Sex Hormones Alter Th1 Responses and Enhance Granuloma Formation in the Lung

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Key Words

Granuloma · Sex hormones · Th1/Th2 balance ·
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Abstract

Background: The lung is one of the sites of granulomatous responses, which are characterized by the recruitment and organization of activated macrophages and lymphocytes. There have been several reports that have shown that some pulmonary granulomatous diseases, such as sarcoidosis and nontuberculous mycobacterial disease, are likely to be characterized by a preponderance in postmenopausal females. Although sex hormones have been shown to play an important role in the regulation of the immune system, the influence of sex hormones on pulmonary granuloma formation is still unclear. **Objectives:** The purpose of this study was to assess whether sex hormones are involved in granulomatous inflammation and to evaluate how sex hormones modulate this response in the lung. **Methods:** Ovariectomized rats were used as an experimental postmenopausal model

in which chronic pulmonary granulomatous inflammation was induced by intravenous injection of complete Freund's adjuvant. **Results:** Histological analysis of lung tissues demonstrated enhancement of granuloma formation in the ovariectomized group. Such enhanced granuloma formation was significantly associated with generalized Th1-biased cytokine production in the bronchoalveolar lavage fluid. **Conclusion:** These results indicate that sex hormones play an important role in pulmonary granuloma formation by altering the Th1 responses. Copyright © 2011 S. Karger AG, Basel

Introduction

Granuloma formation, which is characterized by the recruitment and organization of activated macrophages and lymphocytes in discrete lesions, is a consequence of chronic antigenic stimulation and is one of the important defense mechanisms against infectious microbes such as fungi and mycobacteria [1]. Nontuberculous mycobacte-

rial disease is one of the infectious granulomatous diseases in the lung [2–5]. It is characterized by the formation of various degrees of granulomas. It is important to note that patients with nontuberculous mycobacterial disease are predominately female and postmenopausal [4, 6, 7].

The lung is also a site of noninfectious granulomatous responses, such as sarcoidosis, which affects the pulmonary and lymphatic systems [8]. This disease shows a consistent predilection for adults less than 40 years of age, peaking in subjects of 20–29 years. Interestingly, for males, there is no second peak of disease incidence, but there is a second peak of disease incidence in females older than 50 years of age in Scandinavian countries and Japan [9–12]. This suggests that age-related changes in females, such as the hormonal dysregulation associated with menopause, may contribute to sarcoidosis. Although significant heterogeneity in disease presentation and disease severity occurs among different ethnic and racial groups [13–16], both of these pulmonary granulomatous diseases, sarcoidosis and nontuberculous mycobacterial disease, are likely to be characterized by a preponderance in postmenopausal females. Therefore, it is likely that fluctuations of sex hormones may be associated with enhanced granuloma formation in the lung.

Analyses of the experimental animal models of granulomatous diseases have suggested that humoral mediators, such as IL-4, IL-12, IFN- γ , TNF- α and CXC3 ligand, play crucial roles [17–19]. In addition, there is ample evidence that sex hormones can act as immunomodulators [20–22]. Indeed, a variety of immune cells, including T cells, B cells, monocytes and macrophages, have been shown to possess functional sex hormone receptors [23–26]. However, the role of sex hormones in pulmonary granuloma formation is still unclear. Moreover, there are no studies showing the effect of sex hormones on pulmonary granuloma formation by histological findings.

In the present study, we established an experimental postmenopausal model in which female rats were ovariectomized (OV) and treated by intravenous injection of complete Freund's adjuvant (CFA) to induce chronic pulmonary granulomatous inflammation. To our knowledge, the present study is the first report to reveal that granuloma formation in rats treated by ovariectomy was significantly greater than that in sham-operated rats on the basis of histological findings. We also show that sudden withdrawal of sex hormones enhances pulmonary granuloma formation and increases the local Th1 cytokine responses.

