

CD4⁺, CD8⁺ and CD4⁺CD25⁺ T lymphocytes in peripheral blood and peritoneal fluid of women with endometriosis - preliminary report

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Abstract

Introduction: CD4⁺CD25⁺ cells play a major role in maintenance of immunological self-tolerance and negative control of pathological and physiological immunological reactions. Their absence or ill function leads to autoimmunization, immunopathology and allergy.

The aim of this study was to investigate the prevalence of T lymphocyte subpopulations in peripheral blood and peritoneal fluid of women with endometriosis, as well as IL-10 generation by peripheral blood lymphocytes.

Material and methods: The studied group comprised 24 women with endometriosis diagnosed during laparoscopy. The studied group was divided into two parts, depending on progression stage. The control group comprised 12 women without endometriosis. The percentages of subpopulations of T lymphocytes were evaluated via flow cytometry with IMK Plus test (BD Biosciences, USA). IL-10 concentration was analyzed by enzyme-linked immunosorbent assay – ELISA (R&D System, USA).

Results: In the present study we observed higher percentage of CD4⁺CD25⁺ cells in peripheral blood of affected women in comparison with healthy subjects. We did not observe such a difference for the peritoneal fluid between the groups. Changes in number of CD4⁺CD25⁺ affect CD8⁺ shift.

We observed a decreased amount of IL-10 in peripheral blood in women with endometriosis. The increase after PHA (phytohaemagglutinin) stimulation was lower in women suffering from endometriosis than in the control group.

Conclusions: The quantitative disproportion of CD4⁺CD25⁺ T cells between peripheral blood and peritoneal fluid can affect the onset and development of endometriosis. The impaired migration and function of Treg cells alter composition of peritoneal fluid, which favours the onset and development of endometriosis.

Key words: endometriosis, Treg cells, lymphocyte subsets, IL-10.

Introduction

Endometriosis, defined as the presence of endometrial mucosa together with its stroma outside the original site, is a common gynaecological disease observed in 10-15% of women at reproductive age. In women with infertility the prevalence is higher: 50-60% [1].

The aetiology of disease development has not been elucidated. Currently, the most widely accepted is the theory of retrograde menstruation with further implantation of endometrial cells into the peritoneum. Some authors suggest

that disorders of local immune response in the peritoneum favour implantation and development of ectopic endometrium [2]. These abnormalities include adhesion molecule expression, the chemokine-cytokine network, and cell response. Immune homeostasis disorders can facilitate implantation, proliferation and angiogenesis of endometrial tissue, simultaneously disturbing the reproduction process [2, 3].

There have been reports on the autoimmune nature of endometriosis, due to some criteria, characteristic of autoimmune diseases, that it meets: generation of autoantibodies (to endometrium, ovaries, phospholipids and histones) and coexistence of other autoimmune diseases, e.g.: systemic lupus erythematosus, rheumatoid arthritis, immunologically determined habitual miscarriages [3].

The presence of antibodies may suggest generalized autoimmune syndrome. It has been hypothesized that their presence could make the recognition of ectopic endometrium via cytotoxic and helper T cells more difficult, due to receptor sites being blocked. Another hypothesis is that autoantibodies are generated as a result of abnormal immune response to ectopic endometrium changed by inflammation.

Matarese et al. [3] suggest that endometriosis is an autoimmune disease with genetic, hormonal and environmental factors having a role in its pathogenesis.

Autoimmune diseases affect about 5% of the general population and are more common in women. Their essence is abnormal recognition of self-antigens as foreign antigens, which leads to incorrect and excessive response of the immune system. Only in recent years have the cells responsible for immune response regulation been successfully isolated.

CD4⁺CD25⁺ regulatory cells were first described by Sakaguchi et al. in 1995. From experiments on mice he learned that the Treg cell population is responsible for suppression of autoimmunization [4].

These cells suppress the proliferation and function of self-reactive T cells. Deficiency, absence or abnormal functioning of these cell populations leads to development of autoimmune diseases. CD4⁺CD25⁺ cells play a major role in the maintenance of immune self-tolerance and negative control of pathologic immune reactions. Their absence or dysfunction could lead to autoimmunization, immunopathology and allergy [5, 6, 7, 8, 9].

The aim of this study was to investigate the prevalence of T lymphocyte subpopulations in peripheral blood and peritoneal fluid in women with endometriosis, as well as IL-10 generation by peripheral blood lymphocytes. The number of deliveries and the main disorders in the groups have been analysed additionally.

