Profiling of peritoneal fluid of women with endometriosis by chemokine protein array

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Abstract

Purpose: Chemokines play essential role not only in controlling leukocyte function and trafficking but also in the angiogenesis and modulation of inflammatory responses.

Material and methods: A novel array-based enzyme-linked immunosorbent assay was used to quantitate peritoneal fluid chemokines of 25 infertile women with endometriosis and 18 controls. For our preliminary studies we chose mini-array containing nine different chemokines: MDC/CCL22, TARC/CCL17, MCP-1/CCL2, RANTES/CCL5, MIP-1α/CCL3, -1β/CCL4, -1δ/CCL15, -3α/CCL20, and -3β/CCL19.

Results: We found significantly higher MIP-3β/CCL19 (P=0.0036) concentrations in peritoneal fluid of women with endometriosis as compared to patients with primary infertility without any signs of disease.

Conclusions: Our preliminary results suggest that MIP-3β/CCL19 might play a role in the pathogenesis of endometriosis but its precise role remains to be established. Novel types of screening methods based on high-throughput technologies offer great opportunities to study immunobiology of endometriosis. It will hopefully provide new possibilities for discovery of new markers and potential drug targets.

Key words: endometriosis, chemokines, peritoneal fluid, protein array.

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Introduction

The pathogenesis of endometriosis, a condition in which the foci of endometrial tissue including epithelial and stromal components are present outside uterus, most often in pelvic cavity, ovaries but also in various distant organs, still remains largely unknown. Since in majority of the cases endometriosis implants are found in the pelvic peritoneum, it was postulated that retrograde menstruation with subsequent implantation of endometrial tissue is a main causative factor [1]. Alternatively metaplasia theory has also been advocated [2] and the evidence of the rare cases of endometriosis in men who are receiving estrogens seems to support it [3].

Regardless of the pathogenesis, which also include other than mentioned-above alternative hypotheses [4], it is evident that in the pelvic peritoneum endometriotic implants are bathed in the peritoneal fluid (PF). A subsequent inflammatory reaction in the pelvis changes the characteristics of the peritoneal fluid and its cellular components [5].

In particular, evidence supports the hypothesis that peritoneal fluid cytokines orchestrate many of the complex processes underlying the initiation and survival of nascent endometriosis implants [6]. Numerous secreted products of the cellular components, such as chemokines, growth factors, matrix metalloproteinases and others have been reported in the PF of women with endometriosis [7-9].

Chemokines (CHEMOtactic cytoKINES), a large multifunctional family of cytokines are structurally related, with most containing four invariant cysteine residues. Depending on the arrangement of the first two of these cysteines, chemokines are divided into four subfamilies: CXC (α), CC (β), C (γ) and CX3C (δ). Chemokines are produced as pro-peptides and are cleaved during secretion to produce an active mature protein that functions by activating G-protein-coupled receptors.

Chemokines have often been co-discovered by multiple investigators and therefore have numerous names in the literature, which can be a source of confusion. A new standardised systemic nomenclature has therefore been adopted [10] and is

used in the article with systemic name following ligands being first mentioned.

It is becoming increasingly evident that chemokines of different types appear to be profoundly involved in the pathogenesis of different diseases of reproductive system, including endometriosis [11]. In our study we intended to perform profiling of nine different chemokines in peritoneal fluid from women with endometriosis by a novel high-throughput mini-array enzyme-linked immunosorbent proteomic assay. Macrophage-Derived Chemokine (MDC, a systemic name – i.e. CCL22), Thymus and Activation-Regulated Chemokine (TARC, i.e. CCL17), Monocyte Chemotactic Protein (MCP-1, i.e. CCL2), Regulated upon Activation, Normally T cell-Expressed, and presumably Secreted (RANTES, i.e. CCL5) Macrophage Inflammatory Proteins (MIP) -1 alpha (i.e. CCL3), -1 beta (i.e. CCL4), -1 delta (i.e. CCL15) -3 alpha (i.e. CCL20), and -3 beta (i.e. CCL19) were chosen for the study. The goal of this study was to demonstrate that the novel array-based high-throughput technologies may offer interesting opportunity to study pathogenesis and to possibly search for new markers of endometriosis.