Materials and Methods

Animals

Female, specific pathogen-free DA rats weighing 100–135 g (8 weeks old) were purchased from Charles River Laboratories (Yokohama, Japan). These rats were maintained in a limited access barrier and housed in a humidity- ($55 \pm 10\%$) and temperature- ($24 \pm 2^\circ\text{C}$) controlled room under a 12-hour light/dark cycle. The study protocol was reviewed and approved by the Juntendo University School of Medicine Committee on Animal Care and complies with National Institutes of Health guidelines for animal care.

The Rat Treatment Regimen

To initiate the studies, 62.5 μl /100 g body weight CFA (Sigma, St. Louis, Mo., USA) was injected into the dorsal tail vein under anesthetization by isoflurane (Abbott, Abbott Park, Ill., USA). Seven days later, one group of rats ($n = 12$) was OV. An aseptic surgical procedure was employed for all animals. The rats were anesthetized using isoflurane, then the dorsal part of the lumbar region was shaved, and the site was cleaned with 75% ethanol. A 2-cm incision was made in the skin through the musculature and peritoneum and the ovaries were retracted and removed along with the oviduct. The wound was then closed using a 4-0 sterile suture. The other group of rats ($n = 12$) underwent sham operation (SHAM). The SHAM group underwent the same surgical procedure except that the ovaries were exposed but not removed. At 36 days after injection, the rats were euthanized with an overdose of pentobarbital sodium. Then, bronchoalveolar lavage was performed as described in the next paragraph, and the lungs were prepared for histological evaluation.

Immunoassays of Bronchoalveolar Lavage Fluid and Serum

Rats were euthanized with an overdose of pentobarbital sodium, followed by the collection of blood and bronchoalveolar lavage fluid (BALF). After opening the chest, an incision was made in the neck, and the trachea was cannulated with a plastic catheter secured in place with silk suture. Lavage was performed with calcium- and magnesium-free PBS at a volume of 6 ml. The procedure was performed a total of 3 times. Total cell counts and differential cell counts were performed by Diff-Quick staining. Cytokine levels were measured using ELISA. Prior to the ELISA, the BALF samples were concentrated 12.5 times by Amicon Ultra (Millipore, Billerica, Mass., USA). Rat IFN- γ , IL-12 and IL-13 ELISA kits were purchased from Biosource (Invitrogen Corporation, Calif., USA).

Quantification of Pulmonary Granuloma Formation

After lavage, the lungs were fixed by intratracheal infusion of 10% formalin buffered with PBS at a constant hydrostatic infusion pressure (20 cm H_2O). After 1 h, the lungs were removed from the thoracic cavity and immersed in the fixative for an additional 24 h. The lungs were embedded in paraffin and sliced into 4 transverse sections (apical, upper, middle and lower). Histological sections were prepared at a thickness of 2.5 μm and stained with hematoxylin and eosin. For measurement of the granulomatous areas in the lung parenchyma, a KS 400 image analysis system (Zeiss, Germany) was used. For each section, the whole lung area with 6–27 microscopic fields was examined at a magnification of $\times 20$. The total area of granuloma and lung parenchyma in each rat was calculated by summing all of these measured fields in each section.

Statistical Analysis

Before cytokine analyses were performed, the Smirnov-Grubbs test was used to eliminate outliers. Statistical analysis was performed with an unpaired t test using SPSS statistical package version 15.0F (SPSS Inc., Chicago, Ill., USA).

Results

Decreased Serum Levels of Sex Hormones in OV Rats

In the present study, we used OV rats as a postmenopausal model. To confirm whether ovariectomy mimics the postmenopausal state, which is characterized by decreased levels of sex hormones and increased body weight, we measured and compared the serum levels of estrogen and body weight between OV and SHAM rats 29 days after operation. Serum levels of estrogen were significantly lower in OV than in SHAM rats (fig. 1). Similarly, the body weight of OV rats was significantly higher than that of SHAM rats (data not shown). We conclude that the OV rat is a useful model for investigating immunological reactions in the postmenopausal state.

