Circulating Endometrial Cells: a Tool in Endometriosis Diagnostics and Therapy Management

Eliska Pospisilova[1]*, Imrich Kiss[1,2]*, Helena Souckova[2], Pavel Tomes[3], Jan Spicka[1], Rafal Matkowski[4,5,6], Marcin Jedryka[4,5,6], Simone Ferrero[7], Katarina Kolostova[1,4], Vladimir Bobek[1,4,8, 9]

* the authors contributed equally

1. Department of Laboratory Genetics, Laboratory Diagnostics, Faculty Hospital Královské Vinohrady, Srobarova 50, 100 34 Prague 10, Czech Republic

2. Department of Gynecology, Military University Hospital, U Vojenske nemocnice 1200, 169 02 Prague 6, Czech Republic

3. Department of Obstetrics and Gynecology, University Hospital, Faculty of Medicine Charles University, Alej Svobody 80, 301 66 Pilsen, Czech Republic

4. Cellpeutics Sp. z o.o., Duńska 9, Wrocław, Poland

5. Division of Surgical Oncology, Gynaecological Oncology and Department of Oncology, Wroclaw Medical University, Wybrzeże Ludwika Pasteura 1, 503 67 Wrocław, Poland

6. Lower Silesian Oncology Center, Wroclaw, Plac Ludwika Hirszfelda 12, 534 13 Wrocław, Poland

7. Academic Unit of Obstetrics and Gynecology Ospedale Policlinico San Martino Genoa, Italy Department of Neurosciences, Rehabilitation, Ophthalmology, Genetics, Maternal and Child Health (DiNOGMI) University of Genoa Genoa, Italy Academic Unit of Gynecology and Obstetrics University of Genoa, Genoa, Italy

8. Department of Thoracic Surgery, Krajská zdravotní a.s. Hospital Ústí nad Labem, CZ; 3rd Department of Surgery University Hospital FN Motol and 1st Faculty of Medicine Charles University, V Uvalu 84, 150 06 Prague 5, Czech Republic

9. Department of Histology and Embryology, Wroclaw Medical University, L. Pasteur 1, 503 67 Wroclaw, Poland

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Corresponding author: Katarina Kolostova, PhD, University Hospital, Kralovske Vinohrady, Department of Laboratory Genetics, Srobarova 50, 100 34 Prague, Czech Republic, e-mail: katarina.kolostova@gmail.com, Tel: +420 26716 2287

Abstract:

The focus of the presented work was to isolate and characterize circulating endometrial cells (CECs) enriched from peripheral blood (PB) of patients with diagnosed endometriosis to support endometriosis diagnosis and treatment.

Material and Methods

Blood samples (n=423) were tested for CECs presence. Subsequently, gene expression analysis (GEA) was carried out for CECs. In parallel, the presence and character of CECs was tested during phases of menstrual cycle (MC) (n= 11). CECs were isolated by size-based separation from 2x 8ml PB.

Results

CECs were detected in 78.4% of blood samples. In line with the revised American Fertility Society (rAFS) classification CECs presence was confirmed in all the acknowledged endometriosis stages: minimal, mild, moderate and severe. Surprisingly, the highest CEC negativity rate was reported for severe disease (21.1%).

The highest CEC numbers were detected in the mid-secretory periods of MC, which corresponds with uterine lining decidualization. The cytomorphology of CECs captured during MC is changing between the epithelial, stromal and stem cell-like. CECs captured in mid-secretory period expressed KRT18, NANOG and VIM in higher amounts when compared to the genes in the proliferative phase of MC when KRT19 and ESR1 were mostly observed.

GEA of the super-positive CECs samples (1000 CECs/8mL PB) revealed high expression of KRT18, VIM, NANOG and FLT1. The expression of these genes was elevated in the endometriosis tissue samples and endometrioma, too.

Conclusion

The panel of the identified CECs - genes could be tested in a prospective manner to confirm the predictive value of CECs in endometriosis diagnostics.