Material and methods

A total of 42 regularly menstruating women who underwent laparoscopy at Department of Gynaecology of the Medical University in Bialystok, Poland, participated in the study. None of the women had medications that ameliorate endometriosis or modulate the immunological status at least over 3 months prior to laparoscopy. Endometriosis was diagnosed both laparoscopically and histologically. The study was approved by institutional ethics committee and informed consent was obtained from all the patients.

24 patients (group I) were selected by the existence of visible peritoneal endometriotic lesions, characterized as red- or gland-like lesions as well as red vesicles and ovarian endometriomas (stage I-IV) [12]. Of these women, 14 (58.3%) were scored as minimal and mild (I and II stage) of which 11 were those with primary (those who were never pregnant) and 3 with secondary (at least once pregnant previously) infertility. 10 patients (41.7%) were scored as moderate and severe stage (III and IV) of which 5 were primary and 5 secondary infertile women. Eighteen (group II) infertile women (10 with primary and 8 with secondary infertility), without any sign of endometriosis presented at laparoscopy, were used as a control. All laparoscopies were performed in the first phase of menstrual cycle (days 8-12).

Peritoneal fluid samples were collected, cleared of cells and cell debris by means of centrifugation at 800 g for 10 min and then stored at -80°C. We excluded patients with blood-contaminated peritoneal fluid.

A multiplexed, mini-array, sandwich-type, enzyme-linked immunosorbent assay was used to measure in duplicate the concentration of nine different human chemokines: MDC/CCL22, TARC/CCL17, MCP-1/CCL2, RANTES/CCL5, MIP-1α/CCL3, -1β/CCL4, -1δ/CCL15, -3α/CCL20, and -3β/CCL19. The determination (Pierce Biotechnology Laboratory, Rockford, USA) was performed as previously described [13]. The mini-array was produced by spotting monoclonal antibodies (mAbs) in a 3 x 3 pattern in the bottom of the wells of 96-well polystyrene plates. Each antibody captured specific chemokine present in the standards and samples added to the plate. The bound proteins were then detected with a biotinylated detection antibody, followed by the addition of streptavidin-horseradish peroxidase (HRP) and lastly, SuperSignal® ELISA Femto Chemiluminescent substrate. The plate was imaged using SearchLight® Imaging System which is a cooled charge-coupled device (CCD) camera. ArrayVision® customized software was used to quantify the signal from each spot of the array. The amount of luminescent signal produced was proportional to the amount of each protein present in the original standard or sample. Concentrations were extrapolated off a standard curve.

The specificity of the mini-array was demonstrated by adding samples containing only individual chemokines to different wells of a mini-array plate. In each case, the chemokine in the sample produced a signal only at the spot containing the complementary antibody. Low background in the absence of target, and no cross reactivity was observed between the different chemokines and their arrayed antibodies. The dynamic range for the detection of the target chemokines in the array was demonstrated to be: 0.8-800 pg/mL for MCP-1; 3.2-3200 pg/mL for MIP-1α/CCL3 and MIP-1δ/CCL15; 8-800 pg/mL for MIP-1β/ /CCL4; 0.56-560 pg/mL for MIP-3α; 3.2-3200 pg/mL for MIP-1δ; 0.4-400 pg/mL for RANTES/CCL5, TARC/CCL17, MDC/CCL22 and MIP-3β/CCL19.

Total protein in peritoneal fluid was quantified as previously described [14].

As data did not fulfil the normality assumption (assessed by Shapiro-Wilk test) the comparison of studied chemokines was performed by the use of Wilcoxon test. The comparison between subgroups of patients with minimal/mild vs moderate/severe endometriosis vs primary infertile patients without endometriosis was performed using Kruskal-Walis test, whereas Dunn's multiple comparison test was used to show differences between three combination of subgroups i.e minimal/mild primary vs moderate/severe primary, minimal/mild primary vs primary control and moderate/severe primary vs primary control. The comparison between age, BMI as well as duration of infertility was performed by Student t-test. The significance level was equal to 0.05. All tests were two-sided. The statistical analysis was performed by the use of SAS STAT package.

