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Activation of the IL-6/JAK2/STAT3 pathway induces plasma cell mastitis in mice

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ABSTRACT

Plasma cell mastitis (PCM) is a chronic mastitis with limited treatment options and common recurrence. A histopathological hallmark of PCM is the infiltration of numerous plasma cells surrounding the mammary duct. Our previous study showed that the activity of the IL-6/STAT3 signaling pathway was elevated in patients with PCM. However, the etiology of PCM remains largely unclear. In this study, we sought to explore the effects of IL-6/JAK2/STAT3 signaling pathway in the pathogenesis of PCM. Histological analysis showed that the mammary glands of mice that received human breast tissue homogenates, followed by an injection of IL-6, exhibited features of PCM similar to human PCM. The IL-6/JAK2/STAT3 signaling activity was significantly elevated and Bcl-2 was highly expressed in CD138 + plasma cells in the mammary glands of mice with PCM. Furthermore, treatment with AG-490, an inhibitor of JAK family kinases, suppressed activation of the IL-6/JAK2/STAT3 signaling cascade, in turn resulting in a decreased number of plasma cells in the mammary gland and reversing the pathogenesis of PCM. Taken together, our study indicated that a PCM mouse model was successfully established through activation of the IL-6/JAK2/STAT3 pathway by injecting IL-6 into the mammary gland of mouse that had received homogenates of human breast tissue. Thus, the IL-6/JAK2/STAT3 signaling pathway plays a critical role in orchestrating the pathogenesis of PCM.

1. Introduction

Plasma cell mastitis (PCM) is a unique type of mastitis and is often misdiagnosed as inflammatory breast cancer [1,2]. The incidence of PCM is approximately 5% of breast cancer and has increased gradually in recent years [3,4]. However, treatment options for PCM are limited. Surgical excision is the only current curative treatment for PCM when the disease is definitively diagnosed [5–7]. Notably, PCM frequently recurs after surgery and treatment is greatly hampered by recurrence and fistula formation [5]. To develop effective therapeutic treatments for PCM, it is critical to investigate the mechanism underlying the pathological progression of PCM. In order to study the mechanism, it is necessary to generate an animal model that can faithfully mirror the features of the disease and be used to study potential therapies. Although a study has previously shown that a PCM model could be generated by injecting homogenates of pathological breast tissue from PCM patients into mice [8], the components of these pathological tissues were very complex when analyzing factors involved in the pathogenesis of PCM, and it was difficult to reproducibly examine the precise mechanisms of PCM development in mice.

Plasma cells, derived from B lymphocytes, are rarely present in normal breast tissue. The infiltration of plasma cells, B lymphocytes, and T lymphocytes surrounding the mammary ducts is the diagnostic criteria of PCM [1,4]. Interleukin-6 (IL-6), initially designated as a B cell differentiation factor, is a multifunctional cytokine that regulates immune and inflammatory responses [9]. Once bound to a specific receptor complex, IL-6 can activate downstream kinases such as Janus kinase 2 (JAK2) and subsequently trigger the phosphorylation and nuclear localization of signal transducer and activator of transcription 3 (STAT3) [10,11]. The IL-6/JAK2/STAT3 signaling pathway is crucial not only for the differentiation of plasma cells, but also for their survival [12,13], as plasma cells only survive a short time after being isolated *ex vivo* [14] or generated *in vitro* [15]. In our previous study, we found that IL-6/STAT3 signaling activity was activated in PCM, suggesting that it might play a role in the pathogenesis [4]. However, the effect of the activated IL-6/JAK2/STAT3 pathway on the

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pathological process of PCM remains largely unknown.

In the current study, our data showed that the mammary glands of mice that were injected with homogenates of human breast tissue followed by IL-6 exhibited PCM characteristics, which resembled human PCM. The mammary tissue of these mice had elevated activity of the IL-6/JAK2/STAT3 signaling pathway, and plasma cells within the mammary tissue had high expression of Bcl-2. An inhibitor to the IL-6/JAK2/STAT3 pathway, AG-490, reversed the histological manifestation of PCM in the mouse mammary glands. Thus, our study demonstrates that the IL-6/JAK2/STAT3 signaling pathway plays an important role in regulating the pathogenesis of PCM.

2. Materials and methods

2.1. Murine model of PCM – animal and material preparation

Fresh normal mammary tissues were excised from voluntary breast fibroma patients. The research was approved by the Ethical Committee of Xi'an Jiaotong University and informed consents were obtained from all enrolled patients. Tissues were homogenized in physiological saline at a ratio of 1:3. The homogenate was centrifuged at 12,000*g* for 15 min at 4 °C, and the supernatant was collected. A water-in-oil emulsion was made by mixing the supernatant with an equal volume of complete Freund's adjuvant (Sigma-Aldrich Co., Ltd., U.S.A.). Recombinant murine IL-6 (rIL-6) was purchased from PeproTech (Rocky Hill, NJ, U.S.A.) and was dissolved in double-distilled water (DDW).

