Evidence of Two Ciliated Epithelial Cell Subsets in Mouse Airways

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Patients with asthma-associated airway epithelial damage exhale increased levels of nitric oxide (NO). However, the distribution of endothelial NO synthase (eNOS) in mouse airways remains to be controversial. In the present study, mouse lung sections were stained using antibodies against secretoglobin 1A member [1] (Scgb1a1), acetylated tubulin (ACT), and eNOS. We found that club cells in the mouse airways are immunoreactive toward eNOS. In addition, to the best of our knowledge, for the first time, two subsets of ciliated cells that differ in their expression of eNOS were observed to reside in the mouse airways. Both subsets of ciliated cells survived naphthalene-induced lung injury. These data will help clarify a controversial issue of whether ciliated cells contribute to epithelial maintenance in the airways.

Abstract

Keywords: Mouse airway; Ciliated cells; eNOS; Scgb1a1; Naphthalene

Introduction

Nitric oxide (NO), an important messenger molecule in cells, is formed endogenously in the airways of the lung. NO, generated by various isoforms of NO synthase (NOS) plays crucial and diverse physiological roles and has been implicated in several diseases including asthma and chronic obstructive pulmonary disease [1]. Endothelial NOS (eNOS), originally identified in the endothelium of vascular tissue, is responsible for the majority of the NO produced in this tissue [2]. NO that is produced by eNOS acts as a bronchial vasodilator [3]. In addition to its expression in vasculature tissues, eNOS has been found in the epithelia of airways and is important for their functions. For example, eNOS localizes to the basal membrane of ciliary microtubules in rat lungs and stimulates ciliary beat frequency, which determines the functions of the ciliated cells [4,5]. However, it remains controversial whether ciliated cells contribute to epithelial regeneration in the airways.

Ciliated cells are a terminally differentiated population that is replenished by club cells and/or stem cells residing in the airway epithelia under steady state or during lung injuries [6,7]. Several studies have shown that ciliated cells are capable of giving rise to goblet cells under certain conditions. For example, the epidermal growth factor receptor is activated in ciliated cells during Sendai virus infection, leading to the expression of goblet cell markers in these cells [8]. Turner and colleagues have provided evidence that human ciliated cells convert to goblet cells in culture [9]. In these studies, ciliated cells are characterized by only a few markers including acetylated tubulin (ACT) and Forkhead box protein J1 (FoxJ1). Thus, two or more functionally distinct ciliated cells possibly exist in the airway epithelia, and they cannot be distinguished using the ACT or FoxJ1 markers. In this study, we present preliminary immunofluorescent staining evidence showing that the ciliated cells of the mouse airway include two subsets that are distinguished by their expression of eNOS. These data help clarify the contribution of ciliated cells to epithelial maintenance in airways.

Materials and Methods

Animals

C57BL/6 mice were maintained in pathogen-free conditions in the Tianjin animal facility. Mice were exposed to a 12-h light/dark cycle and had free access to food and water. Adult mice between the ages of 2 and 4 months were sacrificed for experiments according to a protocol approved by the Nankai University Animal Care and Use Committee.

Reagents

Naphthalene, mouse IgG2b anti-ACT (1:8,000) antibody, and goat polyclonal anti-Scgb1a1 antibody (1:50) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse monoclonal IgG1 anti-eNOS (1:500) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Naphthalene Administration

Naphthalene was dissolved in Mazola corn oil and injected intraperitoneally at a dose of 275 mg/kg. All the injections were performed between 8:00 and 10:00 a.m. to normalize injury responses.
Untreated animals were used as controls. At the day post-naphthalene injection indicated, the mice were sacrificed for analysis.

**Immunofluorescence Staining**

Lung lobes were processed for histological analysis by using standard methods described previously [10]. Briefly, lung tissues were fixed by instillation of 10% neutral buffered formalin, followed by immersion in 10% neutral buffered formalin for a total of 2 h at 4°C. Five-micrometer sections were collected from the lung tissues. Sections were incubated with primary antibodies at 4°C overnight, washed with phosphate-buffered saline, and then incubated with the appropriate fluorochrome-conjugated secondary antibody for 2 h at room temperature. Slides were mounted in Fluoromount G containing 4',6-diamidino-2-phenylindole (DAPI). Staining was visualized using a Zeiss Axiovert40 inverted fluorescent microscope.