Results

The mean age, BMI as well as duration of infertility of patients in group I and II was similar and did not differ significantly (*Tab. 1*).

The mean volume of peritoneal fluid was 6.1±2.1 ml in study group and 5.0±1.9 ml in controls – a non-significant difference.

When group I and group II were compared by Wilcoxon test no differences were found between women with endometriosis and infertile controls. It was only after division of patients into subgroups, based on the status of infertility (primary vs secondary), when among nine studied chemokines we were able

Table 1. **Clinical characteristics of patients**

	Group I* (mean ±std)	**Group II**** (mean ±std)	**P*****
BMI (body mass index)	23.15±3.17	22.5±4.3	0.48
Age (years)	29.6±4.18	31.8±2.9	0.15
Duration of infertility (years)	4.22±3.56	3.65±1.77	0.64

* – patients with endometriosis (n=24); ** – infertile patients without endometriosis (n=18); *** – p-value of Student t-test for comparison of group I and II

Table 2. **Chemokine levels in primary infertile women with minimal/mild (subgroup I) and moderate/severe (subgroup II) endometriosis as well as primary infertile patients without any signs of disease (subgroup III)**

	Minimal/Mild endometriosis (n=14)*	**Moderate/Severe endometriosis** (n=10)*	**Primary infertility** (n=10)	**P****
MDC	193; **279.4**; 338.1***	268.6; **283.8**; 286.6	213; **237.4**; 269.5	0.25
TARC	16.3; **28.7**; 30.7	17.5; **19.5**; 26.4	21.1; **23.25**; 34.9	0.68
MCP1	704.6; **1289.7**; 2086.5	563.3; **594.9**; 1337.8	566.7; **1178.4**; 1833.2	0.79
RANTES	10.7; **13.8**; 22.4	5.8; **9.6**; 11	11.3; **17.05**; 23.3	0.1
MIP-1α	44; **49.6**; 59.4	50.7; **56**; 66.3	48.4; **51.05**; 61.5	0.58
MIP-1β	24.2; **43.7**; 61.1	23.9; **45.3**; 47.1	33.1; **40.25**; 56	0.82
MIP-1δ	4638.3; **5222.5**; 6296.5	3296.7; **4483.7**; 7021.1	5927.4; **7554.95**;11194.6	0.15
MIP-3α	25.8; **33.6**; 35.6	20.7; **26.9**; 28	27.6; **31.15**; 40.2	0.44
MIP-3β	113.7; **124.5**; 175.8	135.7; **141.8**; 239.9	68.9; **77.05**; 88.55	0.0007#

* – Primary infertile patients; ** – P-value for Kruskal-Walis test; *** – Data in pg/mL: Q1; Median; Q3 (Q1-Q3 are interquartile values); # – Statistically significant differences

to observe significantly (p=0.0014) higher concentrations of MIP-3β/CCL19 [129.4 pg/mL (interquartile values Q1: 118.05 – Q3: 186)] in patients with endometriosis than those with primary infertility [77.05 pg/mL (interquartile values Q1: 68.9 – Q3: 88.55)]. We also found that the differences are significant when a group I (of patients with endometriosis) was subdivided into minimal/mild (subgroup I) and moderate/severe (subgroup II) - both with primary infertility and then compared with primary infertile women (subgroup III) without any signs of endometriosis (see details *Tab. 2*). By the use of Dunn's multiple comparison test we could show that no significant difference exists between subgroup I and II (p>0.05), however, it does exist between subgroup I and III (p<0.01) as well as II and III (p<0.001). There is also no significant difference (P=0.25) in duration of infertility between subgroup I, II and III (mean ±SD: 3.4±2.33; 6±5.3; 3.65±1.77, respectively for three subgroups).

The concentration of total protein in peritoneal fluid was not different significantly and it was respectively 4.9±0.89 g/dL and 5.3±0.43 g/dL in the study and control groups.

We did not find any statistically significant differences between secondary infertile patients with minimal/mild and moderate/severe endometriosis as compared to secondary infertile women without any signs of disease.

