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Short communication

Little evidence for epithelial-mesenchymal transition in a murine model of airway fibrosis induced by repeated naphthalene exposure

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ABSTRACT

Recent evidence suggests that epithelial-mesenchymal transition (EMT) is involved in the pathogenesis of airway obstructive diseases, such as chronic obstructive pulmonary disease, asthma and bronchiolitis obliterans syndrome after lung transplantation. However, whether EMT occurs in an experimental model of airway fibrosis is not well known. We explored evidence of EMT in a murine model of airway fibrosis induced by repeated exposure to naphthalene. Mice were administered intraperitoneal injections of naphthalene or corn oil vehicle once weekly for 14 consecutive weeks. The animals were sacrificed 5 days after the final injection of naphthalene or corn oil vehicle. EMT was evaluated in lung tissue sections using immunohistochemistry and immunofluorescence. Repeated naphthalene exposure induced loss of club cells, hyperplasia of epithelial cells and peribronchial fibrosis. However, we did not find any loss of E-cadherin expression or any acquisition of vimentin, S100A4 or αSMA in epithelial cells in control or naphthalene-exposed mice. These results suggest that EMT does not contribute significantly to naphthalene-induced airway fibrosis in mice.

1. Introduction

Airway fibrosis is a process of tissue remodeling that occurs in a variety of obstructive lung diseases, such as chronic obstructive pulmonary disease (COPD), asthma and bronchiolitis obliterans syndrome (BOS) after lung transplantation. This process causes fixed bronchial obstruction and leads to a progressive loss of lung function. Recent evidence suggests that fibroblasts in fibrotic diseases arise from at least three cell populations: resident fibroblasts, circulating mesenchymal progenitor cells (fibrocytes) and epithelial cells (Duffield et al., 2013; Wynn and Ramalingam, 2012). Epithelial-mesenchymal transition (EMT) is a phenomenon characterized by the loss of normal epithelial integrity and the gain of a mesenchymal phenotype (Thiery, 2003). During EMT, epithelial cells undergo the gradual loss of E-cadherin, a hallmark of EMT, and the simultaneous acquisition of mesenchymal markers, such as vimentin, α-smooth muscle actin (α-SMA) and S100A4 (Bartis et al., 2014; Zeisberg and Neilson, 2009). In experimental models of renal and hepatic fibrosis, epithelial cells have been identified as important precursors of fibroblasts via EMT, accounting for up to 40% of fibroblasts in fibrotic areas (Thiery, 2003; Wynn and Ramalingam, 2012). However, whether EMT contributes to lung disease is controversial (Bartis et al., 2014). In particular, the incidence of EMT in fibrotic airway disease has not yet been fully elucidated. We have recently established a murine model of airway fibrosis induced by repeated naphthalene exposure (Aoshiba et al., 2014). In this model, repeated naphthalene exposure caused prolonged loss of club cells, triggering activation of aberrant local epithelial repair mechanisms and a peribronchial fibrotic response. In this study, we have explored the evidence of EMT in the airways of lung tissues obtained from mice subjected to this model.

Abbreviations: COPD, chronic obstructive pulmonary disease; BOS, bronchiolitis obliterans syndrome; EMT, epithelial-mesenchymal transition; α-SMA, α-smooth muscle actin; CC10, Clara cell 10 protein 10; HRP, horseradish-peroxidase; DAPI, 4′,6-diamidino-2-phenylindole.

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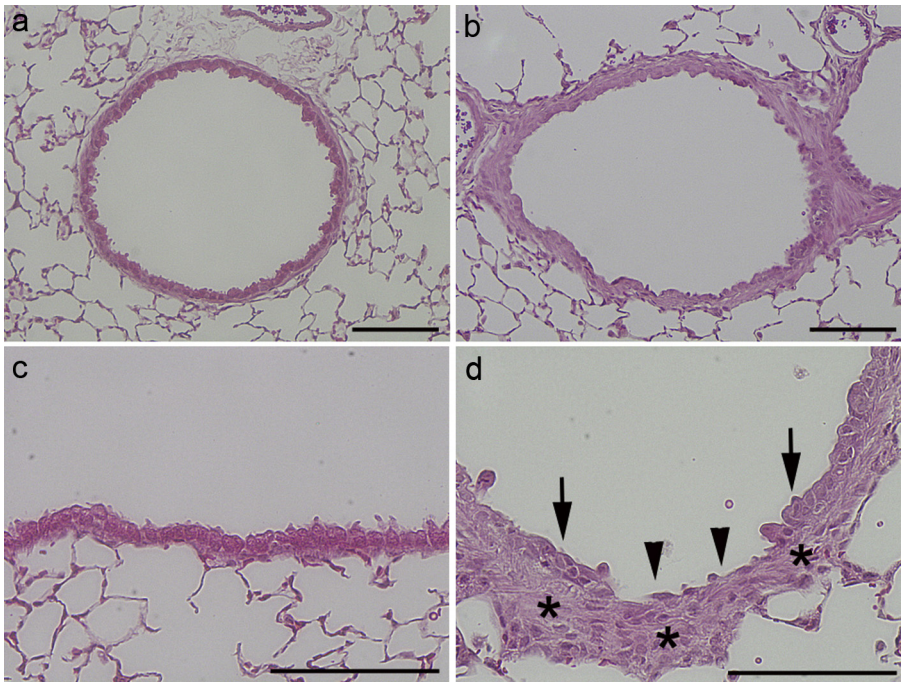

