The estimation of IL-2 and IL-2 receptors in peritoneal fluid of infertile patients with endometriosis

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Abstract

Background: *The aim of the study was to investigate the concentrations of interleukin-2 (IL-2), soluble interleukin-2 receptors (sIL-2R) and the expressions of CD25 antigen (IL-2Rα) on T CD3⁺ lymphocytes in peritoneal fluid of infertile patients with endometrisis in comparison with the reference group of patients with benign ovarian tumors.*

Material and Methods: *Twenty four infertile patients with endometriosis and 18 patients from reference group were included in the study. The concentrations of IL-2 and soluble IL-2 receptors were measured with the use of ELISA immunoenzymatic method. The expressions of surface molecules were estimated using the two-color flow cytometric method.*

Results: *The concentrations of IL-2 in peritoneal fluid of infertile patients with endometriosis were significantly higher when compared to the reference group (2.83±2.6 pg/ml vs. 1.27±1.58 pg/ml; p<0.05). The concentrations of soluble IL-2 receptors and the expressions of CD25 antigen on T CD3⁺ lymphocytes in the study group did not differ significantly when compared to the reference group (sIL-2R: 463.9±200.4 U/ml vs. 461.35±209.7 U/ml; NS; T CD3⁺CD25⁺: 6.77±8.18% vs. 5.7±6.43%; NS). However, we have found significant differences in immunological parameters between individual stages of endometriosis. The expressions of CD25 antigen were significantly higher in the second and third stages of endometriosis when compared to the first stage of the disease.*

Conclusions: *The excessive concentrations of IL-2 in peritoneal fluid of patients with endometriosis may be responsible for an infertilty in these group of patients. Higher expressions of CD25 antigen on T CD3⁺ lymphocytes which are observed in the third and second stage of endometriosis can suggest the acivation of T CD3⁺ cells during the progression of the disease.*

Key words: *CD3⁺25⁺ T cells, endometriosis, infertility, IL-2, soluble IL-2 receptors.*

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Introduction

Endometriosis is characterized by the presence and growth of endometrial tissue in the locations other than uterine cavity. The disease affects about 10-20% of women at reproductive age and up to 45% of infertile patients. Despite many efforts of the investigators to find factors which cause the disease, the etiopathology of this disorder remains unknown. Recent data suggest that aberrant immunologic mechanisms are involved in its pathophysiology. There is a general concept that endometriosis is a local pelvic inflammatory process with altered function of immune cells in the peritoneal environment. Many studies support the hypothesis that endometriosis is associated with an activation of peritoneal macrophages and higher concentrations of these cells in peritoneal fluid. In

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fact, peritoneal cavity of patients with endometriosis contains a fluid with a large amount of activated macrophages. The secretory activity of these cells is increased and it is suggested that they play an important role in the development and maintenance of endometriosis [1]. The role of macrophages in the pathogenesis of endometriosis is well proved, but the engagement of T cells in the pathogenesis of the disease has not been yet established. There are the concepts that the secretion of Th1 and Th2 type cytokines is altered in women with endometriosis, suggesting that T helper subsets play a role in the immunological responses to endometriosis [2, 3]. Some results suggest that the cytotoxic activity of NK and LAK cells is decreased in patients with endometriosis [4, 5].

The aim of our study was estimate the concentrations of Interleukin-2 (IL-2), soluble IL-2 receptors (sIL-2R) and the expressions of CD 25 antigen (IL-2Rα) on T $CD3^+$ lymphocytes in peritoneal fluid of infertile patients with endometriosis and patients with benign ovarian tumors.

Material and Methods

Patients

The patients participating in the study were admitted to the Department of Gynecology, Skubiszewski Medical University of Lublin. We examined forty two patients. Twenty four patients with endometriosis comprised the study group. A control group could not be selected because a sterilisation is forbidden by law in Poland. Therefore we composed a refference group from women with benign ovarian tumors. The patients included in the reference group had serous ovarian cystadenoma. The diameters of the ovarian cysts were between 3 to 6 cm. The patients from the reference group did not have the signs of inflammation in the peritoneal cavity. We took a peritoneal biopsy in the controls to rule out endometriosis.

The patients included in the study group had endometriosis and a primary infertility. The physical examination and a laboratory evaluation were performed. The diagnostic tests have been performed such as: thyroid stimulating hormone (TSH), thyroxine (T_4), triiodothyronine (T_3), prolactin (PRL) levels and liver function tests. All patients had normal values of TSH, thyroid hormones, prolactin and liver function tests. All patients underwent hysterosalpinography before laparoscopy. All couples also had peripheral blood chromosome assessment using standard techniques. In addition, patients with infections (viral and bacterial), hormonal abnormalities or autoimmune diseases were excluded. They did not have a hormonal therapy in the last three months preceding the laparoscopy.

