keywords: Dexamethasone, bleomycin, pulmonary fibrosis, jagged1/notch1

Introduction

Idiopathic pulmonary fibrosis (IPF) is a severe chronic fibrosis interstitial (noninfectious) lung disease, which is currently untreatable [1]. All the available pharmacological interventions are mainly supportive. Dexamethasone is one of the primary first lines of medicine in treating pulmonary fibrosis, due to its anti-inflammatory effects [2]. A recent compelling study has demonstrated that dexamethasone (DEX) could significantly suppress bleomycin (BLM)-induced pulmonary fibrosis by inhibition of TGF-β, Smad3 and JAK-STAT pathway [3]. However, the further exact mechanism of DEX on pulmonary fibrosis is incompletely understood.

Accumulating evidences indicate that accompanied with inflammation, the appearance and persistence of epithelial-mesenchymal transition (EMT) and activated myofibroblasts which over-expressed α-smooth muscle protein (α-SMA) play essential roles in the pathogenesis of IPF [4].

Notch signaling is a highly conserved signaling pathway to coordinate cell fate and tissue morphogenesis [5]. There are four types of Notch receptor (Notch 1-4) and five ligands (Jagged-1 and -2, and Delta-like-1, -3 and -4) were found in mammals. When a cell-surface expressed ligand binding to the Notch receptor, the Notch intracellular domain (NICD) is released and migrated into the nucleus to interact with transcriptional repressors [6]. Recently, Notch signaling has also been reported to be involved in several kinds of tissue fibrosis through inhibiting transforming growth factor-β1 (TGF-β1)/Smad3-induced fibroblast-myofibroblast transition, such as pulmonary fibrosis [7], hepatic fibrosis [8], and kidney fibrosis [8, 9].

However, there is no study examined the effect of DEX on notch signaling in BLM-induced pul-
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monary fibrosis. This study focused on evaluating the potential effect mechanism of DEX on BLM-induced IPF in an experimental rat model and explored its effect on Jagged1/Notch1/TGF-β1 signaling pathway.

Materials and methods

Experimental animals

Male Sprague Dawley rats (n = 45) weighing 250-300 g were purchased from purchased from Da-Shuo Biological Technology Co, Ltd (Chengdu, China). The rats were allowed free access to a standard diet and sterile water in a restricted access room that was temperature-controlled (20 ± 1°C) and humidity-controlled (60 ± 10%) and under a 12-h light/12-h dark cycle. All the experimental procedures were performed in accordance with the guidelines of the Experimental Research Institute of Sichuan University (Permit Number: 2003-149).

Pulmonary fibrosis models

The rats were randomly allocated into four groups: (1) control group; (2) BLM group; and (3) BLM+DEX group (n = 15), respectively. Bleomycin-induced pulmonary fibrosis model was performed by intratracheal instillation of BLM (Nippon Kayaku Co Ltd, Japan) at a dose of 5 mg/kg dissolved in 150 μL sterile 0.9% sodium chloride. Dexamethasone (DEX, 3 mg/kg) was administered intraperitoneally 1 day after bleomycin injection daily for 28 days. Control animals were injected intratracheally with 150 μL of sterile saline of 0.9%.

All rats were anaesthetized with intraperitoneal 15% Chloral hydrate (0.3 mL/100 g) and killed by abdominal aorta exsanguination 7, 14 and 28 days after the first injection of BLM. Five rats from each group were killed on each day. The left lungs were cut for next histological assays and the right lungs were maintained in a -80°C freezer for RT-PCR and Western blotting analyses.

Histology

The left lungs were fixed in 4% paraformaldehyde for ≥72 h, and embedded in paraffin. Five-micron-thick serial sections were stained with HE to grade the degree of alveolitis. Lung fibrosis was evaluated by Masson staining (Sigma-Aldrich) for collagen accumulation, in accordance with the manufacturers’ protocols.