Enhancement of Pulmonary Granulomatous Inflammation by Sex Hormone Withdrawal

CFA is a well-known adjuvant and itself can induce granuloma formation in the lung when emulsified CFA is administered intravenously [27, 28]. Intravenous injection of CFA induced granulomas in the lungs of both OV and SHAM rats (fig. 2a–d). The granulomas were composed of a central core of macrophages, epithelioid cells and giant cells surrounded by lymphocytes, consistent with the findings in sarcoidosis (fig. 2e, f).

There were no differences in the area of total lung parenchyma between the OV and SHAM groups (fig. 3a). In contrast, the size of the granulomas was significantly larger in the OV group than in the SHAM group (fig. 3b). Likewise, the relative size of the granuloma areas in the whole lung parenchyma in the OV group was significantly larger than that in the SHAM group (fig. 3c). These findings suggest that sex hormone withdrawal favors a Th1-polarized granulomatous response in OV rats.

Alteration of Immune Cell Populations in the Lung by Ovariectomy

To investigate the populations of infiltrated immune cells in the lungs, BALF was collected and the cell populations were compared between the OV and SHAM groups. The total number of immune cells recovered in BALF was comparable between the groups (data not shown). The cells recovered from the BALF of the control

Fig. 1. Effect of ovariectomy on serum levels of estradiol. Serum levels of estradiol were measured by ELISA 29 days after operation. The bars indicate the means (n = 3 for OV, n = 6 for SHAM). * p < 0.05.

rats that received saline were mostly alveolar macrophages (97–99%; data not shown). In contrast, the BALF of the OV and SHAM rats, both of which received CFA, showed increased numbers of lymphocytes (fig. 4). The relative population of macrophages in the OV group was significantly lower than that in the SHAM group. Conversely, the relative population of lymphocytes was significantly higher in the OV rats than that in the SHAM rats. There was no difference in the relative population of neutrophils between the OV and SHAM groups.

Th1 Shift of the Cytokine Profiles in the Serum and BALF by Ovariectomy

To investigate the mechanisms responsible for modulation of the granulomatous responses and immune cell populations in the lungs of OV rats, we compared the cytokine levels in the serum and BALF between the OV and SHAM groups. The Th1-type cytokines IFN- γ and IL-12 and the Th2-type cytokine IL-13 were assayed. There was no difference in the serum levels of these cytokines between the OV and SHAM groups, suggesting that ovariectomy does not alter the systemic cytokine balance (fig. 5).

On the other hand, the levels of IFN- γ and IL-12 in the BALF in the OV group were significantly higher than those in the SHAM group (fig. 6a, b), suggesting the occurrence of a local Th1 shift in the lungs of OV rats. The levels of IL-13 in the BALF of OV rats were not significantly different from those of the SHAM rats (fig. 6c). These findings suggest that a Th1-polarized environment in the lung may be contributing to the altered granulomatous response in OV rats.

Fig. 2. Histological findings of pulmonary granulomas in lungs of rats. **a–d** Representative photomicrographs of pulmonary granulomas in the lungs of OV rats (**a, c**) and SHAM rats (**b, d**). Hematoxylin and eosin. Original magnification: $\times 40$. **e, f** Higher mag-

nification reveals that granulomas in lungs of OV rats (**e**) and SHAM rats (**f**) contain epithelioid cells and numerous lymphocytes. Original magnification: $\times 400$.

Fig. 3. Effects of ovariectomy on pulmonary granuloma formation. **a** The area of lung parenchyma. **b** The area of granuloma. **c** The percentage of the lung parenchyma occupied by granuloma. * $p < 0.05$. The bars indicate the means ($n = 12$ for OV, $n = 11$ for SHAM).

