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Short communication

Neuroepithelial body increases in bleomycin-treated mice

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ABSTRACT

Neuroepithelial bodies (NEBs) serve a niche for lung stem cells and proliferate in a variety of pulmonary diseases. We hypothesize that NEBs play an important role in lung injury repair processes, such as during pulmonary fibrosis. To test this hypothesis, we examined NEBs in a bleomycin-induced lung fibrosis mouse model. We divided FVB/NJ mice into bleomycin-treated (BL) and normal saline-treated (NS) groups. Two weeks after intravenous treatment, we immune-stained NEBs with anti-calcitonin gene-related peptide (CGRP) in whole mount preparations and found that the number of NEBs per unit area of airway almost tripled in the BL group (1.11 ± 0.28 number/mm^2; $n = 5$) compared with the NS group (0.32 ± 0.14 number/mm^2; $n = 4$, $p = 0.001$). The size of NEBs increased significantly in the BL group. Our findings support that NEBs play an important role in the pathogenesis of pulmonary fibrosis.

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1. Introduction

Pulmonary fibrosis (PF) is the end result of diffuse injury to the lungs. Known causes of diffuse lung injury that may lead to fibrosis include autoimmune diseases, and various drug and occupational exposures. PF of unknown etiology is a group of diseases classified as idiopathic interstitial pneumonia. Idiopathic pulmonary fibrosis (IPF) is the most common and has the worst prognosis (Schwartz et al., 1994). IPF accounts for about one third of all lung transplants. PF may result from chronic inflammation in rheumatoid arthritis or through profibrotic mechanisms with little contribution from inflammation in IPF. Despite extensive research efforts, the understanding of underlying mechanisms of IPF is limited and there is no effective treatment. Pulmonary neuroendocrine cells (PNECs) are widely distributed throughout the airway mucosa. They may exist as solitary cells or aggregate to form neuroepithelial bodies (NEBs), which are richly innervated and connected with the central nervous system through the vagus nerve (Yu et al., 2006). NEBs are important for early lung development and associated with stem cells in the airway, and may act as vagal sensory receptors (Yu et al., 2006). Inflammatory mediators and cytokines can be detected by airway sensory receptors. This information is transmitted through vagal afferents to the brain and produces responses that regulate disease processes. Since lung injury is a major component in the development of PF and insults to the lung may stimulate NEBs, we hypothesize that NEBs actively participate in the development of PF. Bleomycin is an anti-tumor antibiotic that has been used successfully to treat malignancies with a major limitation of development of interstitial PF (Limper, 2004). Bleomycin-induced PF animal models have been used extensively to study underlying mechanisms in different animal species including mouse (Buck and Chojkier, 2011). We examined the number and size of NEBs in the well-established PF mouse model induced by bleomycin and found that NEBs increased significantly following bleomycin treatment.

2. Methods

2.1. Animal

Experiments were carried out in 10–12 week-old male FVB/NJ mice (Jackson Laboratory, Bar Harbor, Maine, USA). Study procedures were approved by the IACUCs of the University of Louisville and Robley Rex VA Medical Center.

2.2. Bleomycin treatment

Mice were randomly divided into normal saline-treated (NS, $n = 4$) and bleomycin-treated (BL, $n = 5$) groups. Mice were anesthetized with isoflurane, and then injected with 200 μL of saline or bleomycin (80 mg/kg body weight, dissolved in 0.9% NaCl solution) through the tail vein.

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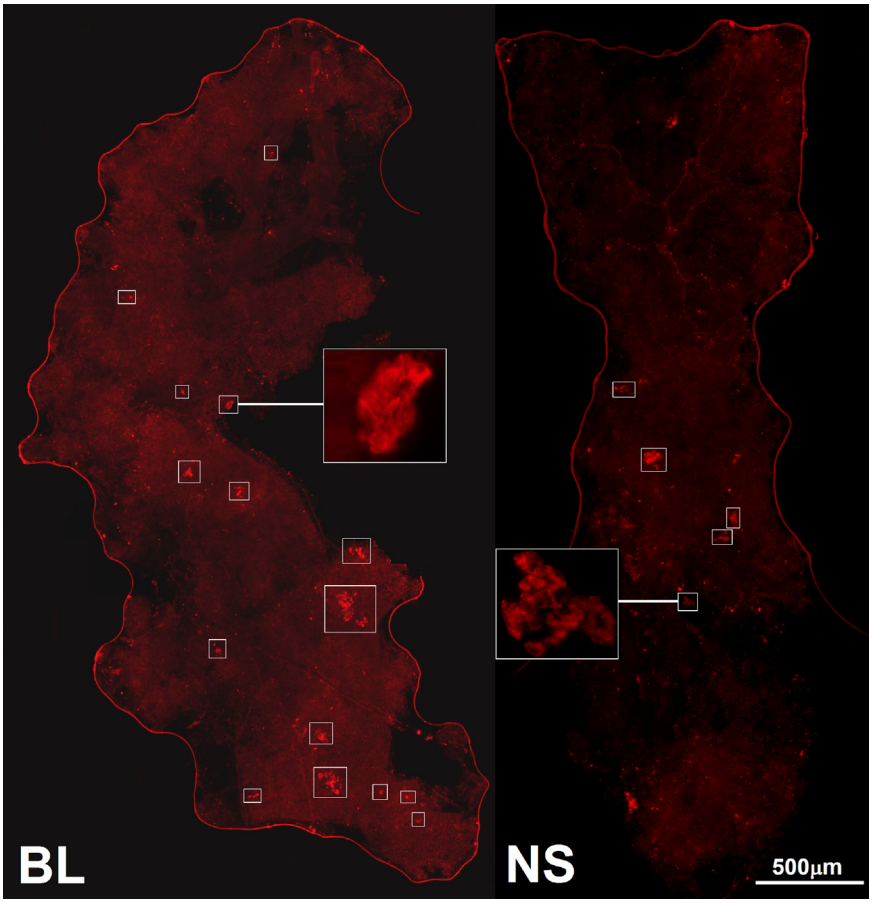

