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Research Article

Progesterone Decreased Cell Infiltration in Airways of Systemic Sclerosis Mice Model

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ABSTRACT

Systemic sclerosis (SSc) is the fibrotic autoimmune disease with a higher incidence in women. Lung fibrosis is the most common cause of death in SSc patients. Sex steroids have crucial role in the induction of autoimmune diseases. Progesterone impacts autoimmunity by direct action on parenchymal cells or through its immunomodulatory effect. This study aimed to investigate the effect of progesterone on the cellularity of airways in an animal model of systemic sclerosis. 6 groups of mice were considered in this study. Systemic sclerosis (SSc) was induced in female BALB/c mice by subcutaneous injection of bleomycin for 28 days. For evaluating the effect of Progesterone in SSc model, Progesterone was administered subcutaneously parallel with bleomycin for 28 days or one week after the first administration of bleomycin for 21 days. Further, three control groups were included in this study. On day 29, under lethal anesthesia bronchoalveolar lavage (BAL) was collected and evaluated for cellularity. Our results indicate the increment of cells in BAL of SSc ($P<0.0001$) mice. Administration of Progesterone for 28 days significantly reduced the infiltrating cells in BALs ($P<0.01$) of SSc mice. The differential count of BALs indicates that Progesterone reduced the number of lymphocytes ($P<0.05$) in SSc mice but did not affect the number of macrophages. Therefore, we conclude that progesterone reduced the inflammatory cells in airways by decreasing the number of lymphocytes.

Introduction

Systemic sclerosis (SSc) is a chronic connective tissue disease with immune system dysregulation, microvascular injury and fibrosis of skin and internal organs. Progressive fibrosis compromises the function of organs by continuous accumulation of extracellular matrix (ECM) components and the replacement of normal tissue with permanent scar tissue [1]. Lung dysfunction because of fibrosis is the leading cause of morbidity and mortality in SSc patients [2]. Fibrosis is initiated by a tissue injury and activation of innate and adaptive immune responses. Activation of the immune system results in activation of M2-type macrophages (alternatively-activated), Th2 cells, and differentiation of fibroblasts to myofibroblasts [3]. An investigation has indicated the increment of collagen synthesis has occurred in fibroblasts adjacent to inflammatory cells. This hypothesis confirms that the necessary signal for activation of fibroblasts and the production of collagen is provided by adjacent inflammatory cells, primarily by lymphocytes [4].

Systemic sclerosis (SSc) is a female predominant autoimmune disease (AD). The prevalence of SSc is three-fold more common in women than men, and its incidence during the reproductive period (ages 15-50) is 15-times higher [5]. The predominancy of autoimmune diseases in females

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has been explained by several theories: The immune regulatory effects of sex hormones, sex-dependent genetic variations, microchimerism and gender-relatedd determinants such as occupation. Estrogen, Progesterone (PG), and androgens are the main hormones involved in sex differences. The effects of estrogen and androgens on autoimmune diseases have been considerably investigated. However, there is a paucity of information concerning the effects of Progesteron on ADs [6].

PG may influence the ADs by its immunomodulatory effects (immune-endocrine interaction) or through its direct effects on parenchymal cells such as brain. Similar to glucocorticoids, PG inhibits the production of TNF-α and IL-6 from cord blood monocytes [7]. PG inhibits the development of Th1 responses; in contrast, it promotes the development of Th2 immune response [8]. PG induces the polarization of M2-type macrophages while it inhibits the functions of M1-type (classically activated) macrophages, e.g., the suppression of TNF-α production [9]. Due to the modulatory effect of PG on inflammation, we established a murine model of scleroderma, and investigate the effect of PG on the cellularity of BAL in SSc mice [10].

Material and Methods

I Animals

8 weeks old female BALB/c mice were achieved from the Pasteur Institute (Tehran /Iran). Prior to the beginning of experiments, mice were retained in the laboratory cages for two weeks to acclimate to the laboratory conditions. Mice were kept in the standard laboratory cages at room temperature and ambient humidity under a 12-hour reverse light-dark cycle. They received ad libitum access to the standard laboratory chow and water [10]. All animal experiments were done according to arrived guideline and were approved by the animal and ethics committee of Iran University of medical sciences (1393.033025094).

Mice were randomly divided into the following 6 groups 1) Control ((Phosphate buffered saline group, PBS), 2) Bleomycin (BLM), 3) Bleomycin+PG for 21days (BLM+PG21), 4) Bleomycin+PG for 28 (BLM+PG28) 5) PG for 21 days (PG21). PG for 28 days (PG28). SSc was induced according to Yamamoto *et al.*, briefly mice received daily Bleomycin (BLM) (Nippon Kayaku, Tokyo, Japan) by a concentration of 1.5 U/mL dissolved in 100 µL Phosphate buffered saline [11]. BLM was injected subcutaneously into the shaved back skin for 28 days. For investigating the effect of PG on recruitment of cells to the airways, mice received daily 1 mg/body PG (dissolved in 40 µL sesame oil) subcutaneously. PG injection started simultaneously with bleomycin injection in group BLM+PG28 and in group BLM+PG21, PG injection started after initiation of the inflammatory phase, with the initiation of fibrotic phase (1 week after bleomycin injection). We included 3 control groups, groups PG21 and PG28 received subcutaneous PG for 21 and 28 days, respectively, and another control group received daily 100µL PBS subcutaneously (PBS). Administration of bleomycin and PG were done using a 30 and 29-gauge needle respectively in various areas of mice back skin.

