The International Journal of Biochemistry & Cell Biology 79 (2016) 231–238

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Klf10 deficiency in mice exacerbates pulmonary inflammation by increasing expression of the proinflammatory molecule NPRA

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ARTICLE INFO

Article history:
Received 3 March 2016
Received in revised form 6 August 2016
Accepted 29 August 2016
Available online 31 August 2016

Keywords:
Krüppel-like factor 10
Atrial natriuretic peptide
Natriuretic peptide receptor A
Pulmonary disease
Inflammation

ABSTRACT

KLF10 is a transforming growth factor (TGF)-β/Smad downstream regulated gene. KLF10 binds to the promoter of target genes and mimics the effects of TGF-β as a transcriptional factor. In our laboratory, we noted that *Klf10* deficiency in mice is associated with significant inflammation of the lungs. However, the precise mechanism of this association remains unknown. We previously identified *NPRA* as a target gene potentially regulated by KLF10 through direct binding; *NPRA* knockout have known that prevented lung inflammation in a mouse model of allergic asthma. Here, we further explored the regulatory association between KLF10 and NPRA on the basis of the aforementioned findings. Our results demonstrated that KLF10 acts as a transcriptional repressor of NPRA and that KLF10 binding reduces NPRA expression *in vitro*. Compared with wild-type mice, *Klf10*-deficient mice were more sensitive to lipopolysaccharide or ovalbumin challenge and showed more severe inflammatory histological changes in the lungs. Moreover, *Klf10*-deficient mice showed pulmonary neutrophil accumulation. These findings collectively reveal the precise site where KLF10 signaling affects pulmonary inflammation by attenuating NPRA expression. They also verify the importance of KLF10 and atrial natriuretic peptide/NPRA in exerting influences on chronic pulmonary disease pathogenesis.

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1. Introduction

Epidemiological studies in humans have revealed that asthma, chronic obstructive pulmonary disease (COPD), tuberculosis, pneumonia, and lung adenocarcinoma are chronic inflammatory lung diseases (Abramson et al., 2014; Glaser et al., 2015; Gomes et al., 2014). However, the mechanisms underlying the involvement of inflammation in the progression of these diseases remain unclear. Information regarding modulation of proinflammatory and anti-inflammatory pathways and involvement of specific cell types in inflammation has facilitated studying and explaining the effects of inflammation on neoplastic growth.

Transforming growth factor (TGF)-β, which is secreted by airway epithelial and inflammatory cells (Sheppard, 2015). *In vivo* studies have suggested contrasting roles of TGF-β and atrial natriuretic peptide (ANP) in the pathogenesis of various chronic pulmonary diseases (Lo et al., 2008). Moreover, previously study (Ishigaki et al., 2009) results indicated that continuous ANP infusion has the useful capacity to suppress the liver fibrosis through reducing TGF-β1 expression. By activating guanylate cyclase-coupled membrane receptors, ANP increases intracellular cyclic guanosine monophosphate (cGMP) levels, thus activating cGMP-dependent protein kinase in various cell types. Three ANP receptor subtypes, namely natriuretic peptide receptor A (NPRA), NPRB, and NPRC, have been identified and characterized through gene sequencing (Zhao et al., 2013). NPRA, expressed in cells of inflamed and injured tissues as well as tumors (Mallela et al., 2013), induces inflamma-

Abbreviations: ANP, Atrial natriuretic peptide; BAL, Bronchoalveolar lavage fluid; cGMP, Cyclic guanosine monophosphate; ChIP-PCR, Chromatin immunoprecipitation-polymerase chain reaction; COPD, Chronic obstructive pulmonary disease; EMSA, Electrophoretic mobility shift assay; IL, Interleukin; KLF10, Krüppel-like factor 10; LPS, Lipopolysaccharide; MPO, Myeloperoxidase; NPR, Natriuretic peptide receptor; OVA, Ovalbumin; PBS, Phosphate-buffered saline; SMAD, Homolog of mothers against decapentaplegic; TNF, Tumor necrosis factor; TGF, Transforming growth factor.

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http://dx.doi.org/10.1016/j.biocel.2016.08.027
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tion; the severity of antigen-induced pulmonary inflammation is significantly lower in *NPRA*-deficient mice than in those expressing NPRA. Moreover, a lack of NPRA can substantially protect C57BL/6 mice from xenografts containing lung cancer, skin cancer, and ovarian cancer cells (Kong et al., 2008; Wang et al., 2011).