Fig. 1. Hematoxylin and eosin staining of lung tissue sections. Mice were administered intraperitonenal injections of either naphthalene (**b** and **d**) or corn oil vehicle (**a** and **c**) once weekly for 14 consecutive weeks and sacrificed 5 days after the final administration. The small airways of the naphthalene-exposed mice showed club cell denudation (*arrowheads*) associated with epithelial cell hyperplasia (*arrows*), and peribronchial fibrosis (*asterisks*). Scale bars = 100 μm.

2. Material and methods

Eight-week-old male C57/BL6J mice were administered intraperitoneal injections of naphthalene (Kanto Chemical, Tokyo, Japan: 200 mg/kg body weight) (n = 7) or corn oil vehicle (n = 7) once weekly for 14 consecutive weeks. Two mice in the naphthalene group died by the end of the experiment, due to intra-abdominal bleeding caused by misinjection of naphthalene into a blood vessel. The animals were sacrificed 5 days after the final injection of naphthalene or corn oil vehicle. The lungs of the mice were inflation-fixed with 10% neutral buffered formalin, embedded in paraffin, sectioned (3 μm) and processed for hematoxylin–eosin staining, immunohistochemistry and immunofluorescence staining. The primary antibodies used were goat polyclonal anti-CC10 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal E-cadherin (Santa Cruz Biotechnology), rabbit polyclonal anti-vimentin (Cell Signaling Technology Japan, Tokyo, Japan), rabbit polyclonal anti-S100A4 (Abcam Biochemicals, Tokyo, Japan), and goat polyclonal anti-α-SMA (Abcam Biochemicals). For immunohistochemistry, the primary antibodies were detected with a secondary antibody conjugated with a horseradish-peroxidase (HRP)-labeled polymer (Histofine® Simple Stain, Nichirei Biosciences, Tokyo, Japan). For antigen retrieval, the sections were autoclaved in a citrate buffer (pH 6.0) for 20 min before application of the primary antibodies. Endogenous peroxidase activity was quenched by exposure to 3% hydrogen peroxide for 20 min. The immunoreactants were detected with a diaminobenzidine substrate or a HistoGreen® substrate (AbCys, Paris, France). For immunofluorescence staining, the primary antibodies were reacted with a secondary anti-IgG antibody conjugated with Alexa Fluor 488 or Alexa Fluor 594 (Invitrogen, Carlsbad, CA) and the cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). The animal protocol was reviewed and approved by the Animal Care, Use, and Ethics Committee of Tokyo Women's Medical University.

3. Results

Hematoxylin and eosin staining of lung tissue sections from control mice (n = 7) showed normal appearance of the airways (Fig. 1). As reported previously (Aoshiba et al., 2014), mice repeatedly exposed to naphthalene (n = 5), a toxicant to club cells, exhibited severe epithelial lesions, including loss of club cells and hyperplasia of remaining epithelial cells, and the development of peribronchial fibrosis and thickening (Fig. 1). In the two groups of mice, however, we did not found any fragmentation of the reticular basement membrane, a likely hallmark of EMT in smokers with COPD (Sohal et al., 2011, 2010).

We further examined whether EMT is involved in the development of peribronchial fibrosis. The process of EMT involves, on the one hand, the epithelial expression of mesenchymal proteins, such as vimentin, S100A4 and α-SMA, and, on the other hand, the loss of epithelial polarity resulting from the disassembly of junctional proteins, such as E-cadherin (Pain et al., 2014; Zeisberg and Neilson, 2009) Immunohistochemical staining of lung tissue sections obtained from the naphthalene-exposed mice showed increased numbers of peribronchial mesenchymal cells that express vimentin (Fig. 2d) or S100A4 (Fig. 2f). However, bronchial epithelial cells in control and naphthalene-exposed mice were not stained for vimentin or S100A4. Smooth muscle cells in control and naphthalene-exposed mice, and some mesenchymal cells in the naphthalene-exposed mice, expressed α-SMA (Fig. 2e and f). However, bronchial epithelial cells in the two groups of mice were not stained for α-SMA. E-cadherin was markedly expressed in bronchial epithelial cells in both groups of mice (Fig. 2g and h). No reduction in epithelial E-cadherin expression was observed in the naphthalene-exposed mice compared to control mice. We next performed double immunofluorescence staining for CC10, a marker of club cells, and for mesenchymal cell markers. Neither CC10-positive club cells nor CC10-negative epithelial cells expressed vimentin or S100A4 in control mice and naphthalene-