Biochemical and Biophysical Research Communications 461 (2015) 642–647

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Ovary-dependent emphysema augmentation and osteopontin induction in adult female mice

Yuichi Niikura [a], Takashi Ishii [a, b], Keisuke Hosoki [a, b], Takahide Nagase [b], Naomi Yamashita [a, *]

[a] Department of Pharmacotherapy, Research Institute of Pharmaceutical Sciences, Musashino University, 1-1-20 Shinmachi, Nishitokyo-shi, Tokyo 202-8585, Japan
[b] Department of Pulmonary Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8654, Japan

ARTICLE INFO

Article history:
Received 11 April 2015
Available online 23 April 2015

Keywords:
COPD
Elastase
Emphysema
Osteopontin
Ovary
Purinergic receptor

ABSTRACT

Biological differences between the sexes greatly impact the development and severity of pulmonary disorders such as emphysema. Recent studies have demonstrated crucial roles for osteopontin (OPN, also known as SPP1) in lung inflammation and alveolar destruction in human and experimental emphysema, but the impact of gender on OPN action remains unknown. Here, we report ovary-dependent induction of *Opn* mRNA with augmentation of experimental emphysema in adult female mice. Both male and female mice developed emphysematous lungs following intra-tracheal administration of porcine pancreatic elastase; however, compared with male mice, female mice developed more severe injury-related inflammation and pathologic alterations of the lungs. Notably, we observed female-specific induction of the *Opn* gene upon lung injury. Ovariectomy blocked this induction, with attenuation of lung inflammation and alveolar destruction, demonstrating the essential role of ovaries in injury-related *Opn* induction and augmentation of emphysema in adult female mice. Lastly, pre-treatment of adult female mice with pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid, which blocks ATP-mediated wound response, suppressed *Opn* mRNA induction upon lung injury, resulting in attenuation of enhanced lung inflammation. Together, our findings define a novel, ovary-dependent mechanism underlying gender-specific augmentation of emphysema through transcriptional control of the *Opn* gene.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is an incurable, progressive respiratory illness characterized by limited airflow due to lung inflammation and structural matrix destruction [1]. Emphysema, a major component of COPD, is defined as the enlargement of alveolar air spaces, accompanied by bronchiolar fibrosis in the proximal airways. These pathological features result from low-grade inflammation elicited by chronic exposure to cigarette smoke (CS). Because smoking habits strongly influence the prevalence rate of COPD, gender effect was initially believed to have only a minor effect on COPD development and severity. However, studies comparing female and male smokers demonstrate that women tend to exhibit more severe symptoms including larger reductions in FEV_1 (the forced expiratory volume in one second) and higher mortality, after adjustment for smoking intensity [2–4]. In experimental emphysema, CS-exposed female mice exhibit more severe phenotypes in terms of inflammation, alveolar destruction, and respiratory dysfunction of the lung when compared with male counterparts [5]. Therefore, a female-specific mechanism underlying augmentation of COPD might exist.

Osteopontin (OPN) is a pleiotropic cytokine involved in a wide range of biological functions including bone metabolism, immune response, and cancer metastasis [6]. Elevated OPN levels have been reported in the lungs of patients with asthma, COPD, or pulmonary fibrosis, or a combination of the above [7–11] and are linked to pathological features of these diseases, including eosinophil and neutrophil infiltration [12,13], goblet cell hyperplasia [14,15], airway hyper-responsiveness [13,14], and fibrosis [11,15,16]. Shan et al. have recently demonstrated in mice that chronic exposure to CS induces *Opn* mRNA expression in lung dendritic cells, which in turn stimulates differentiation of Th17 cells and thus interleukin 17A (IL-17A)-driven inflammation, eventually leading to emphysema [17,18]. In contrast, *Opn* gene deficiency in mice attenuates

* Corresponding author. Fax: +81 42 468 8647.
E-mail address: naoyama@musashino-u.ac.jp (N. Yamashita).

http://dx.doi.org/10.1016/j.bbrc.2015.04.081
0006-291X/

CS-induced immune cell infiltration, IL-17A production, and alveolar destruction [17], demonstrating that OPN actively contributes to emphysema pathogenesis by modulating both innate and adaptive immunity.

Extracellular ATP is a danger-signaling molecule receiving extensive attention in the field of pulmonary medicine. Lung injury caused by chemicals and mechanical ventilation triggers ATP release, which recruits immune cells and fibroblasts through the activation of purinergic receptors, leading to inflammation and fibrosis of the lungs, respectively [19,20]. Like elevated OPN levels, elevated extracellular ATP concentrations have been reported in the lungs of smokers and former smokers, especially those suffering from COPD. Pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), a potent purinergic receptor antagonist, ameliorates lung inflammation and alveolar destruction in CS-exposed mice [21], indicating that the purinergic receptor–mediated wound response actively contributes to emphysema pathogenesis. Despite the functional importance of OPN and purinergic receptors in emphysema pathogenesis, their roles in disease severity and gender difference have not yet been defined.