Material and methods

The study group

The studied group comprised 24 women with endometriosis diagnosed during laparoscopy in the

Department of Gynaecological Surgery of the Polish Mother's Health Centre Research Institute, in the years 2003-2005. The laparoscopy was performed as a diagnostic means in infertility, small pelvic pain and tumours of adnexa. In each case the in-operation diagnosis was verified by histopathology. The severity of endometriosis was defined according to the improved American Fertility Society classification. The studied group was divided into two parts, depending on progression stage of the disease – I/II° (12 women) and III/IV° (12 women). The control group comprised 12 female patients without endometriosis as observed in laparoscopy. Age range of the patients treated for endometriosis was between 22 and 52 years old with the average age 34 years old. Accordingly in the control group the age ranged from 17 to 47 years old – average 36 years old. Among women with endometriosis 91.7% inhabited urban areas, whereas 8.3% inhabited rural areas. In the control group respectively 83.4% and 16.6%.

Peripheral blood was obtained before surgery with lithium heparin as an anticoagulant; peritoneal fluid was collected during laparoscopy into sterile apyrogenic test tubes.

Written, informed consent from each patient was obtained before the study.

The study was approved by the Polish Mother's Health Centre Research Institute Bioethical Committee and it was financed with the Department's own funds.

Analysis of peripheral blood and peritoneal fluid lymphocyte populations

The percentages of CD4⁺, CD8⁺ and CD4⁺CD25⁺ subpopulations of T lymphocytes were evaluated via flow cytometry with IMK Plus test (BD Biosciences, USA) and using anti-CD4 (FITC, SK3 clone) and anti-CD25 (PE, 2A3 clone) monoclonal antibodies (BD Pharmingen, USA), in peripheral blood and peritoneal fluid. 100 µL of blood or peritoneal fluid cells suspension was incubated at room temperature together with the appropriate amount of monoclonal antibodies. In evaluation, flow cytometer FACSCalibur with 488 nm argon laser (BD Biosciences, USA) was used. To analyze the results, SimulSET and CellQuest software (BD Biosciences, USA) was applied. The results are given as percentages of double-positive cells in an analyzed sample.

Analysis of IL-10 production by peripheral blood mononuclear cells (PBMC)

IL-10 production level was analyzed in supernatants obtained after 72 hours of incubation of isolated PBMC (1x10⁶ cells/mL) in CO₂ (5 %) atmosphere at 37°C. The cells were cultured on a culture medium composed as follows: RPMI 1640 with addition of 10% v/v inactivated foetal calf serum (FCS, 56°C, 30 min), 100 U/mL penicillin and 100 µg/mL streptomycin, or with addition of phytohaemagglutinin – PHA (5 µg/mL). IL-10 concentration was analyzed in enzyme-linked

immunosorbent assay – ELISA (R&D Systems, USA), using the commercially available Endogen kit.

Statistical analysis

For all analyzed parameters within examined groups mean value, standard deviation (SD) and standard error of mean (SEM) were calculated (in IL-10 release level analysis).

Statistical verification was performed by Student's t-test, and Pearson's correlation coefficient significance test. $P \leq 0.05$ was assumed to be statistically significant. Statistical analysis was performed using STATISTICA 5.0 PL software (StatSoft, Poland).

Results

The number of deliveries and the main disorders in the groups have been analysed additionally. The most numerous part of this group is nonpartum women: 12 patients altogether (50%), 4 in the control group (33.33%). Together with the advance of endometriosis the number of nonpartum women rises. In the group of women with I/II° of endometriosis were 4 nonpartum women (33.33%) and in the group with III/IV° there were 8 (66.66%). Irrespective of the degree of endometriosis, among women who gave birth, the most common were those who gave birth once - 10 women (41.66%). In comparison with the control group there are noticeable differences in the figure of sequent pregnancies: multipara from the examined group - 8.33%; in the control group - 25%.

The main disorder because of which a women reported to a gynaecologist was pelvic pain - it occurred in 17 patients (70.83%). It is worth noting that it is an atypical disorder as in the control group the problem was reported by 9 women (75%). Difficulties with fertility were another problem - 11 women (45.83%) were treated for infertility. 4 women (16.66%) reported dyspareunia and 5 women (20.83%) reported dysmenorrhoea. The two last mentioned disorders coexisted with pelvic pain or

infertility. The detailed clinical profiles of the examined groups are shown in Table I.