II Bronchoalveolar Lavage and Cell Counting

On day 29, mice were anesthetized intraperitoneally with ketamine (87.5 mg/kg) and xylazine (12.5 mg/kg). Tracheas of lethally anesthetized mice were cannulated and lavaged, as described by Mojtabavi *et al.* [12]. In brief, the airways were washed with 2 X 800 µl PBS and the volume of recovered fluid was determined. Total leukocytes in the bronchoalveolar lavage (BAL) were counted with a hemocytometer then cytospin slides were prepared and stained with May-Grünwald- Giemsa to determine the cell differential count.

III Statistics

Statistical analyses were performed using GraphPad Prism software. Groups greater than two were analyzed with One-Way ANOVA, tukey multiple comparisons test. All data are expressed as mean ± SEM. Differences were considered significant when $p < 0.05$ (denoted as *), $p < 0.01$ (denoted as **), $p < 0.001$ (denoted as ***) and $p < 0.0001$ (denoted as ****).

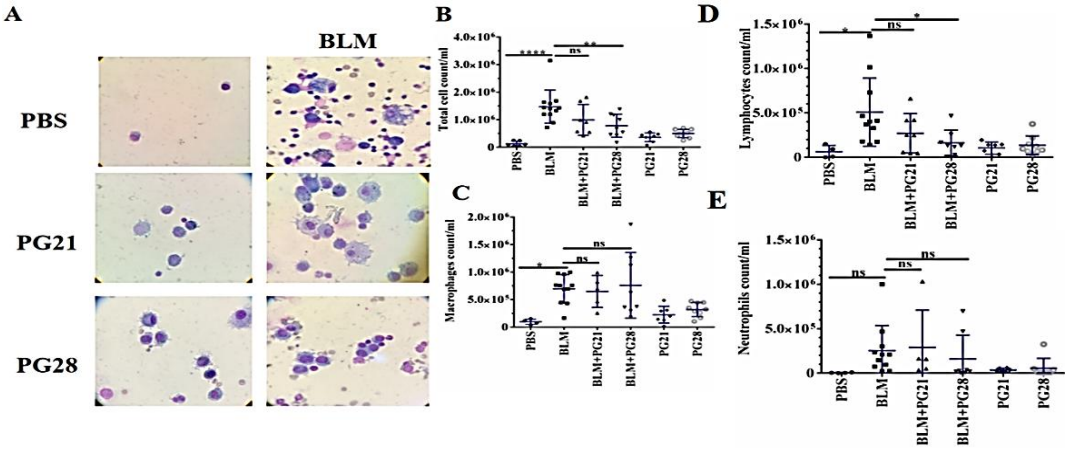

Figure 1: Progesterone changes the cellularity of BAL in SSC mice. Mice were treated with bleomycin alone or bleomycin and PG for 28 or for 21 days. On day 29 mice were lethally anesthetized and airways were cannulated and washed with PBS. Bronchoalveolar lavage (BAL) were collected. Cells were counted and transferred to the slides via cytospine and stained with May Grunwald- Giemsa for cell differential and 1000X magnification **A)** Total cell count in BAL **B)** number of macrophages **C)** number of lymphocytes **D)** number of neutrophils **E)** results were expressed as a mean ±SEM. N=9-10 mice in each group. Data were analysed with One–way ANOVA.
BLM: Bleomycin; PG: Progesterone, PBS: Control; BLM+PG: Bleomycin+Progesterone; ****: p<0.0001; ***: p<0.001; **: p<0.01, *: p<0.05, ns: p>0.05.

Results

Immune Cell Trafficking in Airways

Bleomycin injection induced fibrosis in skin and lung of mice as we reported in our previous study [13]. BLM injection seriously increased cellularity in airways compared to Control mice; PBS ($P < 0.0001$). The total cell numbers in bronchoalveolar lavage (BAL) of mice received bleomycin and PG for 28 days (BLM+PG 28group) decreased significantly in comparison with mice treated only with bleomycin ($P<0.01$). PG alone did not affect cellularity in airways (Figures 1A & 1B). In all groups, macrophages were predominant cells (Figure 1C). In groups BLM+PG lymphocyte count decreased in comparison with BLM group and was significant in BLM+PG28 group ($p<0.05$) (Figure 1D). Neutrophil count variation was not significant between groups (Figure 1E).