BALB/c mice (6–8 weeks old, body weight 18–20 g) were purchased from Laboratory Animal Center of Xi'an Jiaotong University (Xi'an, China). All animals were fed with standard laboratory chow and water, and kept at a temperature of 24 ± 1 °C, with a relative humidity of 40–80%. Thirty female mice were randomly divided into five groups: group A (saline + DDW), group B (saline + rIL-6 (1 μg/mL)), group C (water-in-oil emulsion + DDW), group D (water-in-oil emulsion + rIL-6 (1 μg/mL)) and group E (water-in-oil emulsion + rIL-6 (2 μg/mL)). A chloral hydrate solution (0.3%) was used for anesthesia via intraperitoneal injection. The saline or water-in-oil emulsion (50 μL) was injected subcutaneously to the #4 inguinal mammary gland after sterilizing the surrounding area with 75% ethanol. After 3 weeks, the mammary gland was injected with DDW or rIL-6 (50 μL) at indicated concentrations. Animals were sacrificed 1 week after injection of DDW or rIL-6. All animal experiments were conducted in accordance with the principles and procedures outlined in the Guideline for the Care and Use of Laboratory Animals of the Xi'an Jiaotong University.

2.2. AG-490 treatment

Female BALB/c mice were randomly divided into two groups (8 mice/group): group AG-490 and group DMSO. All mice were treated under the same conditions as group D. After the injection of rIL-6, mice were treated daily with AG-490 (0.5 mg, Selleck Chemical, TX, USA) or DMSO (1%) for 1 week through intraperitoneal injection. Animals were sacrificed 1 day after the last treatment.

2.3. Histological analysis

Following euthanasia, the mammary tissue of mice in each group were harvested and fixed in 10% formalin overnight. Fixed tissue was processed and embedded in paraffin. For hematoxylin and eosin (H&E) staining, tissue sections (5 μm) were deparaffinized with xylene, rehydrated with graded alcohol, and stained according to the manufacturer's instructions. The stained tissue sections were visualized under a microscope (Olympus BX51, Japan).

2.4. Immunohistochemical analysis

The tissue sections (5 μm) were deparaffinized, rehydrated, and washed in phosphate-buffered saline (PBS). Endogenous peroxidase was blocked by 3% hydrogen peroxide at room temperature for 20 min. After antigen retrieval, the sections were incubated overnight at 4 °C with IL-6 polyclonal antibody (1:500, Abcam, Cambridge, UK), CD138 monoclonal antibody (1:500, Abcam, Cambridge, UK), CD20 polyclonal antibody (1:200, Bioss, Beijing, China), phospho-JAK2$^{Y1007/1008}$ monoclonal antibody (1:200, Abcam, Cambridge, UK), phospho-STAT3^{Y705} monoclonal antibody (1:200, Cell Signaling, MA, USA) or Bcl-2 polyclonal antibody (1:100, Bio-World, Ohio, USA). After rinsing three times with PBS, sections were incubated with a biotinylated secondary antibody for 30 min at 37 °C, followed by staining with diaminobenzidine.

The stained sections were blindly evaluated by two experienced pathologists. Cells were counted in five randomly selected areas with a 40× objective. For immunohistochemical (IHC) scoring, both the percentage of positive cells and the intensity of staining were considered [16]: percentage score = 0 if < 1%, 1 if 1–25%, 2 if 25–50%, 3 if 50–75%, 4 if > 75%; intensity score = 1 (weak), 2 (moderate), and 3 (strong). A final classification was obtained by multiplying percentage and intensity.

2.5. Immunofluorescence analysis

For immunofluorescence (IF) analysis, frozen sections (10 μm) were incubated in primary antibodies against CD138 (1:500, Abcam, Cambridge, UK) and Bcl-2 (1:100, Bio-World, Ohio, USA) overnight at 4 °C. Alexa 488 and Alexa 594-conjugated secondary antibodies (1:400, Invitrogen, CA, USA) were used for 1 hr at 37 °C, and the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) for ten minutes. The stained sections were observed under a microscope (Olympus BH2, Japan) equipped with a camera.

2.6. Cytokine enzyme-linked immunosorbent assays

Blood was centrifuged at 2000*g* for 10 min and subsequently the serum was collected for assay. Mammary gland tissue was weighed, homogenized with PBS (1/7 w/v) on ice and centrifuged at 2000*g* for 40 min at 4 °C. The supernatants were collected for the measurement of IL-6. IL-6 levels in the serum or the tissue homogenate were quantified using the enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Ray Biotech Inc., GA, USA).

2.7. Western blot analysis

The mammary tissues were homogenized and total proteins were extracted by Tissue Protein Extraction Reagent (T-PER), according to the manufacturer's instructions. The protein concentration was determined by BCA protein assay kit (Heart, Xi'an, China). The proteins were separated on a 10% SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% nonfat dry milk solution, the membrane was incubated overnight at 4 °C with primary antibodies against JAK2 (1:1000, Cell Signaling, MA, USA), phospho-JAK2$^{Y1007/1008}$ (1:500, Cell Signaling, MA, USA), STAT3 (1:1000, Cell Signaling, MA, USA), phospho-STAT3^{Y705} (1:1000, Cell Signaling, MA, USA) and Bcl-2 (1:500, Bio-World, Ohio, USA). GAPDH was used as a loading control. The membranes were subsequently incubated with a secondary antibody for 1 hr at room temperature. Membranes were washed and processed with Immobilon Western HRP Substrate (Millipore, MA, USA).

2.8. Statistical analysis

Statistical analyses were performed using the SPSS 17.0 software package (IBM, IL, USA). All values were expressed as the mean ± standard error of mean (SEM). Fisher's exact test was used to compare the positive pathological findings of PCM among groups. One-way

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