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A murine model of airway fibrosis induced by repeated naphthalene exposure

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ABSTRACT

The airway epithelium serves as a biological barrier essential for host defense against inhaled pollutants. While chronic epithelial injury, commonly associated with chronic obstructive pulmonary disease and bronchiolitis obliterans syndrome, often results in airway fibrosis, limited animal models of airway fibrosis have been established. Club cells (Clara cells) in the small airways represent an important population of epithelial progenitor cells and also the principal site of localization of the cytochrome P-450 monooxygenase system, which metabolically activates xenobiotic chemicals such as naphthalene by converting them to toxic epoxide intermediates. We hypothesized that repeated exposure to naphthalene may cause prolonged loss of club cells, triggering aberrant local epithelial repair mechanisms that lead to peribronchial fibrosis. We administered intraperitoneal injections of naphthalene to C57/BL6J mice once a week for 14 consecutive weeks. Repeated club cell injury caused by naphthalene triggered regional hyperproliferation of epithelial progenitor cells, while other regions remained denuded or squamated, resulting in fibroblast proliferation and peribronchial collagen deposition associated with upregulation of the fibrogenic cytokines transforming growth factor-β and connective tissue growth factor. The total collagen content of the lung assessed by measurement of the hydroxyproline content was also increased after repeated exposure to naphthalene. These results lend support to the relevance of repeated injury of airway epithelial cells as a trigger for resting fibroblast proliferation and airway fibrosis. This model of airway fibrosis is simple and easy to reproduce, and may be expected to advance our understanding of the pathogenesis and potential treatment of airway fibrotic disorders.

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1. Introduction

The airway epithelium represents a biological barrier that serves multiple functions essential for tissue homeostasis, such as mucociliary clearance, hydration and host defense against invading microorganisms and inhaled pollutants (Rackley and Stripp, 2012; Tam et al., 2011). Acute injury to the airway epithelium is repaired rapidly through a highly regulated process involving epithelial cell spreading, proliferation and differentiation (Gardner et al., 2010). However, chronic epithelial injury, commonly associated with chronic obstructive pulmonary disease (COPD), severe bronchial asthma, and bronchiolitis obliterans syndrome often results in structural remodeling involving airway fibrosis. Although airway fibrosis is the final common pathway for many chronic lung diseases, there is a paucity of effective treatment options (Borok et al., 2011). To understand the pathogenesis of airway fibrosis and develop optimal therapies, the establishment of suitable animal models is necessary.

Current animal models of tissue fibrosis suggest that repeated epithelial injury and defective repair lead to an aberrant fibrotic response within the lung (Hagimoto et al., 1997; Sisson et al., 2010; Lawson et al., 2011; Palmer et al., 2011; Hardie et al., 2010), liver (Hayashi and Sakai, 2011), and kidney (Grigic et al., 2012). While the cascade from epithelial cell injury to fibrosis is a complex subject of intensive research, airway fibrosis has been reported to occur as a result of altered crosstalk between epithelial cells and interstitial fibroblasts (Wynn, 2011).

Nonciliated bronchiolar epithelial cells (club cells, Clara cells) represent an important population of progenitor cells that contribute to epithelial repair after airway injury (Evans et al., 1978; Chen et al., 2009). Club cells are the predominant epithelial cell type in the small airways of mice and other rodents (Plopper et al., 1991), and represent the principal site of metabolism of xenobiotics by the cytochrome P-450 monooxygenase system

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(Plopper et al., 1987), which metabolically activates lipophilic xenobiotic chemicals such as naphthalene by converting them to toxic epoxide intermediates (Mahvi et al., 1977). Several previous studies in mouse models have shown that acute exposure to naphthalene given via inhalation or the intraperitoenal routes causes club cell swelling, vacuolization, and cellular exfoliation into the lumen of the airways within 24 h. However, the extensive airway epithelial injury is rapidly repaired through activation of the surviving progenitor cells, such as naphthalene-resistant club cells (variant club cells) and local tissue stem cells residing at selected niches associated with tracheo-bronchial glands, neuroepithelial bodies (NEBs) and the bronchioalveolar duct junction (BADJ) (van Winkle et al., 1995; Stripp et al., 1995; Reynolds et al., 2000; West et al., 2001; Hong et al., 2001, 2004; Giangreco et al., 2002, 2009; Phimister et al., 2004; Rackley and Stripp, 2012).