Fig. 1. Immunofluorescent staining of neuroepithelial bodies with CGRP antibody in the whole mount bronchiole (luminal views) from FVB/NJ mice. Please note that NEBs are more numerous in the bleomycin-treated (BL) than in the normal saline-treated (NS) tissue. The two large insets are amplified by 6 times.

2.3. Lung tissue preparation

On day 14 after treatment, mice were euthanized by anesthesia. Lung tissues were fixed overnight in a 0.1 mol/L phosphate-buffered solution containing 4% paraformaldehyde (pH 7.4). Lung parenchyma was removed carefully under a dissecting microscope to obtain trachea–bronchial trees for whole mount staining.

2.4. Immunofluorescent staining

Tissues were washed thoroughly, incubated at 4 °C overnight with rabbit anti-calcitonin gene related peptide (anti-CGRP, Sigma, 1:200), and then washed and incubated 1 h at room temperature with goat anti-rabbit IgG labeled with Alexa Fluor 555 (Life Technologies, NY, USA, 1:200). They were then mounted on glass slides with Fluoromount-G (SouthernBiotech, Alabama, USA), examined under a fluorescent microscope (Olympus SZ61) and analyzed with CellSens software. The number of NEBs (more than 4 neuroendocrine cells) in whole trachea–bronchiole trees was counted, their sizes were measured and then analyzed in percent area of the tissue.

2.5. Statistical analysis

Statistical analyses were carried out by Independent-Samples *t*-Test with SPSS 14.0 for Windows Evaluation Version. $p < 0.05$ was considered statistically significant.

3. Results

We examined the number and size of NEBs in the trachea–bronchiole tree after treatment with bleomycin or saline (Fig. 1). NEBs were more numerous and larger in the BL group than the NS group (Fig. 2). The number of NEBs per tissue area was 1.11 ± 0.28 number/mm^2 in BL lungs and was 0.32 ± 0.14 number/mm^2 in NS lungs ($p = 0.001$). The percent area of NEBs in lung tissue was greater in BL lungs ($0.15 \pm 0.06\%$) than in NS lungs ($0.04 \pm 0.02\%$, $p < 0.05$). In addition, the spectrum curve in the BL group was right upward shifted in comparison with that of the NS group. Large NEBs (>2700 μm^2) appeared in the BL group, but not in the NS group (Fig. 2). Furthermore, we examined NEB distribution pattern and found no specific differences between bleomycin- and saline-treated mice.

4. Discussion

In the current study, our data show that NEBs increased significantly in number and size in BL mice. It provides a potential link between NEBs and PF, and is consistent with our hypothesis that NEBs play an important role in the pathogenesis of PF.

The NEB has a strategic location and complex structure. Due to their neural epithelial nature and rich innervation, NEBs possess the cellular machinery to achieve both neural and humoral control of the local environment and to communicate bidirectionally with the central nervous system through the vagus nerve. NEBs