Notably, ANP signaling has a proinflammatory effect on the anti-inflammatory TGF-β1 signaling pathway through blockade of TGF-β1-induced Smad2/3 nuclear translocation (Li et al., 2007). However, such counterregulatory mechanisms involving both signaling pathways remain speculative. Krüppel-like factor 10 (*Klf10*), a TGF-β/Smad inducible early gene, is expressed in specific cell types among numerous tissues and is involved in repression of cell proliferation and inflammation (Subramaniam et al., 2010). Studies have indicated that KLF10 binds to SP/KLF DNA sequences and activates or represses the transcription of numerous genes, suggesting the importance of KLF10 as a factor mediating several specific types of signaling. A chromatin immunoprecipitation (ChIP)-chip assay was used to screen for possible targets of KLF10 on the basis of specific binding sites required by KLF10 for transcription regulation (Hsu et al., 2011; Wu et al., 2015; Yang et al., 2013): NPRA was identified as a gene regulated by KLF10 binding to the *NPRA* promoter. Given their importance in inflammation and cancer risk, we investigated the potential interrelated regulatory mechanisms associated with *KLF10* and *NPRA*. In the present study, we detected specific transcriptional regulation of NPRA by KLF10. Furthermore, by using *Klf10*-deficient mice, we confirmed the counterregulatory effects and role of NPRA in lung inflammation.

2. Materials and methods

2.1. Ethics statement

All animal experiments were conducted in accordance with the guidelines of the Laboratory Animal Center of Taipei Medical University (TMU), Taiwan. The animal use protocols were reviewed and approved by the TMU Institutional Animal Care and Use Committee. The *Klf10*-deficient mice were created as described in our previous manuscript (Yang et al., 2013). All homozygotes used in our experiments were commonly backcrossed to C57BL/6 over 8 generations, an inbred strain that has been well characterized.

2.2. Lipopolysaccharide-induced acute lung injury

We included 8-week-old wild-type (WT; control) and *Klf10*-deficient C57BL/6 mice; these mice were divided into two groups according to the concentration of lipopolysaccharide (LPS) treatment received—untreated and 40 μg/kg body weight. The mice were intratracheally instilled with each concentration of LPS dissolved in 40 μL of phosphate-buffered saline (PBS). For blood and bronchoalveolar lavage fluid (BAL) collection, all mice were sacrificed through isoflurane asphyxiation 24 h after LPS administration.

2.3. Ovalbumin-induced allergic airway inflammation

The 8-week-old C57BL/6 mice were sensitized through intraperitoneal injection of ovalbumin (OVA; 20 mg) emulsified in 2 mg of aluminum hydroxide in a total volume of 200 mL of PBS on day 0; the dose was supplemented with 50 mg of OVA emulsified in 2 mg of aluminum hydroxide on days 14 and 28. For OVA challenge, all mice were administered OVA (100 μg in a total volume of 40 μL PBS) intranasally on days 40, 41, and 42. Blood and BAL were collected 24 h after the final OVA challenge.

2.4. BAL collection

All experimental mice were sacrificed 24 h after LPS or final OVA challenge. Their chest cavities were opened carefully and their tracheas were exposed; BAL was collected by delivering 0.8 mL of cold PBS into the airway through a tracheal cannula and then gently aspirating the fluid. The BAL samples were stored at 4 °C for further analysis.

2.5. Inflammatory cell count

The total cell number was counted through hemocytometry. BAL cell smears were prepared and stained with Giemsa stain for differential cell counts. Three locations were randomly selected for differential cell counting, and the average leukocyte number from the three locations was used as the representative.

2.6. Total protein concentrations in BAL

The total protein concentration in BAL was determined using trichloroacetic acid precipitation (5%), followed by washing and resolubilization (Lowry et al., 1951). The readings were collected on a microplate reader (EZ Read 400, Biochrom) by using bovine serum albumin as the standard.

2.7. Myeloperoxidase assay

Myeloperoxidase (MPO) activity was determined using a mouse MPO duo set (R&D systems) as per the manufacturer instructions. MPO activity was measured on a spectrophotometer (EZ Read 400; Biochrom) by using absorbance at 450 nm. The MPO concentration was calculated by plotting the average optical absorbance of duplicated samples in the standard curve.

2.8. OVA-specific immunoglobulin e detection

Serum levels of OVA-specific immunoglobulin E (IgE) were measured using the LEGEND MAX™ Mouse OVA Specific IgE ELISA Kit with Pre-coated Plates (Biolegend) as per the manufacturer instructions. The concentration of OVA-specific IgE was calculated by plotting the average optical absorbance of duplicated samples in the standard curve.

2.9. Neutrophil-specific immunohistochemical staining

For immunohistochemical staining of mouse migratory neutrophils, we used rat antimouse neutrophil antibodies (AbD Serotec). We applied alkaline phosphatase-conjugated antirat IgG (Sigma Aldrich) as a secondary antibody. The signals were detected using fast red (Sigma Aldrich).

2.10. Plasmid construction and promoter luciferase assay

DNA fragments of interest, covering nearly 2 kb upstream of the mouse *NPRA* promoter region, were cloned into the pGL3-Enhancer vector (Promega) separately. Before being cloned, each fragment was amplified using polymerase chain reaction (PCR) with specific primers (forward: 5′-CTGGTACCGTGTGCGTGCGTGTTAG-3′; reverse: 5′-GAGGTACCGCAATCTCGGCTCGTTG-3′). The product was then separately inserted into the pGL3-basic vector by using the Kpn1 and Xho1 cutting sites. Luciferase activity was determined using a Dual-Luciferase Reporter Assay (Promega) after transfection.

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