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Differential Expression Profile of Immunological Cytokines in Local Ovary in Patients with Polycystic Ovarian Syndrome: analysis by Flow Cytometry

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ABSTRACT

Objective: Immune dysregulation may play an important role in the pathogenesis of polycystic ovary syndrome (PCOS). The purpose of this study was to investigate the Th1 and Th2-related cytokine profile in local ovary of women with PCOS.
Study Design: The T lymphocytes of follicular fluid (FF) were obtained at the time of oocyte retrieval before *in-vitro* fertilization (IVF) in woman with or without PCOS. After culturing with PMA, Ionomycin and Golgi stop agent, cells were detected for the intracellular cytokine production by flow cytometry. The profile of Th1 (IFN-γ, IL-2) and Th2 (IL-4, IL-10) cytokines of $CD3^+$ $CD4^+$T lymphocyte subsets were analyzed through invert gating. These cytokines in FF were also evaluated by ELISA.
Results: Flow cytometry analysis showed that the production of Th1 (IFN-γ, IL-2) cytokines in FF lymphocytes in PCOS patients were significantly higher than those in controls; ELISA result also demonstrated that the concentration of Th1 cytokines (IFN-γ, IL-2) in FF in PCOS patients is significantly increased compared with those in controls.
Conclusion: It is concluded that the immune dominance of Th1 may be the immunological feature of the ovary in PCOS patients. It might participate in the immune pathogenesis in the ovary of PCOS patients. These results suggest that chronic inflammation maybe one of the underlying mechanism for the pathogenesis of PCOS.

Introduction

The polycystic ovary syndrome (PCOS) is one of the most common endocrinopathies in women. It is considered as the most common cause of female infertility and affects 4–10% of reproductive-age women [1,2]. The disorder is characterized by hyperandrogenism, chronic oligoovulation or anovulation and polycystic ovaries [3,4]. Other manifestations include hyperinsulinism, atherogenichyperlipidaemia, diabetes mellitus, obesity, hirsutism, acne and an increased risk of endometrial cancer. [5]. Although the etiology of PCOS remains unclear, previous studies show that hyperandrogenism and insulin resistance(IR) as well as their interaction have been thought to accelerate the development of PCOS [6,7]. In addition, most PCOS patients are accompanied with obese, which is strongly associated with insulin resistance and hyperglycemia [8–10].

Recently, immunological mechanisms have been reported to participate to the regulation of folliculogenesis [11]. Bukulmez et al. (2000) found an increased number of white blood cells in

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polycystic ovaries, and Shi et al (2013) confirmed their results [12,13] . It has been found that PCOS is associated with low-grade systemic inflammation as evidenced by elevated level of multiple markers of inflammation such as C-reactive protein, IL-18, IL-6, monocyte chemoattractant protein-1 and white blood cell count as well as endothelial dysfunction and increased oxidative stress [14,15]. These results indicate a possible involvement of the autoimmune inflammation in the pathogenesis of PCOS [16,17]

The follicular fluid (FF) cells are made up of granulosa cells, leukocytes, macrophage, among other cells [18]. Granulosa cells and leukocytes can secrete numerous cytokines and chemokines implicated in mediating diverse functions in ovarian processes such as folliculogenesis, ovulation and corpus luteum formation and regression [19,20]. In the past decades, these immunocompetent cells including monocyte, macrophages, B cells, T cells, as well as active cellular interactions among T lymphocyte subpopulations have been described in human preovulatory follicles [21]. These cells seem to exert an effect in the immunoregulation of hormones involved in folliculogenesis [22,23].

Accumulating evidence shows that the inflammation and immune regulation may be involved in the etiology of PCOS. However, the underlying mechanisms remain unclear. This study was designed to investigate the cytokine profiles of $CD3^+$ $CD4^+$ T lymphocyte subset of FF in ovaries of women with PCOS undergoing assisted reproductive technologies (ART) through flow cytometry analysis and ELISA, respectively. We attempted to explore the role of Th1 and Th2-type cytokine in the development of PCOS and to describe a multiparametric graph of the levels of Th1 and Th2-associated cytokines in PCOS through invert gating pattern by flow cytometry.