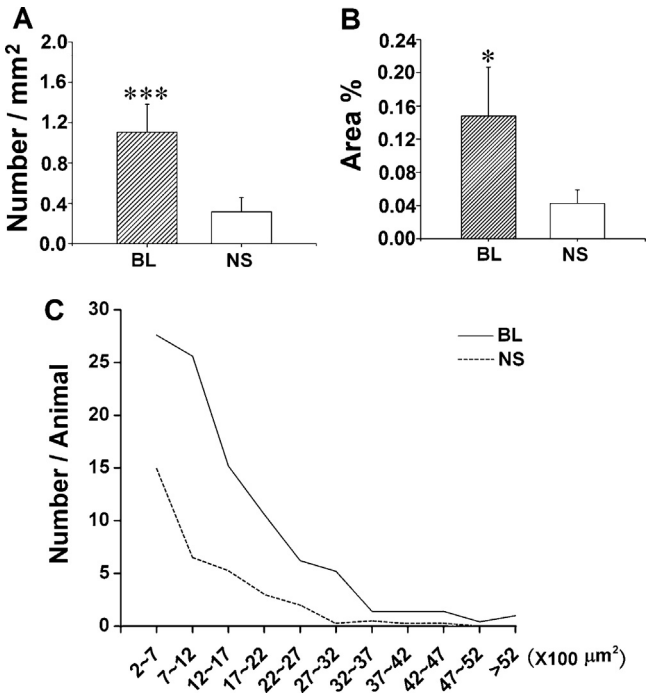

Fig. 2. Number, size and distribution of NEBs in the trachea–bronchiole tree after treatment with bleomycin (BL; $n = 5$) or normal saline (NS; $n = 4$). (A) Number of NEBs per unit area (mm^2) was significantly higher in the BL group than the NS group ($^{***}p = 0.001$). (B) Percent area of NEBs in the lung was also greater in the BL group than the NS group ($^{*}p < 0.05$). (C) Distribution of NEBs (average number/animal) with different sizes ($2–52 \times 100\ \mu m^2$). There were more NEBs, especially the smaller ones, in the BL group.

contain α-7nAChR (Fu et al., 2003). Vagotomy alters NEB content. NEBs release bioactive mediators and are closely associated with varied neural sources (vagal, spinal, and parabronchial) (Adriaensen et al., 2006). NEBs may be directly connected with airway sensory afferents in the lung (Yu et al., 2006). PNECs and NEBs contain many bioactive substances with growth factor and mitogenic properties, such as gastrin-releasing peptide (GRP), bombesin, calcitonin gene-related peptide (CGRP), and serotonin (5-HT) (Cutz et al., 2007). These substances play an important role in lung morphogenesis. NEBs are prominent in fetal and neonatal lungs and may promote lung development, a process much like tissue repair. NEBs have been proposed as oxygen sensors and may be involved in pulmonary hypertension (Gosney et al., 1989; Cutz et al., 2007), because they proliferate in pulmonary hypertensive patients and produce large quantities of 5-HT, a powerful pulmonary vasoconstrictor. Increasing evidence indicates that the vagus nerve plays an important role in asthma, chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome (ARDS), and lung cancer (Song et al., 2012). In addition, NEBs displayed hypertrophy and hyperplasia in a variety of lung diseases, such as sudden infant death syndrome (SIDS) (Cutz et al., 2007), lung cystic fibrosis, naphthalene-induced acute pulmonary injury (Stevens et al., 1997) and bronchopulmonary dysplasia. In addition, the vagus nerve may promote PF (Song et al., 2012). NEBs may be closely related to pulmonary neuroendocrine tumors, such as small-cell carcinoma, large-cell neuroendocrine lung carcinoma, and typical as well as atypical carcinoids (Rydzewska-Rosolowska et al., 2001). The typical pulmonary blastoma, a neuroendocrine tumor, exhibits the structural pattern of early lung development, consistent with the function of NEBs.

Tissue remodeling is an important step in lung disorders, including PF. NEB secretory products, such as GRP and 5-HT, may exert airway remodeling effects, including proliferation of fibroblasts, epithelial and smooth muscle cells (Cutz et al., 2007). For example, bombesin induces alveolar myofibroblast proliferation and increased alveolar wall thickness (Ashour et al., 2006). 5-HT stimulates collagen synthesis by fibroblasts, which is attenuated by blocking its receptors (Fabre et al., 2008). In addition, NEBs are thought to be stem cells (Cutz et al., 2007) capable of cell repair after injury or as a stem cell niche, a microenvironment that protect stem or progenitor cells from an injury and so they can proliferate afterwards. Following naphthalene-induced injury, airway epithelial cells regenerate from cells that reside in the NEB (Stevens et al., 1997). Significant hyperplasia of bombesin/GRP immunoreactive PNECs/NEBs has been reported in bronchopulmonary dysplasia, in which airway epithelium is repeatedly injured. Intraperitoneal injection of bombesin into mice increases proliferation of interstitial myofibroblasts and thickness of alveolar septa.

In summary, based on growing evidence, stimulation of NEB may promote development of PF. We have shown that the number and size of NEBs increase following intravenous bleomycin treatment in mice. This supports our hypothesis that NEBs play an important role in the pathogenesis of PF. Delineation of the underlying mechanisms may lead to new strategies to treat the devastating disease.

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