Immuno histochemical (IHC) assay of α-SMA in lung tissue

The paraffin-embedded sections of lung tissue slide were routinely deparaffinized and endogenous peroxidase was quenched with 3% H2O2 in 1x PBS. Epitope retrieval was performed by heating sections for 10 min in 10 mM citrate buffer (pH 6.0) in a water bath at 95-100°C. Nonspecific reactions were blocked with blocking solution of the Zymed kit (Zymed Laboratories, USA). The sections were incubated with primary antibodies including anti-α-SMA antibody (1:100, Santa Cruz, USA) and isotype control. After a 30-min incubation with the primary antibody at room temperature, the slides were washed twice with 1x PBS (5 min per wash), and then incubated with the secondary antibody solution (Zymed Laboratories, USA) for 35 min at room temperature. The slides were then counterstained in a hematoxylin solution. α-SMA positive cells were visualized using an ABC staining system (Santa Cruz Biotechnology). Negative controls for all staining were performed by omitting the primary antibodies or the isotype control antibodies. The sections were evaluated by Image-Pro Plus 6.0 software, which quantified the number of positive cells at 200× magnification.

RNA isolation and qRT-PCR

Total RNA (1 μg), obtained using Gene JET RNA Purification Kit (Thermo scientific Inc, MA, USA), was subjected to reverse transcription with a Revert Aid First Strand cDNA Synthesis Kit (Thermo scientific Inc, MA, USA). Realtime RT-PCR was performed with BIO-RAD S1000 Thermal Cycler (Thermo scientific Inc, MA, USA). The conditions for amplification of β-actin, Jagged1, Notch1, and Tgf-β1 were: 95°C for 40 sec for 1 cycle; 95°C for 10 sec, 56°C for 10 sec, and 72°C for 20 sec for 45 cycles; and 95°C for 10 sec for 1 cycle. Negative controls performed with no RT yielded no detectable fragments with either primer pair. The sequences of primers used for rat β-actin (263 bp) were: 5′-GAGACCTTCAACACCCCAGC-3′ (sense) and 5′-ATGTCACGCACGATTTCCC-3′ (antisense). The sequences of primers used for rat Jagged1 (190 bp) were: 5′-AACTGTACCGTGGCGAA-3′ (sense) and 5′- TGATGCAAGATCTCCCTGAAAC-3′ (antisense). The sequences of primers used for rat Notch1 (267 bp) were: 5′-ATGATGCCACACCCCTTCTG-3′ (sense) and 5′-CCAGCAACACT-
Figure 1. Effect of Dexamethasone on histopathological changes of bleomycin-induced pulmonary fibrosis. (A) Representative images of HE staining and Masson’s trichrome staining on Day 7, 14 and 28 after bleomycin injection (magnification, 400×). Pathohistological scores of (B) alveolitis and (C) pulmonary fibrosis in bleomycin-induced lung fibrosis in rats. Data are expressed as mean ± SEM of three independent experiments. *P<0.05, significantly different compared with the control group; #P<0.05, significantly different compared with the BLM group.

TTGGCAGTC-3’ (antisense). The sequences of primers used for rat Tgf-β1 (215 bp) were 5’-TCCATGACATGAACCGACCC-3’ (sense) and 5’-GAAGTTGCGATGTAGCCCT-3’ (antisense). Pri-
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Protein isolation and western blot

Lung tissues were homogenized in lysis buffer (RIPA 500 µl and PMSF 5 µl) and centrifuged at 12,000g for 30 min at 4°C. Then, the proteins were resolved by electrophoresis in a 10% SDS-PAGE gel and transferred to a polyvinylidene difluoride (PVDF) membrane (BIO-RAD, USA). The PVDF membrane was incubated in 5% non-fat milk for 50 min at room temperature. After being washed with 0.1% Tween 20-Tris-buffered saline (TBS) 3 times, 5 min/time, the PVDF membrane was incubated serially in anti-Notch1 monoclonal antibody from rat (1:1000, Santa Cruz, USA), anti-Jagged1 monoclonal antibody from rat (1:1000, Santa Cruz, USA), and anti-TGF-β1 monoclonal antibody from rat (1:1000, Santa Cruz, USA) overnight at 4°C. Between incubations with the primary antibody, the PVDF membrane was washed in 0.2% Tween 20-TBS thrice, 10 min/time, and incubated in HRP goat anti-rat IgG (1:6000, Zhongshan-Beijing, China) at room temperature for 2 h. Expressions of Notch1, Jagged1, and TGF-β1 were detected by an enhanced chemiluminescence system (Millipore, USA) and exposure to film (Kodak, USA). β-actin (1:10000; Abcam) was included in all samples as a control against false negative results. Expression levels of the proteins were compared with β-actin based on the relative intensities of the bands. Band density was quantified using the software program Bio-Rad Quantity One v4.6.6.