In the analysis of percentages of each T lymphocyte population in peripheral blood of patients with endometriosis and healthy subjects a decrease in percentage of CD8⁺ lymphocytes in affected women was observed (Table II). Because

Table I. The clinical data of the examined groups

Parameter examined	Control group	Endometriosis
AGE		
range /years/	17-47	22-52
average /years/	36	34
PLACE OF RESIDENCE		
urban areas /%/	83.4	91.7
rural areas /%/	16.6	8.3
NUMBER OF DELIVERIES		
0 [#]		
n	4	12
%	33.33	50
1 [#]		
n	5	10
%	41.66	41.66
≥2 [#]		
n	3	2
%	25	8.33
DISORDERS		
pelvic pain		
n	9	75
%	17	70.83
infertility		
n	2	16.66
%	11	45.83
dyspareunia		
n	1	4
%	8.33	16.66
dysmenorrhoea		
n	2	5
%	16.6	20.83

n – number of patients

– number of deliveries

Table II. The distributions of T lymphocytes CD8⁺, CD4⁺, CD4⁺CD25⁺ in peripheral blood and peritoneal fluid of women with endometriosis and healthy subjects (control group). Data are presented as means ± standard deviation (SD)

Parameter examined	Control group (n=12)	Endometriosis (n=24)	Endometriosis (I-II°) (n=12)	Endometriosis (III-IV°) (n=12)
Peripheral blood				
CD8 ⁺ (%)	34.2 ± 5.71	28.0 ± 5.74 [#]	25.9 ± 7.20 [#]	27.8 ± 7.50 [#]
CD4 ⁺ (%)	45.8 ± 7.30	45.1 ± 8.19	46.2 ± 6.98	45.2 ± 7.97
CD4/CD8-index	1.38 ± 0.35	1.78 ± 0.51 [#]	1.91 ± 0.54 [#]	1.87 ± 0.82 [#]
CD4 ⁺ CD25 ⁺ (%)	7.0 ± 3.06	8.3 ± 3.58	7.8 ± 2.30	8.7 ± 4.51 [*]
Peritoneal fluid				
CD8 ⁺ (%)	50.1 ± 11.00	50.4 ± 13.20	50.2 ± 17.80	50.0 ± 5.74
CD4 ⁺ (%)	22.2 ± 9.40	18.8 ± 7.80	17.8 ± 7.30 [#]	20.0 ± 8.51
CD4/CD8-index	0.40 ± 0.170	0.38 ± 0.150	0.36 ± 0.130	0.40 ± 0.180
CD4 ⁺ CD25 ⁺ (%)	6.5 ± 2.89	6.3 ± 2.42	5.9 ± 1.83	6.67 ± 2.39

[#] - $p < 0.05$ statistically significant differences as compared to control group

^{*} - $p = 0.06$ statistically significant differences as compared to control group

n - number of performed investigations

the percentage of CD4⁺ lymphocytes was similar in all examined groups, the CD4/CD8 ratio turned out to be higher in affected subjects. Although

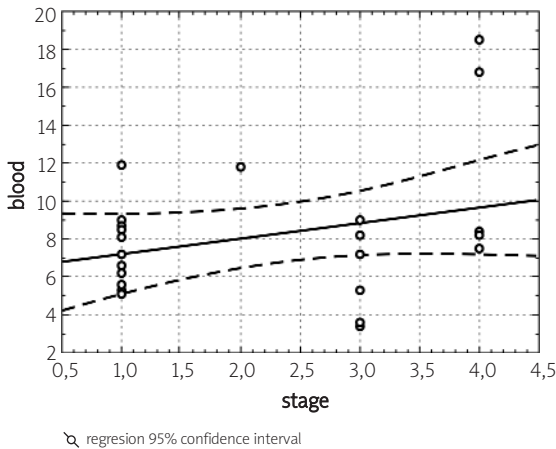


Figure 1. Analysis of Pearson's linear correlation ratio between progression stage of the disease and percentage of Treg lymphocytes (CD4+CD25+) in peripheral blood

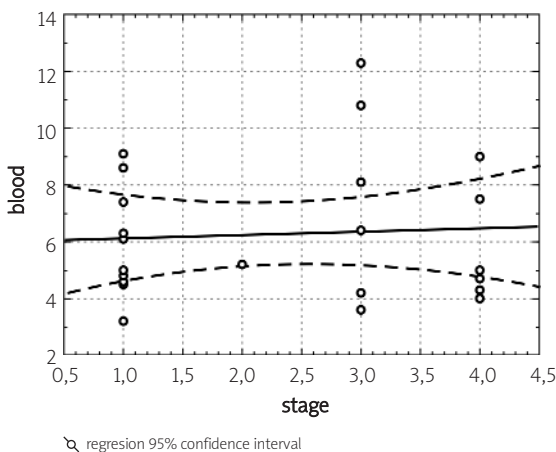


Figure 2. Analysis of Pearson's linear correlation ratio between progression stage of the disease and percentage of Treg lymphocytes (CD4+CD25+) in peritoneal fluid