Fig. 4. Effect of ovariectomy on cell populations of BALF. BALF was collected from the lungs of the OV and SHAM rats. Cell populations were identified on air-dried cytocentrifuged smears (800 rpm for 5 min) before staining with Diff-Quick stain. **a, b** Representative photomicrographs of air-dried cytocentrifuged smears from BALF of OV rats (**a**) and SHAM rats (**b**). Diff-Quick stain. Original magnification: $\times 400$. **c** Differential cell counts were performed by counting at least 300 cells to identify macrophages (Mac), lymphocytes (Ly) and neutrophils (Neutro). The bars indicate the means. * $p < 0.05$.

Discussion

In the present study, we established an experimental postmenopausal model using OV rats, which can mimic the postmenopausal state. In fact, OV rats present decreased levels of serum estrogen and increased body weight similar to those in postmenopausal women. Using this animal model, we examined the pathogenesis of pulmonary granulomatous inflammation, which is observed in postmenopausal women. We demonstrated that the size of the granulomatous areas in the lungs of OV rats was significantly greater than that in SHAM rats. These results indicate that enhancement of pulmonary granulomatous inflammation is associated with decreased levels of sex hormones.

Menopause, which is accompanied by a decline in the production of sex hormones, affects granuloma formation [3, 4, 6, 9, 29, 30]. However, the histopathological status of the granulomatous lesions found in those studies is unclear. Our histopathological examination revealed enhancement of pulmonary granulomatous lesions induced by CFA in OV rats. The lesions evoked in this experimental model are pathologically similar to those observed in

sarcoidosis, suggesting that CFA injection followed by OV in rats may be a good experimental model for sarcoidosis. We would anticipate that ovarian dysfunction in humans is likely to accelerate the granulomatous response in the lung.

We demonstrated that pulmonary infiltration with lymphocytes was significantly increased in the BALF of the OV rats, consistent with observations in OV rats treated with heat-killed bacillus Calmette-Guérin [29]. It is worth noting that the lymphocyte population varies between blood and BALF when examined in healthy rats; in the lung, lymphocytes marginate to the vascular endothelium (marginal pool) and enter the interstitial tissue (interstitial pool), where they can be recovered in BALF [31]. Therefore, it is likely that a decrease in sex hormones provokes local inflammatory responses, resulting in the recruitment of lymphocytes.

The levels of Th1-type cytokines in the BALF of OV rats were significantly higher than those in SHAM rats, while the levels of Th2-type cytokines in both BALF and serum were comparable between OV and SHAM rats. These findings suggest that a decline in the levels of sex hormones does not alter the systemic immune status but

Fig. 5. Effect of ovariectomy on the production of Th1 and Th2 cytokines in the serum. IL-12 (a), IFN- γ (b) and IL-13 (c) levels in the serum were measured by ELISA. The bars indicate the means.

Fig. 6. Effect of ovariectomy on the production of Th1 and Th2 cytokines in BALF. IL-12 (a), IFN- γ (b) and IL-13 (c) levels in BALF were measured by ELISA. The bars indicate the means. * $p < 0.05$.

does affect the local immune responses at the site of inflammation in a Th1-polarized manner. In agreement with this, Th1-polarized local immune responses accompanied by infiltration of lymphocytes have been highlighted not only in sarcoidosis but also in a number of other granulomatous diseases [32, 33].

There is ample evidence suggesting that the sex hormones modulate the Th1/Th2 balance [34–38]. Kamada et al. [39] demonstrated that the levels of Th1 cytokines are augmented in women after menopause, and hormone replacement therapy prevents this augmentation, thereby improving the aberration of the Th1/Th2 balance. Recently, sex hormones were shown to modulate expression of the transcription factors T-bet and GATA3, which are

likely associated with Th1 and Th2 responses, respectively [40, 41]. Although further analyses are necessary to identify the molecular clues responsible for the cytokine shift provoked by modulation of sex hormones, our experimental animal model provides potential for understanding the roles of sex hormones in the pathogenesis of menopause-associated granulomatous diseases, such as sarcoidosis.

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