Because chronic exposure to CS activates innate immunity mediated by neutrophils and macrophages, both of which secrete proteinases, including elastase, which disrupts the lung extracellular matrix, the process leading to emphysema can be mimicked by a single intra-tracheal administration of porcine pancreatic elastase (PPE) in experimental animals [22–24]. In the current study, we demonstrated *Opn* mRNA induction, and enhanced inflammation and alveolar destruction, following PPE-induced lung injury in adult female mice. Both ovariectomy and PPADS pretreatment successfully repressed the injury-related *Opn* mRNA induction in adult female lungs, resulting in reduced lung inflammation and reduced structural alterations. These findings suggest that ovary-dependent transcriptional control of the *Opn* gene plays a role in the female-specific augmentation of experimental emphysema in mice and that it may have clinical implications for emphysema in female patients.

2. Materials and methods

2.1. Animals

Wild-type C57BL/6N mice (6- to 8-wk old) were obtained from the Sankyo Lab Service Corporation, Inc. (Tokyo, Japan). Mice were housed in a specific pathogen-free environment and allowed access to food and water ad libitum. Bilateral ovariectomy was performed on 6-wk-old mice, which were then housed for 3 wk to eliminate endogenous ovarian hormones, confirmed by uterine regression, before experiments were conducted. Emphysema mice were generated by intra-tracheally administering 5 units of porcine pancreatic elastase (PPE; Elastin Products, Co. Inc., Owensville, MO, USA) in a total volume of 25 μL saline by using a microspray (Penn-Century, Inc., Wyndmoor, PA, USA) under anesthesia. As the control, mice were given saline using the same procedure described above. To block purinergic receptor signaling, mice were administered 4.8 μg of pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid dissolved in 20 μL of saline (400 μM solution) intra-nasally 30 min before PPE administration. Bronchoalveolar lavage fluid (BALF) and lungs were collected at 24 h or indicated time points after PPE administration for further analysis.

2.1.1. BALF cell analysis

BALF cell analysis was performed as previously reported [24]. Briefly, BALF (2 mL) was centrifuged at 540 × *g* for 10 min at 4 °C. The supernatant was collected and stored at −80 °C until further use, and the cell pellet was suspended in 1 mL of PBS. The total cell number in BALF was determined by using a hemacytometer. To obtain cell counts of particular types of inflammatory cells, less than 50,000 cells from each BALF were spun at 640 rpm for 2 min at room temperature onto glass microscope slides by using a Shandon Cytospin 4 (Thermo Electron, Waltham, MA, USA), and the cells were stained with Diff Quik (International Reagents Corporation, Osaka, Japan). At least 200 cells per mouse were counted under bright-field microscopy for this differential cell analysis.

2.1.2. Lung histology

Four weeks following the elastase administration, lungs were collected and fixed with 4%(w/v) buffered paraformaldehyde phosphate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 24 h at 4 °C, and then embedded in paraffin. Coronal sections, 6-μm thick and encompassing the left lobes, were cut with a microtome from the paraffin-embedded lungs. To determine the extent of alveolar destruction, the sections were de-paraffinized, rehydrated, and then stained with H&E. The mean linear intercept, as a measure of intralveolar wall distance, was calculated as described previously [24].

2.1.3. Real-time PCR

Right lung lobes were frozen in liquid nitrogen immediately after isolation and were then fractured with a Multi Bead Shocker (Yasui Kikai Co., Osaka, Japan). Total RNA was extracted from the fractured lung powder by using TRI reagent (Invitrogen, Carlsbad, CA, USA), followed by DNase treatment (TURBO DNase; Ambion, CA, USA) to eliminate contaminated genomic DNA. For cDNA synthesis, 1.5 μg of total RNA was reverse-transcribed by using PrimeScript (Takara, Tokyo, Japan). Real-time PCR was performed for genes encoding osteopontin by using SYBR Premix Ex Taq II (Takara) in a total volume of 20 μL β-actin was used as the internal control. For calculating absolute copy numbers of target genes, serially diluted plasmids containing target gene cDNAs were used to generate a standard curve of each target gene. Melting temperature was used to confirm the specificity of the reactions. Forward and reverse primers used were as follows:

β-actin (5′-CTC CTA GCA CCA TGA AGA TCA-3′, 5′-CCT GCT TGC TGA TCC ACA TC-3′)
Osteopontin (5′-AGA ATC TCC TTG CGC CAC AG-3′, 5′-ATC GTC ATC ATC GTC GTC CAT-3′)

2.1.4. Statistics

Data from experimental replicates were pooled and are presented as means ± SEM. All data were statistically analyzed by 2-tailed Mann–Whitney *U* tests (for comparisons of two groups) or Kruskal–Wallis tests with Dunn's post-tests (for comparisons of multiple groups). Statistical significance was set at $P < 0.05$.

2.1.5. Study approval

All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee at the Musashino University, Japan, and were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

3. Results and discussion

3.1. Enhanced acute neutrophil infiltration to lungs upon elastase treatment in adult female mice

To gain insight into the mechanisms underlying gender differences in the pathogenesis and disease severity of emphysema, we generated elastase-induced experimental emphysema in male,