Statistical analysis

Statistical analyses were performed with SPSS statistics 17.0 software. All values were expressed as mean ± standard error of the mean. Statistical comparisons were made via one-way ANOVA with Tukey’s post-hoc test. P-values <0.05 were considered statistically significant.

Results

Dexamethasone alleviates BLM-induced pulmonary fibrosis in rat

In this study, Hematoxylin-eosin and Masson staining of lung tissues were used to examine the histopathological changes in BLM-induced pulmonary fibrosis rat model. As shown in Figure 1A, lung tissue from the saline treated control group showed normal alveolar spaces and normal thickening of the alveolar septa. Compared with the control group, BLM induced significant airway inflammatory response at Day 7 after the initial administration, reaching a maximum on Day 14 after the initial injection, which manifested obvious more interstitial infiltration by inflammatory cells. Besides, Masson staining showed BLM induced the deposition of microfibrils in thickened alveolar walls, and fibroblast accumulation in areas where alveolar spaces had collapsed at Day 14 after the initial injection, reaching a maximum on Day 28 after the initial injection. Such changes were significantly alleviated by dexamethasone pretreatment.

The pathological scoring of BLM-induced lung inflammation and pulmonary fibrosis by semi-quantitative histological analysis is shown in Figure 1B and 1C. The results show that the alveolitis and pulmonary fibrosis scores were significantly increased in the BLM model group (P<0.001), as compared to control group. Compared with BLM model group, administration of dexamethasone obviously decreased the scores of alveolitis (P<0.05) and lung fibrosis (P<0.01).

Dexamethasone alleviated bleomycin induced epithelial-mesenchymal transition (EMT) in lung

Alpha-SMA is accepted as a marker of epithelial-mesenchymal transition (EMT). Immunohistochemistry (IHC) was performed to detect the expression of α-SMA in lung. As our expected, BLM induced obviously α-SMA up-regulated in the lung tissue. As shown in Figure 2A and 2B, α-SMA-positive cells were significantly elevated in the lungs of BLM model rat at Day 7 after the initial injection (P<0.0001). Moreover, BLM induced up-regulation of α-SMA in the lungs was markedly inhibited by dexamethasone treatment (P<0.0001). As shown in Figure 2B, the number of α-SMA-positive cells in the lungs was significantly reduced in BLM + DEX-treated rats as compared with BLM-treated rats (P<0.0001).

Dexamethasone inhibited the mRNA and protein expression of Jagged1, Notch1 and TGF-β1 expression in lung tissues of rat

To further confirm the mechanism of dexamethasone on bleomycin induced EMT in lung of rat, the mRNA and protein expression of Jagged1,
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Notch1 and TGF-β1 expression in lung tissues on Days 7, 14, and 28 were evaluated by qRT-PCR and western blotting, respectively. As shown in Figure 3E-G, compared to the saline-treated control rats, real-time PCR showed that the mRNA expression of Jagged1, Notch1 and TGF-β1 significantly increased in BLM-treated rats as compared with saline-treated control rats, especially at Day 28 after the initial injection (P<0.0001). Compared to BLM-treated rats, treatment with dexamethasone significantly decreased the mRNA expression of Jagged1, Notch1 and TGF-β1 in lung tissues on Days 14, and 28, but not on Day 7 (P<0.0001).
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A similar tendency was detected by western blot analysis (Figure 3A-D). The protein levels of Jagged1, Notch1 and TGF-β1 markedly up-regulated in BLM-treated rats as compared with saline-treated control rats at Day 7 after the initial injection, reaching a maximum on Day 28 after the initial injection (Figure 3E-G). The protein level of Jagged1, Notch1 and TGF-β1 after dexamethasone pretreatment dramatically decreased from Day 7 to Day 28, as compared to BLM-treated rats (P<0.0001).