All procedures were performed between seventh and ninth day of the menstrual cycle with ovulation confirmed by midphase luteal progesterone concentrations. The study design was accepted by the local Ethics Committee. Informed consent from the patients for peritoneal fluid sampling was obtained. Patients with endometriosis were classified according to the Committee on Terminology of the American Fertility Society.

Peritoneal fluid sampling and cell preparation

Peritoneal fluid samples from patients with endometriosis were taken during laparoscopy. Ten milliliters of peritoneal fluid were taken from each patient and every woman from the reference group and collected in sterile heparinized tubes. Peritoneal fluid samples were isolated using the lymphocyte separation medium – Lymphoprep (Nycomed, Torshov, Norway). Mononuclear cells were centrifuged for 30 min at 600 g at 4°C, collected from the interface with a Pasteur pipette and washed twice in 2 ml of PBS by centrifugation for 5 min at 250 g at 4°C. After washing, the cells were resuspended in PBS with 2% FCS (Fetal Calf Serum, GibcoBRL, UK) and the total number of cells was determined using the microscope. The cells were counted under the microscope with the use of the chamber of Neubauer. The cell density was adjusted to 10^7 cell/ml in PBS with 2% FCS. Next, the cells were labelled by direct staining with monoclonal antibodies. One hundred microlitres of the cell suspension were added to 10 µl of appropriate solution of fluorescein isothiocyanate (FITC) and phycoerythrin (PE) – conjugated antibodies (Becton Dickinson) in the following combinations: 1. negative control – anti-IgG_1 (FITC) and anti-IgG_{2a} (PE); 2. anti-CD 45 (FITC) and anti-CD14 (PE); 3. anti-CD3 (FITC) and anti-CD25 (PE). The cell and antibody mixture was incubated for 30 min at 4°C in the dark, it was centrifuged, washed twice by adding 1 ml of cold PBS to each tube with 1% sodium azide and 1% FCS and centrifuged again at 400 g for 10 min. Next, the supernatant was separated. Before a cytometric analysis, each sample was resuspended in 200 µl of PBS.

Flow cytometric analysis

The cell phenotype characterization was performed using the two-colour flow cytometric method (cytometer – Facs Calibur Becton Dickinson with an argon laser operating at 488 nm, equipped with analysis software-Immunocount II). The results were presented as percentage of cells stained with the antibody. The percentages of cells that were antibody positive were calculated by comparison with the appropriate control.

The estimation of IL-2 and sIL-2R concentrations

Sandwich enzyme immunoassay test kits were used for the quantitative determination of IL-2 (Endogen Human IL-2 ELISA kit, ENDOGEN Inc, MA, USA) and soluble receptors for IL-2 Endogen Human IL-2 ELISA kit, ENDOGEN Inc, MA, USA).

For Endogen Human IL-2 ELISA kit the standard curve was 0 – 1500 pg/ml, the detection limit – 6 pg/ml, inter – assay and intra – assay variation were less than 10%.

Assays for IL-2 and soluble IL-2 receptors were performed in the following manner – similar for each cytokine ELISA kit. The assays were performed on the microwell strips. Examined samples and the biotinylated antibody reagent were pipetted into the wells coated by anti-cytokine antibody (or anti-soluble IL-2 receptor antibody) and the plate was incubated in room temperature for 3 hours. Next, the plate was washed three times with wash solution, the streptavidin-HRP conjugate was added to each well and then the plate was again incubated at 20-25°C for 30 min. After washing, the substrate solution was pipetted into each well and the plate incubated under exclusion of light. The reaction was terminated by addition of the stop solution. Adsorbance was read at 450 nm using ELISA reader. All determinations were performed in duplicate. Cytokine (or soluble IL-2 receptor) concentrations were read from the standard calibration curve. The concentrations of IL-2 were presented in pg/ml and the concentrations of sIL-2R in U/ml.

Statistical analysis

Statistical differences between the groups were estimated using a standard non-parametric test (Mann-Whitney U test). The results of IL-2, sIL-2R concentrations and the expressions of CD25 anitgen on T $CD3^+$ cells were presented as the mean with SD and ranges. The correlations between different parameters were analyzed by Spearman rank correlation test. Differences at $p<0.05$ were considered as statistically significant.