In humans, repeated naphthalene exposure can occur from environmental exposures, since naphthalene is a prominent constituent of mainstream tobacco smoke and also abundant in side stream smoke and automobile emissions (U.S. Department of Health and Human Services, 2010). In this study, we hypothesized that repeated naphthalene exposure would cause prolonged loss of club cells, triggering activation of aberrant local epithelial repair mechanisms and a peribronchial fibrotic response. While airway epithelial tolerance has been described after daily exposure of mice to naphthalene by inhalational or intraperitoneal administration (O'Brien et al., 1989; Lakritz et al., 1996; West et al., 2000, 2003; Sutherland et al., 2012) we have demonstrated previously that airway epithelial cells remained susceptible to naphthalene when the mice received intraperitoneal administration of naphthalene once weekly for three weeks (Zhou et al., 2011). In this study, we demonstrated that intraperitoneal administration of naphthalene once weekly for 14 weeks is useful for successfully establishing a mouse model of airway fibrosis.

2. Materials and methods

2.1. Animal protocol

The animal protocol was reviewed and approved by the Animal Care, Use, and Ethics Committee of Tokyo Women's Medical University. Eight-week-old male C57/BL6J mice were administered intraperitoneal injections of naphthalene (Kanto Chemical, Tokyo, Japan: 200 mg/kg body weight) 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. The lungs of the mice were inflation-fixed in situ for 5 min with 10% neutral buffered formalin (NBF) at 25 cm water pressure, removed, and immersion-fixed in NBF for 24 h. Formalin-fixed tissue was embedded in paraffin, sectioned (3 μm), and processed for hematoxylin–eosin staining, picrosirius red staining, immunohistochemistry, and immunofluorescence staining.

2.2. Immunohistochemistry and immunofluorescence

The primary antibodies used were goat polyclonal anti-CC10 (sc9772: Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-β-tubulin IV (ONA1A6: BioGenex, San Ramon, CA), mouse monoclonal anti-pancytokeratin (CK502: BioLogo, Kronshagen, Germany), rabbit polyclonal anti-S100A4 (ab27957: Abcam Biochemicals, Tokyo, Japan), rabbit polyclonal anti-vimentin (#5741: Cell Signaling Technology Japan, Tokyo, Japan), rat monoclonal Mac-3 (M3/84: Japan Becton Dickinson, Tokyo, Japan), rabbit polyclonal anti-CD3 (ab5690: Abcam Biochemicals), mouse monoclonal anti-$p21^{CIP1}$ (sc6246: Santa Cruz Biotechnology), rabbit polyclonal anti-Ki67 (ab15580: Abcam), mouse monoclonal anti-p-H3 (ab14955), anti-rabbit monoclonal anti-β-catenin (ab32572), rabbit polyclonal anti-TGFβ (sc146: Santa Cruz), and goat polyclonal anti-CTGF (sc14939: Santa Cruz). 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 double staining, the prior antibody complexes were removed by immersing the slides in glycine–HCl buffer (pH 2.2) for 1 h, and the sections were then immunostained for the secondary antigen. Cell nuclei were counterstained with nuclear fast red, as needed. For immunofluorescence staining, the primary antibodies were reacted with a secondary anti-IgG antibody conjugated with Alexa Fluor 488 (Invitrogen, Carlsbad, CA) and the cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Images were acquired using an Olympus IX71 microscope (Olympus Optical Co., Ltd., Tokyo, Japan) equipped with a digital camera, and processed using a computerized color image analysis software system (Lumina Vision ver. 2.0; Mitani Corporation, Fukui, Japan) and Adobe Photoshop software (San Jose, CA).

2.3. Morphometric analyses for the airways

Morphometric analyses for airway histopathology were preformed in cross-sectional areas of the rounded airway wall (defined as having a ratio between the perpendicular diameters of 0.8 or greater) (Wright et al., 2011). Tangential sections were excluded from the analysis. Ten airways with an internal perimeter (Pi) ranging from 0.8 to 2.0 mm were evaluated and the obtained measurements were averaged for each animal.

2.3.1. Epithelial injury

The number of CC10-positive club cells per millimeter of basement membrane of the airway was determined. The extent of epithelial denudation was expressed as the percentage of airways that were not covered by pan-cytokeratin-positive cells.

2.3.2. Airway inflammation

Airway infiltration by macrophages and T-lymphocytes was examined by immunohistochemistry. The number of individual cells staining positive for Mac-3 or CD3 in the peribronchial space were counted using a light microscope. Results were normalized to the length of the basement membrane of the adjacent epithelium.

2.3.3. Airway fibrosis

Fibrotic wall remodeling was examined in picrosirius red-stained lung tissue sections under a light microscope. The wall area of each airway was calculated by outlining the lumen at the level of the basement membrane and the external area at the adventitial border, and expressed as a proportion of the total airway area (wall plus lumen) (Churg et al., 2009). The collagen content of each airway was determined by color segmentation with the Lumina Vision software, selecting red color as the color of interest to separate collagen fibers from the background color (i.e., other tissue or air space), and expressed as a proportion of the total airway area (Hendzel et al., 1997). The number of S100A4-positive fibroblasts in the peribronchial space was counted under a light microscope and normalized to the length of the basement membrane of the adjacent epithelium.

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