Discussion

In our study, Progesterone decreased the cellularity, especially lymphocytes count in bronchoalveolar lavage (BAL) of systemic sclerosis (SSc) mice. Interstitial lung disease is a profound problem in SSc patients and the main cause of morbidity and mortality in these patients [2]. Therefore, investigation of the BAL fluid for disease prognosis and detection of biomarkers was in consideration of researchers [14]. Our results indicated that subcutaneous administration of bleomycin in BALB/c female mice causes lung and skin fibrosis, which closely resemble human SSc [13]. BLM injection seriously increased cellularity in airways of SSc mice, and the main infiltrating cells in SSc BALs were macrophages and lymphocytes. Progesterone injection in fibrotic mice reduced cellularity, especially lymphocyte count in airways.

Most of the studies suggested that fibrosis is initiated by inflammation and activation of innate and adaptive immunity; therefore, they supposed that without inflammation, there would be no fibrosis [3]. In SSC patients and animal model of SSC, damage of endothelium and epithelium plus inflammation of lung tissue, alveolitis, and finally, accumulation of collagen causes lung fibrosis [1]. Neutrophils and macrophages will be recruited to the damaged tissue. The presence of macrophages in this scenario is necessary to uptake the apoptotic cells, and this action will divert their classical phenotype to an alternative phenotype. When the repair system is insufficient, these macrophage stay in tissue and produce growth factors which would participated in fibrosis process [15].

Gene analysis studies revealed that responsible genes in tissue repair and fibrosis are the same genes that are involved in Th2 response, including IL-4, IL-5, and IL-13. Studies indicated that infiltrating T cells to fibrotic tissues are heterogeneous and controversial results were obtained based on the production of IFN-Ɣ or IL-4 cytokines. The effect of polarized cells can have a major role in the metabolism and differentiation of fibroblasts, whereas Th1 cells inhibit collagen production and induce MMPs through IFN-Ɣ or direct contact with fibroblasts. In contrast, Th2 cells increased collagen accumulation in tissues and inhibited MMPs through IL-4 and IL-13 [3, 16]. In our observation, bleomycin injection damages cells in the lung tissue and causes injury, apoptosis, and cellular trafficking in airways, which manages milieu for the occurrence of the fibrosis as mentioned above scenario [10].

According to the report from Braun *et al.*, macrophages are the major cell types in the BAL of bleomycin model. The number of macrophages increased and reached the highest on day 28 however, the number of neutrophils remained constant in all assessed time points. The number of lymphocytes on early time points was low but reached its maximum on day 28, and the majority of them were CD4 positive cells [17]. In agreement with this, in our research, macrophages were prominent cells in BAL of bleomycin treated mice. Bleomycin injection increased the number of lymphocytes in airways, but the number of neutrophils was constant. Increment of neutrophils number in BAL of SSC patients is accompanied by bad prognosis, however, in an animal model of SSC and our study, neutrophils do not increase, and this could be a reason why we didn't have any mortality in our mice [14].

Injection of PG for 28 days decreased the total cell number in BAL. Nonetheless, macrophages count remained unchanged. PG in the injured lung with reducing inflammation and inducing alternatively-activated macrophages in the early phase of bleomycin injury may accelerate lung fibrosis; however, further studies are necessary to investigate the macrophages phenotype.

It is known that PG is an anti-inflammatory hormone; this steroid can inhibit inflammation, inflammatory macrophages, and can cause the induction of alternative macrophages [18, 19]. In this study, we postulate that PG with decreasing airway cellularity and increasing macrophages percent may reduce the inflammation in airways and divert inflammatory macrophages toward alternative macrophages. Induction of alternative macrophages progresses the tissue repair and fibrosis, which can explain why women are more prone to systemic sclerosis. Nevertheless, to prove this hypothesis, further studies are required for identifying the phenotype of macrophages in BAL and lung tissue. Epidemiological studies support this hypothesis that atopic diseases such as allergic asthma are more prominent in young boys before puberty and increased in girls after puberty and confirm the effect of sexual steroids in those diseases [6].

PG is the main hormone of luteal phase and pregnancy which provokes Th2 responses and decreases the cytotoxicity of lymphocytes [20]. Lee *et al.* indicated the reduction of CD4 cells in the luteal phase in comparison to the follicular phase and increment of IL-4 in this phase [21, 22]. Therefore, consistent with the effect of PG in blood lymphocytes, PG decreased lymphocytes in BAL of SSc mice. Animal models discovered different roles for lymphocytes in the induction of fibrosis. Depletion of lymphocytes by antibodies reduced the fibrosis; however, in SCID mice, lung fibrosis was induced, which indicates that induction of fibrosis by bleomycin administration is not lymphocytes dependent [23, 24]. In our study, we did not investigate the impact of lymphocytes reduction on lung fibrosis, which required further studies.
In this study, we did not determine the cell phenotype, notably the phenotypes of macrophages in BAL and also the impact of PG on those phenotypes, which would be included in our feature studies. In conclusion, Progesterone reduced the infiltration of cells, especially lymphocytes, in airways of SSc mice model.

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