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Microvesicles released from ectopic endometrial epithelium foci as potential biomarker of endometriosis

Objectives: Angiogenesis is one of the key steps engaged in pathogenesis of endometriosis. The purpose was to investigate the presence of MVs with essential angiogenesis mediators, like vasculo-endothelial growth factor (VEGF) and metalloproteinase-9 (MMP – 9) in peripheral blood and peritoneal fluid of women aged 25-45 with endometriosis staged as II-IV stage according to AFS. MVs released from cells of endometriosis foci were analyzed in this project. The research was performed in group of participants who were subjected to surgical treatment due to the suspected endometriotic cyst, deep and superficial infiltrating endometriosis of pelvic peritoneum. MVs presence locally in peritoneal fluid and systematically in blood may be yet unknown mechanism of immune response regulation. Moreover MVs may have influence on immune tolerance and growth of endometriosis foci. "Metastatic" nature of endometriosis in some patients may suggest such a scenario.

Material and Method: The study was conducted on blood samples and peritoneal fluid samples collected from women aged 25-45 with endometrial lesion in pelvic organs diagnosed during laparoscopic surgery. Women undergoing laparoscopic surgical treatment due to benign non-hormonal dependent ovarian lesions (teratomas) will be used as a control. Microvesicles (MVs) were determined in samples of 5 ml blood and samples of 5 ml peritoneal fluid. The blood samples were collected day before operation during taking blood sample to preoperative test. The fluids were collected from the peritoneal cavity during operation. In the study 30 samples of blood were obtained: 23 samples from women with endometriosis and 7 from women with teratomas and 27 samples of peritoneal fluid were obtained: 19 samples in the test group and 8 samples in the control group. The blood samples and the peritoneal fluid samples were dispensed into tubes containing anticoagulants and were undergone the process of getting platelet free plasma (PFP)/ platelet free peritoneal fluid. Thirty minutes after collection the sample was centrifuged (3000g/15minuts) to isolate MVs from the blood and peritoneal fluid sample. PFP and platelet free peritoneal fluid were frozen in -40□. In the next step the samples were thawed at the room temperature and centrifuged in 1000g in 5 minutes. Analysis of isolated MVs was performed by flow cytometry (FACS) with using annexiny V, antibodies for molecules characteristic for cells from endometriosis foci (keratin 18 (K18), CD105, CD146) and antibodies for intraepithelial vascular growth factor VEGF and metalloproteinase - 9 (MMP - 9). There were double "reading" of the sample using flow cytometry (FACSCanto II)

Analysis and Results: In the study we analyzed the results of flow cytometry of 30 plasma samples (23 samples from the test group of women with endometriosis and 7 from the control group with teratomas), 27 peritoneal fluid samples (19 samples from the test group of women with endometriosis and 8 from the control group with teratomas). In 10 patients, tests were performed in both samples (plasma and peritoneal fluids), while the remaining cases had only one of the tests. Statistical analysis was performed using the STATISTICA program. The data generated by flow-cytometers were classified by size: All – number of whole objects, 05-1/024 – number of objects larger than 0.24 μm, 05-1/022 - number of objects larger than 0.22 μm but smaller than 0.24 μm. Objects with dimensions from 0.22 μm to 0.24 μm (220-240 nm) and marked by antibodies were subjected to analysis. Three sets of arrangements were made: set1- CK18 + annexin V + VEGF + MMP-9, set 2 - CD105 + annexin V + VEGF + MMP-9, set3- CD146 + annexin V + VEGF + MMP-9. The results were expressed as a percentage of counts of particular type in relation to the superior category (% parent). It allowed to avoid the influence, of significant differences in the total number of counts between patients, on the

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result. Median (as a measure of the average value in each group) and 1st and 3rd quartiles (as a measure of the results spread) were counted for each type of test in sets (set 1, set 2 and set 3) in the subgroups of patients. Comparisons between patients groups were made with the Mann-Whitney test. The test hypothesis assumed that both analyzed samples (test and control) came from the same population (or population with identical medians).

We compared each of the flow cytometry parameters between the control and endometriosis groups to check which of the cytometry results differ the groups. We were looking for a correlation between plasma and peritoneal fluid to see if there was a relationship between the amount of microvesicles in these media. In plasma samples the statistically significant differences were observed in three cases: set 3 VEGF + / MMP9 - higher percentage of object marked by those antibodies in the control group, a larger percentage of microvesicles with annexinV in the group with endometriosis and higher percentage of microvesicles with MMP9 in the control group. It was also noticed that tests with annexin V plus another marker (antibodies) gave few object to count whereas tests with VEGF plus another possible marker gave much more objects to count. The same criteria were used to analyze samples of peritoneal fluid. There was detected one significant difference between test and control group: set 3 VEGF + / MMP9 - higher percentage of objects in the control group. Like in plasma, microvesicles marked by VEGF, also in combination with other markers, were the higher number of objects. Moreover we observed higher percentage of objects marked by CK18 in peritoneal fluid in both group.

There was a try to estimate the logistic regression to see if it was possible to predict, to which of group patients belonged using microvesicles profile. The logistic regression models ware developed for collected date of plasma and peritoneal fluid analysis. However the odds ratio estimate was impossible due to the small number of samples.

Moreover we tried to cluster patients within the plasma and peritoneal fluid groups to visualize the overall picture of the cytometry results between the groups. Clustering did not properly separate two groups of patients. The results indicated the heterogeneity of the study group. The heterogeneity was observed in both plasma and peritoneal fluid samples. The reason could be clinical factors not included in the analyzed data.

Conclusion: Results of the study did not confirm hypothesis that microvesicles with proangiogenic factors (VEGF, MMP9) are produced in higher amount by endometriosis foci. The research revealed presence of MVs in blood and peritoneal fluid samples in both groups. The higher percentage of MVs with VEGF+/MMP9 and only MMP9 in blood was unexpected result. Those factor are important in angiogenesis. Process, which is more advanced in endometriosis foci, not in teratomas cysts. In peritoneal fluid analysis we also observed more MVs VEGF+/MMP9 in control group. Moreover in peritoneal fluid there were noticed a lot of microvesicles marked by CK18 in both grup. It was supsrising result, because cytokeratine 18 was choosen as e specific marker for endometrial cells.

To sum up, the study indicated single parametrs, which differing group of patients witch endometriosis and control group (patients with teratomas). Most of the measurements did not differentiate the analyzed groups of patients. The tests group was heterogeneity, while the control group was quite small, which made difficulties to obtain statistically significant results. The reason of heterogeneity in group of patient with endometriosis could be many. In this study information of stage of endometriosis advanced and date of menstrual cycle in which was samples collected were not compared with analyzed data. Perhaps these data had a significant impact on variety results

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