Discussion

The genomics and proteomics revolution has introduced a paradigm-shift in the experimental approach, which tends to generate, rather than test, hypotheses. This has become possible by the widespread application of high-throughput technologies such as gene and protein microarrays. The concept of using different types of arrays has been revolutionising biomedicine and its application to proteomics is becoming increasingly popular. Majorities of the hitherto applied protein array systems have, however, demonstrated the ability to detect qualitative interactions between proteins. Only few exhibited the real ability of multiplexed quantification of proteins [15,16]. An interesting option has recently been demonstrated by Moody et al. who applied enzyme-linked immunosorbent assay for the qualitative measurement of different cytokines from a single sample [13].

In our study we used exactly the same method for determination of nine different chemokines in peritoneal fluid obtained from women with endometriosis. The choice of the profiled chemokines was driven not only by the fact that their role in the immunobiology of endometriosis is practically unknown but was also prompted by accumulating evidence for a significant contribution of many different chemotactic cytokines in the pathogenesis of endometriosis [11,17].

In the present study we found higher MIP-3β/CCL19 concentrations in peritoneal fluid of women with endometriosis, regardless of the severity, as compared to patients with primary infertility without any signs of disease. The chemokine MIP-3β/CCL19 has not to our knowledge been previously found to be connected with pathophysiology of endometriosis and the role of MIP-3β/CCL19 in relation to affected women is speculative.

Chemokines, in general, affect a number of different tissues and organs not only by the endocrine mode of action through influencing distant organs but also acting locally, possible also in the peritoneal fluid. MIP-3β/CCL19 is a chemoattractant for different types of cells, including natural killer cells [18] as well as T and B lymphocytes [19].

In the reproductive system, MIP-3β/CCL19 has recently been found to be expressed in endometrium [20], and its lower concentrations we found in serum of women with preterm labor [21]. However the precise role of MIP-3β/CCL19 as a chemoattractant of endometrial natural killer cells or as a specific immunological mediator in endometriosis remains unclear.

It is also speculative what might be the source of MIP-3β/ /CCL19 in the peritoneal fluid. Cells in the PF are mainly mononuclear cells, particularly macrophages (90%); other mononuclear cells that are present include lymphocytes and natural killer [22] cells. It has been generally accepted that in peritoneal fluid of women with endometriosis, the number and activity of macrophages and its cytokines is increased. On the contrary, NK cell number and activity has been found to be decreased in peripheral blood and peritoneal fluid of women with endometriosis [23,24]. It would obviously be of interest to correlate the results obtained for MIP-3β/CCL19 with different cellular subtypes and this is now being contemplated as part of the future studies.

The results of our study might seem somewhat surprising given the fact that two other chemokines i.e: RANTES/CCL5 and MCP-1/CCL2 were previously found to be increased in peritoneal fluid of women with endometriosis [25-27]. This, however, may be owing to at least three inherent drawbacks of our study. First concerns lower number of patients recruited as compared to studies cited above. Most of the patients in the moderate/severe group were, however, diagnosed with endometrial cysts and abundant adhesions with lesser visible pelvic lesions. It is well accepted that biological activity of endometriosis tends to weaken in the most advanced cases as described above (IV stage) and immunological milieu of the peritoneal cavity is somewhat different from the III and lower stages thus contributing to lowering of normally increased concentrations of some of the chemokines, possibly RANTES/CCL5 and MCP-1/CCL2.

It was previously found that mean RANTES/CCL5 concentrations in the PF of patients with mild endometriosis are particularly high i.e. 7.8 ng/mL for mild and 29.1 ng/mL for the severe group [28]. In the more recent study Bersinger et al. found significantly lower median PF RANTES/CCL5 levels of 22.5 pg/mL in the mild and 35.5 pg/mL in the severe group of patients [29] whereas in our study the median values were even lower i.e 13.8 pg/mL and 9.6 pg/mL, respectively. The same might apply to MCP-1/CCL2 which was previously found to be increased in women with endometriosis and the median peritoneal fluid MCP-1/CCL2 level was 144 pg/ml (range 54--261) in endometriotic women without adhesions and 336 pg/ml (range 130-2494) in women with adhesions [28]. The ranges and median values of MCP-1/CCL2 were in our studies significantly higher (see *Tab. 2*). Whether the difference in the above studies and ours are owing to the size of study group or might also result from different methodologies (commonly used classical ELISA vs novel array-based ELISA system) remains to be seen in the future expanded studies on larger group of patients who are now ongoing in our department.