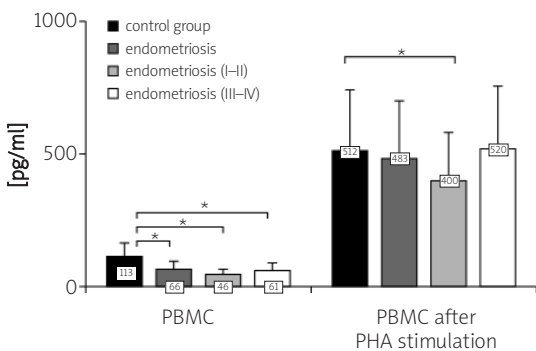


Figure 3. Analysis of IL-10 generation by peripheral blood mononuclear cells (PBMC) in patients with endometriosis in comparison with healthy women

CD4⁺CD25⁺ regulatory lymphocyte percentage in the peripheral blood did not differ significantly between each group, we still noted a higher increase in patients with endometriosis ($p=0.06$). CD4⁺CD25⁺ lymphocyte percentage in peripheral blood rose together with advancing stage of the disease (Figure 1). The analyses did not indicate any shift in proportion of this subpopulation examined in peritoneal fluid in patients with endometriosis in comparison to healthy subjects. The percentage of these cells in peritoneal fluid also did not correlate with disease progression (Figure 2).

IL-10 generation by peripheral blood mononuclear cells was evaluated after the cells were cultured, without and after stimulation with PHA. Significantly lower production of this interleukin was observed in women suffering from endometriosis versus healthy subjects (Figure 3). In PHA-stimulated cultures, significantly lower IL-10 production was observed only in women with mild endometriosis.

Discussion

In the pathogenesis of endometriosis some natural and acquired humoral and cell-mediated immunity disorders have been observed. Humoral response disorders include the synthesis of abnormal antibodies to endometrial and ovary cells, phospholipids and histones. Decreased cytotoxicity of NK cells, quantitative disorders between CD4 and CD8 cells, as well as abnormal transformation of B lymphocytes are among the cellular response disorders [10].

Many authors points to a local inflammatory reaction taking part in development of endometriosis, with a special role of peritoneal fluid macrophages. Cytokines and growth factors produced by these cells affect other morphotic elements, thus favouring ectopic implantation, proliferation and angiogenesis [2, 3].

Szytło et al. [11] investigated the role of T lymphocytes in the pathogenesis of endometriosis. They observed altered T cell redistribution between peripheral blood, peritoneal fluid and endometrial tissues. T lymphocytes in women with endometriosis produce higher amounts of pro-inflammatory cytokines – mainly IL-6 and TNF- α , which can impair Treg cell function, thus causing the lack of defence against autoimmunization.

The isolation of CD4⁺CD25⁺ regulatory cells by Sakaguchi et al. in 1995 brought the beginning of extensive studies on structure and use of these cells. The authors claimed that the Treg cell population is responsible for suppression of autoimmunization [4].

CD4⁺CD25⁺ cells make up 5-10% of peripheral CD4⁺ T cells. The structure and operation of Treg cells have been precisely described in many papers [5, 6, 7, 8, 9]. Strength and mode of stimulation affect the functioning of regulatory cells [5, 6, 8, 12]. Present studies focus on the role of these cells in individual diseases, though there is a lack of manuscripts

describing the role of Treg cells in endometriosis. The peritoneal fluid milieu, specific and having a major role in development of endometriosis, was not investigated with respect to its effect on regulatory functions of the immune system.

Based on the results we can observe a shift of CD4⁺CD25⁺ cells in peripheral blood of affected patients in comparison with healthy subjects. The percentage of Treg cells in peripheral blood increases compared to healthy women. The values are close to the borderline of statistical significance ($p=0.06$). Such a quantitative shift is not observed in the peritoneal fluid between the groups.

It was shown that Treg cells take part in constraining the inflammation [5, 6, 8]. CD4⁺CD25⁺ cells are responsible for effector cell (CD8⁺) regulation. CD8⁺ cells, which are inhibitory in function, are suppressed by CD4⁺CD25⁺ cells.