**Results**

**Expression of eNOS in mouse airway epithelia**

To assay eNOS expression in mouse airways, immunofluorescence staining was performed on lung tissue sections. Immunoreactivities of Scgb1a1 and eNOS indicated that they colocalized in the proximal airway, suggesting that eNOS is expressed in mouse club cells (Figure 1 A–C). Club cells negative for eNOS were not observed in the present study. Consistent with the previous reports [4,11], the immunoreactivities of eNOS and the ciliated cell marker ACT revealed that ciliated cells in the proximal airway expressed eNOS (Figure 1 D–F). We also observed for the first time that the proximal airway epithelium in the mouse contained a subset of ciliated cells that did not express eNOS (Figure 1 J). Analysis of [3–5] individual lung sections indicated that eNOS-positive ciliated cells were more abundant than eNOS-negative ciliated cells in both the proximal and the distal airways of mouse (Figure 1K).

*eNOS*-negative ciliated cells resist naphthalene-induced lung injury

Ciliated cells that express eNOS have been shown to be resistant to naphthalene-induced lung injury [12]. Therefore, we examined whether eNOS-negative ciliated cells had a similar capacity. To address this, naphthalene was injected intraperitoneally into mice. As reported previously [13], most Scgb1a1-expressing club cells were ablated 1 day post-naphthalene (Figure 2A), except for a few Scgb1a1-expressing cells residing in the distal airway that survived the naphthalene-induced injury. These surviving Scgb1a1-expressing cells were immunoreactive for eNOS (Figure 2B). Moreover, we observed that both eNOS-positive (Figure 2C-D) and eNOS-negative ciliated cells (Figure 2C-D) survived naphthalene injury. By day 10, during the post-injection repair process, a significant number of Scgb1a1-expressing cells were regenerated in the airway epithelium (Figure 2F-I). During the injury process (day 1 post-naphthalene), greater than 70% of the surviving ciliated cells in both the proximal and distal airways were negative for eNOS (Figure 2J). However, during the repair process (day 10 post-naphthalene), the relative abundance of eNOS-positive ciliated cells in the total ciliated cell population returned back to normal levels in both the proximal and the distal airways (Figure 2K).

Further, more alveolar cells exhibited immunoreactivity for eNOS in the naphthalene-injected group compared to the controls, suggesting that the expression of eNOS can be induced by naphthalene injury.

**Figure 1:** Expression of eNOS in mouse airway epithelial cells. Lung sections from C57BL/6 mice were stained using antibodies for the epithelial club cell marker secretoglobin 1A member 1 (Scgb1a1) (A), endothelial nitric oxide synthase (eNOS) (B, E, H), or airway ciliated cell marker acetylated tubulin (ACT) (D,G), followed by fluorochrome-conjugated secondary antibody. 4',6-Diamidino-2-phenylindole (DAPI) was included in the mounting medium to mark the cell nuclei (blue). Merged images are shown in C, F and I. Negative control without Scgb1a1 and ACT antibodies but with secondary fluorescent antibodies and DAPI was included (J). Arrows indicate ciliated cells stained with eNOS, while arrowheads mark ciliated cells lacking eNOS expression. Images shown are representative of five individual mice. The proportions of eNOS+ and eNOS- ciliated cells analyzed in 3–5 individual images of the proximal or distal airways are summarized in K.
attributed to the source of the eNOS antibodies used in the different studies. Our data clarify this issue by showing that eNOS clearly colocalized with Sgcb1a1, a marker of epithelial club cells.

Discussion

The airway epithelium plays critical roles as a protective physical and functional barrier between the external environment and underlying tissues, and as a central element in the initiation and regulation of immune responses in the lung [14]. Epithelial repair is initiated quickly after injury by club cells, which act as endogenous progenitors [15]. During the airway reparative process, club cells exhibit differential potential to become ciliated and mucous cells [16,17]. Hierarchically, club cells are replenished in the distal airway bodies of pulmonary airways serve as a reservoir of progenitor cells capable of epithelial regeneration. Am J Pathol 156: 269-278.

Acknowledgements

This work was supported by the Natural Science Foundation of Tianjin City (Nos. 13JCYBJC40000, 13JCYBJC22400).

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Figure 2: Both subsets of ciliated cells survive naphthalene injury. C57BL/6 mice were intraperitoneally injected with naphthalene and then sacrificed on days 1 and 10 post injection. Lungs were collected and processed for Sgcb1a1 (A and F), eNOS (B and G), and ACT (C and H) staining. Merged images are shown in D and I. Nuclei were visualized using DAPI (white). Negative control without Sgcb1a1 and ACT antibodies but with secondary fluorescent antibodies and DAPI was included (E). Data are representative of four individual mice. Proportions of eNOS+ and eNOS- ciliated cells were analyzed in 3–5 individual images of the proximal and distal airways from mice collected from day 1 post-naphthalene (J) and day 10 post-naphthalene (K).
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