Materials and Methods

Subjects

Forty women with PCOS (20 lean and 10 overweight women and 10 obese women) between 20–33 year and 40 weight-matched female (20 lean and 10 overweight women and 10 obese women) who underwent IVF or ICSI for other causes of infertility, including tubal disease, male factor and unexplained infertility between 20 and 33 year participated in the study. Obesity and overweighed were in accordance with WHO obesity definition, patients with BMI between 25 and 30 are considered overweighed and higher that 30 are considered obese. While lean subjects had a BMI between 18 and 25 kg/m^2. All the control group and PCOS group are in line with normal distribution when tested with SPSS test. PCOS was diagnosed according to Rotterdam criterion, which include at least two of the three criteria: 1. Oligo- and/or anovulation; 2. Clinical and/or biochemical signs of hyperandrogenism; 3. Polycystic ovaries (presence of 12 or more follicles in each ovary measuring 2 ± 9 mm in diameter, and/or increased ovarian volume), and exclude nonclassic congenital adrenal hyperplasia, Cushing's Syndrome, hyperprolactinemia and thyroid diseases [3]. All PCOS patients received primary treatment, including Diane-35 treatment for 3–6 months and appropriate dosage of metformin drug to improve insulin resistance if the patients show different status of insulin resistance. All subjects were screened for diabetes or inflammatory illnesses, and no one was taking medications that would affect immune function for at least 6 weeks before study participation. The process was approved by the Ethics Committee of the Sichuan University, China, and all subjects signed and received a copy of a written informed consent form.

Lymphocyte Isolation and Culture

Folicularfluid (FF) which was not contaminated by visible blood or aspiration buffer was aspirated and pooled at the time of oocytes retrieval for each patient. After washing with Hanks' balanced salt solution, cells were resuspended with PBS and mixed over with 6% hetastarch in 0.9% sodium chloride at the ratio of 1:6 (v:v) for 1 h at 20 °C to enrich the nucleated cells. The upper suspended layer was collected and the cells were washed with PBS for three times, then subjected to erythrocyte removal using red blood cell lysis solution (Biolegend, San Diego, CA, USA). Cells were resuspended in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (ICN), 2% L-glutamine, and 1% penicillin–streptomycin at the concentration of 2×10^6 cells/ml, then cultured with PMA (25 μg/L) (Phorbol 12-Myristate 13 Acetate, Sigma) and Ionomycin (1 μg/L, Sigma) at 37 °C, 5% CO_2 for 72 h. Golgi stop agent Monensin (2 mg/L, Sigma) was added 6 h before harvesting the cells.

Flow Cytometry Analysis

Cells were harvested from the culture, then 4×10^5 cells were incubated with a panel of monoclonal antibodies directly conjugated with different fluorochromes (three-color direct immunofluorescence, MultiTEST CD3 APC/CD4 PE-cy5/CD8 PE-cy7, eBioscience, San Diego, CA, USA) at 4 °C for 15 min for surface antigen detection. After washing cells were supplemented with Fixation Medium Reagent A (Caltag Laboratories, Invitrogen, CA, USA) for 15 min, then followed by the addition of Permeablilization Medium Reagent B (Caltag Laboratories, Invitrogen, CA, USA) for determination of intracellular cytokine production. FITC-conjugated anti-human IFN-γ, PE-conjugated anti-human-IL-4 as well as FITC-conjugated anti-human IL-2, PE-conjugated anti-human-IL-10 was added, respectively (eBioscience). Cells were washed and resuspended into 0.5 ml PBS. The immunofluorescence reactivity was then detected by cytofluorometry. 2×10^5 cells were counted by flow cytometer (FACSAria, BD, San Jose, USA) for each sample. Data were analyzed using FACSDiVa Software (BD, San Jose, USA). The invert gating is employed, in which $CD3^+$, $CD8^-$cells were considered as $CD3^+$ $CD4^+$ population as CD4 molecules would shielded after incubating with PMA[24]. Staining pictures of the surface antigens(CD3/4/8) without intercellular antigens were shown in Fig. S1.

Enzyme-Linked Immunosorbent Assays

FF was obtained from first puncture follicles (range 18–24 mm) at oocyte collection from each patient. Samples were centrifuged at 1,200 rpm for 10 min, and then stored at -20 °C. Th1 and Th2-type cytokine levels were measured by a solid-phase enzyme-linked immunosorbent assay using a commercially available immunoassay kits (Bender Medsystems, Vienna, Austria) according to manufacturer's instruction. Inter- and intra-assay variability was 6.8 and 7.9% for IFN-γ, 6.7 and 7.8% for IL-4, respectively. And inter- and intra-assay variability was 5.4 and 6.1% for IL-2, 8.9 and 9.0% for IL-10.

Statistics

A unpaired Student's *t*-test was used to compare two groups (means ± SD). Hypothesis tests were set as two sides, with probability values of less than 5% were used to represent statistical significance. SPSS 16.0 (SPSS Inc., Chicago, USA) software was used for statistical analysis.