Another drawback may also concern both the structure of control group, which is limited only to infertile patients, and the collection of studied patients which was restricted to the follicular phase of the cycle. It is therefore difficult to evaluate whether control group is a healthy one alone since infertility might to some degree influence different chemokine levels. Nevertheless, in Poland it is a difficult task to collect patients who undergo diagnostic laparoscopic procedure for other reasons than infertility and/or pelvic pain and the operations in our Department are conducted only in the first phase of the cycle. We are also now in the process of collecting samples from patients with pelvic pain to see whether any differences might exist between two groups of patients.

Regardless of the explanation as to the potential role of MIP-3β/CCL19 we believe that in general the above-presented preliminary findings create a basis for future confirmatory studies that would hopefully add another brick to the ever-rising wall of the knowledge about immunobiology of endometriosis. This is becoming a possible thanks to the use of a novel multiplexed proteomic assay. By relatively small amount of examined factors, nine altogether, we were able to start laying groundwork for one more hypothesis about the role of chemokines in the pathogenesis of endometriosis.

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References

1. Sampson JA. Heterotopic or misplaced endometrial tissue. Am J Obstet Gynecol, 1925; 10: 649-64.
2. Iwanoff NS. Druesiges zysthaltiges Uterusfibromyoma kompliziert druch Sarkom und Karzinom (adenofibromyoma cysticum sarcomatodes carcinomatosum). Mschr Geburtsh Gynecol, 1898; 7: 295-9.
3. Witz CA. Current concepts in the pathogenesis of endometriosis. Clin Obstet Gynecol, 1999; 42: 566-85.
4. Laudanski P. Denervation – reinnervation – hypothesis well-founded but not sufficient. Med Hypotheses, 2005; 64: 1241-42.
5. Koninckx PR, Kennedy SH, Barlow DH. Pathogenesis of endometriosis: the role of peritoneal fluid. Gynecol Obstet Invest, 1999; 47 (Suppl. 1): 23-33.
6. Harada T, Iwabe T, Terakawa N. Role of cytokines in endometriosis. Fertil Steril, 2001; 76: 1-10.
7. Laudanski P, Szamatowicz J, Ramel P. Matrix metalloproteinase-13 and membrane type-1 matrix metalloproteinase in peritoneal fluid of women with endometriosis. Gynecol Endocrinol, 2006; 21: 106-10.
8. Lebovic DI, Mueller MD, Taylor RN. Immunobiology of endometriosis. Fertil Steril, 2001; 75: 1-10.
9. Szamatowicz J, Laudanski P, Tomaszewska I. Matrix metalloproteinase-9 and tissue inhibitor of matrix metalloproteinase-1: a possible role in the pathogenesis of endometriosis. Hum Reprod, 2002; 17: 284-8.
10. Murphy PM, Baggiolini M, Charo IF, Hebert CA, Horuk R, Matsushima K, Miller LH, Oppenheim JJ, Power CA. International union of pharmacology. XXII. Nomenclature for chemokine receptors. Pharmacol Reviews, 2000; 52: 145-76.
11. Garcia-Velasco JA, Arici A. Chemokines and human reproduciton. Fertil Steril, 1999; 71: 983-93.
12. Medicine ASfR. Revised American Society for Reproductive Medicine classification of endometriosis. Fertil Steril, 1997; 67: 817-21.
13. Moody MD, Van Arsdell SW, Murphy KP, Orencole SF, Burns C. Array-based ELISAs for high-throughput analysis of human cytokines. Biotechniques, 2001; 31: 186-90, 192-4.
14. Lowry JOH, Rosenbrough RL, Farr RL, Randal J. Protein meas-

urement with the Folin phenol reagent. J Biol Chem, 1951; 193: 265-75.