Changes in CD4⁺CD25⁺ numbers influence CD8⁺ shift. In the peritoneal fluid of women with endometriosis the percentage of Treg cells is lower than in the peripheral blood, which might explain why the number of CD8⁺ cells in the peritoneal fluid is increased in comparison with the peripheral blood.

An unknown defect of CD4⁺CD25⁺ cells or their impaired migration in the inflammation site (which may be a result of abnormal functioning of chemokines, for example) could lead to the lack of their inflammation-limiting action.

Due to the lack of reports describing Treg cell involvement in endometriosis it is not possible to compare our results with other studies. There is some research on the role of CD4⁺CD25⁺ Treg cells in other diseases: among them we should mention atopic dermatitis [13], rheumatoid arthritis [14], juvenile idiopathic arthritis [15], type-1 diabetes [16, 17], multiple sclerosis [18] and myasthenia gravis [19].

In patients with recurrent aphthous ulcerations (RAU) an increase in IL-6 and TNF- α pro-inflammatory cytokine levels and a decrease in IL-10 and TGF- β anti-inflammatory cytokine levels was observed [20]. There was a decrease in number of Treg suppressive cells in affected patients in comparison with healthy subjects. It favoured inflammation as a response to bacterial antigens of microorganisms colonizing the oral cavity. It is conceivable that a similar situation occurs in the case of endometriosis, where impaired function of Treg cells promotes pro-inflammatory cytokines, growth factors and antigens, which leads to survival, proliferation and development of endometrial tissue in the altered environment.

The number of CD4⁺CD25⁺ cells in the population of patients with myasthenia gravis [19] and multiple sclerosis [18] does not differ from the control group. Suppressing activity of CD4⁺CD25⁺ regulatory cells is weakened, with accompanied lowered FOXP3 expression [13]. Also, the secreted cytokines profile is changed. Some authors claim that high

concentration of IL-6 leads to loss of suppressive properties [21].

In patients with atopic dermatitis it was observed [13] that CD4⁺CD25⁺ Treg expression is increased in peripheral blood in comparison with the control group, with an accompanying rise in FOXP3 expression. Bacterial antigens in the skin led to suppressive activity of Treg being reverted and their function impaired.

Depending on the disease, the percentage of Treg cells in peripheral blood can rise [20], show no change [13, 14, 17, 18] or fall [15, 16] in comparison with healthy subjects.

Many authors describe increased IL-10 level in peritoneal fluid of women with endometriosis in comparison with the control group [22, 23, 24]. They rightly conclude that the main source of IL-10 is peritoneal macrophages. Our studies confirm this assumption, as the lowered number of CD4⁺ and CD4⁺CD25⁺ cells in the peritoneal fluid cannot make up for the main source of the cytokine production.

In the present study we observed a decreased amount of IL-10 in peripheral blood in women with endometriosis. The increase after PHA stimulation was lower in women suffering from endometriosis than in the control group. Apart from anti-inflammatory properties, IL-10 also takes part in suppression. While analyzing Treg action using IL-10 one can conclude that lowered IL-10 level in the peripheral blood results from impaired functioning of CD4⁺CD25⁺ Treg cells in this disease, because in the peripheral blood of women with endometriosis the percentage of CD4⁺CD25⁺ cells increases. In current studies the percentage of Treg cells in peripheral blood increases independently of endometriosis progression, without an accompanying IL-10 rise in the peripheral blood, as was observed in previous studies [22, 23, 24]. Most probably, in this specific disease there are involved some mechanisms upsetting the activation and functioning of CD4⁺CD25⁺ cells in peripheral blood, which can lead to lower levels of IL-10. Its decreased amount in peripheral blood promotes inflammation via lack of suppressive action.

Because of the small size of the investigated groups, further observations are necessary in order to confirm the obtained results.

Conclusions

We analyzed the percentage of CD4⁺CD25⁺ T cells in peripheral blood and peritoneal fluid of women with endometriosis, without the evaluation of activity, which should be determined in further studies. In the present study we observed a higher proportion of CD4⁺CD25⁺ Treg cells in the peripheral blood of affected women in comparison with healthy subjects. We did not observe such a shift in the peritoneal fluid between the groups. The quantitative disproportion of CD4⁺CD25⁺ T cells between peripheral blood and peritoneal fluid can affect the onset and development of endometriosis. The impaired migration

and function of Treg cells alters the composition of the peritoneal fluid, which favors the onset and development of endometriosis. Activity and maturity, confirmed by increased expression of HLA-DR, CTLA-4, GITR markers, as well as FOXP3 expression, require further research.

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