Discussion

Glucocorticoids are the first-line therapy of acute lung injury (ALI) and pulmonary fibrosis [2], especially in China [10]. However, the application of glucocorticoids in ALI and PF were always controversial. The opposite effect of glucocorticoids treatment on pulmonary function has been demonstrated in patients and animal model [11, 12]. The consistent results were low-dose of dexamethasone was benefit for
bronchopulmonary hyperreactivity and systemic inflammation [12]. Therefore, in the present study, BLM induced IPF model of rats was established to investigate the mechanism of low dose of dexamethasone (3 mg/kg) on IPF. This study showed that dexamethasone (3 mg/kg) had a significant inhibitory effect on BLM-induced pulmonary fibrosis. In detail, after treatment of DEX, the scores of alveolitis and lung fibrosis and expression of α-SMA protein were obviously decreased. So DEX treatment not only suppressed BLM-induced acute inflammatory injury but also attenuated the fibrotic changes such as collagen accumulation and epithelial-mesenchymal transition (EMT).

Transforming growth factor β (TGF-β) is a well-known stimulant of extracellular matrix production [24], which is involved in the initiation and progression of fibrosis [13]. TGF-β1 is in BLM induced animal model, BLM stimulated alveolar macrophages secreting large quantities of active TGF-β, and initiated the transformation of fibroblast into myofibroblasts. Finally, deposition of excessive ECM causes the development of pulmonary fibrosis [14]. Recently, Shi et al. [3] reported that the possible mechanism of dexamethasone in treating pulmonary fibrosis in mice model is inhibiting TGF-β pathway. Consistently, in this study, we found that TGF-β1 increased markedly from Day 7 to 28 in BLM-treated rats together with a severe IPF, which was also obviously reduced by DEX treatment. In line with these studies, we observed increased lung mRNA levels of TGF-β1 after endotracheal instillation of BLM, which was significantly decreased by DEX treatment, too.

Interestingly, TGF-β is the Notch pathway-related molecules [15]. Notch, a kind of transmembrane protein which was first found in flies, plays a crucial role in coordinated cell fate determination and tissue morphogenesis [16], including the lungs [17]. Among the five Notch ligands (Delta-like1, 3 and 4 and Jagged1, 2), Jagged1 was expressed in bronchial and bronchiolar epithelial cells and contributed to control proximal airway cell fate and differentiation [18]. In addition, several lines of evidence suggest that Notch1 was highly localized to the basal cell population [19] and normal activity of Notch1 is required for repair of the airway epithelium [20]. Recently, a report of Aoyagi-Ikedaa et al. in 2011 demonstrated that Notch1 was also expressed in SMA positive myofibroblasts both in a rat model of BLM-induced pulmonary fibrosis and in patients [7]. Furthermore, in pulmonary fibrosis, Notch signaling can be activated in cross-talk with other signaling pathways, such as TGF-β to induce myofibroblast differentiation through activates SMA gene transcription [7, 21]. Together, all these suggested that Jagged1/Notch1 signal pathway is a potential therapeutic target in IPF. Our results demonstrated that the mRNA and protein expression of Jagged1 and Notch1 also increased after BLM treatment in the current study. DEX treatment significantly prevented the increase of Jagged1 and Notch-1 expression, accompany with alleviation of collagen deposition and fibrosis.

In conclusion, BLM administration induced a progressive and significant inflammation, exacerbated fibrosis, and severe alveolar destruction in rat lungs with significant increase of α-SMA and elevation of Jagged1, Notch1 and TGF-β1. Besides, Lung mRNA levels and protein levels of Jagged1, Notch1 and TGF-β1 were down-regulated after dexamethasone (3 mg/kg) administration. The protein level of α-SMA was down-regulated after dexamethasone (3 mg/kg) administration, too. Overall, our results suggested that dexamethasone (3 mg/kg) alleviated BLM induced IPF in rat via suppressing Jagged1/Notch1 signaling pathway.

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Disclosure of conflict of interest
None.

Address correspondence to: Zhongwei Zhang, Department of Intensive Care Unit, West China Hospital, Sichuan University, Chengdu 610041, Sichuan, P. R. China. Tel: 86-28-85423525; E-mail: 71646-1751@qq.com

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