15. Mendoza LG, McQuary P, Mongan A, Gangadharan R, Brignac S, Eggers M. High-throughput microarray-based enzyme-linked immunosorbent assay (ELISA). Biotechniques, 1999; 27: 778-80, 782-6, 788.

16. Schweitzer B, Wiltshire S, Lambert J, O Malley S, Kukanskis K, Zhu Z, Kingsmore SF, Lizardi PM, Ward DC. Inaugural article: immunoassays with rolling circle DNA amplification: a versatile platform for ultrasensitive antigen detection. PNAS USA, 2000; 97: 10113-9.

17. Szamatowicz J, Laudanski P, Tomaszewska I, Szamatowicz M. Chemokine growth-regulated-alpha: a possible role in the pathogenesis of endometriosis. Gynecol Endocrinol, 2002; 16: 137-41.

18. Kim CH, Pelus LM, Appelbaum E, Johanson K, Anzai N, Broxmeyer HE. CCR7 ligands, SLC/6Ckine/Exodus 2/TCA4 and CKbeta-11/MIP-3beta/ELC, are chemoattractants for CD56+CD16- NK cells and late stage lymphoid progenitors. Cell Immunol, 1999; 193: 226-35.

19. Kim CH, Pelus LM, White JR, Applebaum E, Johanson K, Broxmeyer LP. CK beta-11/macrophage inflammatory protein-3 beta/EBI1-ligand chemokine is an efficacious chemoattractant for T and B cells. J Immunol, 1998; 160: 2418-24.

20. Daikoku N, Kitaya K, Nakayama T, Fushiki S, Honjo H. Expression of macrophage inflammatory protein-3β in human endometrium throughout the menstrual cycle. Fertil Steril, 2004; 81 (Suppl. 1): 876-81.

21. Laudanski P, Lemancewicz A, Pierzynski P, Akerlund M, Laudanski T. Decreased serum level of macrophage inflammatory chemokine-3β/CCL19 in preterm labor and delivery. Eur J Obstet Gynecol Reprod Biol, 2005; 124: 23-6.

22. Witz CA, Monotoya-Rodriguez IA, Dey TD, Schenken RS. Characterization of lymphocyte subpopulations and T cell activation in endometriosis. Am J Reprod Immunol, 1994; 32: 173-9.

23. Braun DP, Dmowski WP. Endometriosis: abnormal endometrium and dysfunctional immune response. Curr Opin Obstet Gynecol, 1998; 10: 365-9.

24. Dmowski WP, Steele RW, Baker GF. Deficient cellular immunity in endometriosis. Am J Obstet Gynecol, 1981; 141: 377-83.

25. Akoum A, Lemay A, McColl S, Turcot-Lemay L, Maheux R. Elevated concentration and biologic activity of monocyte chemotactic protein-1 in the peritoneal fluid of patients with endometriosis. Fertil Steril, 1996; 66: 17-23.

26. Hornung D, Bentzien F, Wallwiener D, Kiesel L, Taylor RN. Chemokine bioactivity of RANTES in endometriotic and normal endometrial stromal cells and peritoneal fluid. Mol Hum Reprod, 2001; 7: 163-8.

27. Khorram O, Taylor RN, Ryan IP, Schall TJ, Landers DV. Peritoneal fluid concentrations of the cytokine RANTES correlate with the severity of endometriosis. Am J Obstet Gynecol, 1993; 169: 1545-9.

28. Zeyneloglu HB, Senturk LM, Seli E, Oral E, Olive DL, Arici A. The role of monocyte chemotactic protein-1 in intraperitoneal adhesion formation. Hum Reprod, 1998; 13: 1194-9.

29. Bersinger NA, von Roten S, Wunder DM, Raio L, Dreher E, Mueller MD. PAPP-A and osteoprotegerin, together with interleukin-8 and RANTES, are elevated in the peritoneal fluid of women with endometriosis. Am J Obstet Gynecol, 2006; Apr 22; (Epub ahead of print).