Results

The concentrations of IL-2 in peritoneal fluid of infertile patients with endometriosis were significantly higher when compared to the reference group (IL-2: 2.83±2.6 pg/ml vs. 1.27±1.58 pg/ml; $p<0.05$). The concentrations of soluble IL-2 receptors in peritoneal fluid of patients with endometriosis did not differ significantly when compared to a reference group (sIL-2R: 463.9±200.4 u/ml vs. 461.35±209.7 u/ml; NS). The expressions of CD 25 antigen on T $CD3^+$ lymphocytes cells in peritoneal fluid of the study group did not differ in comparison with the reference group (T $CD3^+CD25^+$: 6.77±8.18% vs. 5.7±6.43%; NS). But we have found significant differences between individual stages of endometriosis. The expressions of CD25 antigen on T $CD3^+$ cells in the third stage of endometriosis were significantly higher when compared to first stage of the disease. Similarly, the expressions of CD25 antigen in second stage of endometriosis were significantly higher in comparison with the first stage of the disease. The concentrations of IL-2, sIL-2R and the expressions of CD25 antigen on T$CD3^+$ lymphocytes in perotoneal fluid in the group of patients with endometriosis depending on stage of the disease are presented in table 1.

Discussion of results

Many investigators observed decreased content of cytokines which reflect the function of T and NK cells, such as IL-2 and IFN-γ in peritoneal fluid of patients with endometriosis [6, 7].

The results of Hernandez-Guerrero at al. showed smaller intracellular synthesis of IL-2 and IFN-γ in peritoneal fluid (PF) and peripheral blood (PB) of patients with endometriosis. The decrease was associated with smaller percentage of activated, cooperative T lymphocytes and NK cells in PF of patients with endometriosis. This phenomenon was more stressed at the III and IV pathology degree. The studies of Polish group confirmed that the concentrations of IFN-γ were significantly decreased in the group of patients with endometriosis [8].

Hsu et al. did not observed the differences in mRNA or protein level of IL-2 and IL-10 in PF and PB of patients with endometriosis. The levels of IL-4 in PF and PB of patients with endometriosis were significantly higher than those of normal patients [4].

We observed the increased concentrations of IL-2 in peritoneal fluid of infertile patients with endometriosis. Similar results were obtained by Chinese researchers who have found the increased content of IL-2 in the cases of infertile patients with endometriosis [2].

In contrast to these results, Ho et al. observed the decreased concentrations of IL-2 in patients with endometriosis [9]. IL-2 is well known Th1 type cytokine responsible for infertility as direct embriotoxic agent. The results of our study suggest that endometriosis complicated by infertility is associated with the Th1/Th2 imbalance with predominance of Th1 type immunity. It seems possible that increased concentrations of IL-2 are the main factors which are

Table 1. The concentrations of IL-2, sIL-2R and the expressions of CD25 antigen on T $CD3^+$ lymphocytes in peritoneal fluid of patients with endometriosis depending on stage of the disease

	IL-2 (pg/ml)	sIL-2R (U/ml)	$CD3^+25^+$ (%)
I stage (n=10)	2.01±2.92	598.33±212.52	1.38±1.02
II stage (n=8)	3.77±2.69	361.06±145.65	6.00±2.68
III stage (n=6)	1.97±1.41	450.73±194.25	12.14±10.83
p	II vs. I (NS) III vs. II (NS)	II vs. I ($p<0.05$) III vs. II (NS)	II vs. I ($p<0.05$) III vs. I ($p<0.01$)

n – number of patients; p – statistical significance.

responsible for the infertility in the group of patients with endometriosis.

In our study we did not find the differences between the concentrations of soluble IL-2 receptors (sIL-2R) between the group of infertile patients with endometriosis and reference group. The concentrations of sIL-2R in the first stage of endometriosis were significantly higher when compared to second stage of the disease. In the literature we did not find the information about the concentrations of sIL-2 receptors in peritoneal fluid of infertile patients with endometriosis. Koumantakis et al. estimated serum concentrations of sIL-2R. They have found that the concentrations of sIL-2R were significantly higher in women with endometriosis when compared to controls [10].

They have made similar observations in the three year follow-up of a patient with severe endometriosis. They observed increased serum concentrations of sIL-2R before treatment which have been suppressed after Danazol administration [11].

The percentage of T $CD3^+25^+$ cells did not differ significantly in the group of infertile patients with endometriosis when compared to the reference group. But we observed significant differences between individual stages of endometriosis. The numbers of T lymphocytes with the expression of CD25 antigen were significantly higher in the third stage of endometriosis when compared to first stage of the disease. Similarly, the levels of T $CD3^+25^+$ cells in the second stage of endometriosis were significantly higher in comparison with the first stage of the disease.

Sotnikova et al. observed that endometriosis was associated with high levels of expressions of CD25 and HLA-DR molecules and diminished expressions of Fas antigen on peritoneal T $CD3^+$ lymphocytes. Furthermore, the supernatant of 24h culture of peritoneal macrophages from women with endometriosis induced the expressions of CD25 antigen on T $CD4^+$ but not $CD8^+$ lymphocytes and decreased Fas expressions by both cell subsets [12].

The observed increase in T $CD25^+3^+$ cells in association with the disease severity may reflect an increase in the number and activation of T regulatory (Treg) cells in the peritoneal cavity.

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