Introduction

Endometriosis is a common disease among women in reproductive age and a major contributor to pelvic pain and subfertility causing disability and significantly compromised quality of life [1]. It affects up to 10% of women of reproductive age, 50 to 60% of women and 2/3 teenage girls with pelvic pain and dysmenorrhea, and up to 50% of women with infertility [2], [3]. Because of its wide and non-specific clinical symptoms and difficult diagnosis, endometriosis is frequently underdiagnosed or diagnosed in later, more severe stages [4]. Diagnostic lead marks could be put together from the patient's history, gynecological examination containing ultrasound and few specific laboratory markers such as CA 125. Up to this point there has been no non-invasive biomarker from the endometrium, blood or urine or combined non-invasive tests specific enough to be used in clinical practice. Therefore, laparoscopy remains the gold standard for the diagnosis of endometriosis and using non-invasive tests should only be undertaken in a research setting [1], [5], [6], [7], [8].

As the aetiology and pathophysiology of endometriosis is still not fully understood, and more theories are being studied, it is challenging to discover a highly specific and sensitive preoperative diagnostic tool. Furthermore, it is necessary to validate the diagnostic accuracy of every promising test prospectively in an independent symptomatic patient population with subfertility and/or pain without clear ultrasound evidence of endometriosis and with a clinical indication for surgery, divided into cases with laparoscopically and histological confirmed endometriosis and controls with laparoscopically confirmed absence of endometriosis [9].

To ensure full understanding of the hypothesis of circulating endometrial cells (CECs) the lymphovascular spread (also called embolization, metastasis, transplantation) theory must be introduced first. It was first published by Halban in 1925 [10] who detected endometrial cells in the lymphatic system of the uterus in patients with endometriosis. Meanwhile, Sampson studied the volume and shape of the uterine cavity in normal and pathologic conditions. When injecting the uterus with a suspension, he found the injected mass to escape from uterine veins, which led him to believe that the endometrial cells would enter circulation in the same way [11]. This theory was further studied in an experiment in 1940 when Hobbs and Bortnick injected endometrial cells into circulation of rabbits. He found endometrial lesions in the lungs and pleura of these animals later during dissection [12]. In 1952 Javert followed up on Sampson's work and detected endometrial cells in the pelvic veins of patients with endometriosis [13].

More than half a century later CECs were described by Bobek et al. [14] in accordance with the same vascular spread theory. Endometrial cells from peripheral blood (PB) and peritoneal washings (PW) in patients with endometriosis were successfully isolated by a size-based separation method (Metacell®).

The endometrial origin of the captured cells was proved by immunohistochemistry [14]. Later, Chan et al. used immunofluorescence staining and separation via microfluidic chips for CECs detection. The results indicated that CECs could be a promising biomarker with great potential in diagnostics of endometriosis [15].

The aim of this study was to isolate CECs in patients with different types of endometriosis and clinical symptoms and to characterize these cells by molecular analysis. The results of cytomorphological analysis and gene expression profiling were correlated with patients' clinical data. Further analysis was conducted in patients with multiple sampling throughout the menstrual cycle (MC) to understand the character of CECs in each phase of MC. New information on the possible pathophysiology and development of endometriosis was found through comparisons of molecular profiles of endometrial lesions obtained during gynecological surgeries and CECs.

Material and Methods

A multi-center prospective study was initiated to collect blood samples from women with endometriosis. The inclusion criterion was that all the patients have a histologically proven anamnesis of endometriosis. A form was filled for each patient containing her detailed data i.e. information about the menstruation cycle, hormonal therapy if any, type of endometriosis (ovarian, peritoneal, RV septum, adenomyosis, extragenital), classification by revised American Fertility Society (rAFS), symptoms and signs (pelvic pain, dysmenorrhea, dyspareunia, metrorrhagia, hypermenorrhoea, sterility, infertility, gastrointestinal problems). Samples were obtained from 423 patients. As the samples were collected from various centers nationally and internationally, a possible bias in results due to different transport conditions was considered. The sample was marked CEC positive if endometrial-like cells were detected. Subsequently, we divided the positive samples to categories based on CECs quantity (low positivity – up to 10 cells, medium positivity up to 100 cells, high positivity more than 100 cells). Samples with high positivity (n=13) were subjected for molecular analysis. To be able to analyze the molecular behavior of CECs during the MC, we obtained multiple samples from 11 patients during menstrual cycle (2x48 samples in total). A minimum of 4 samples were taken for every patient to correspond with different phases of the cycle (menstruation, proliferative phase, ovulation, secretory phase). The phase was calculated from the last menstrual bleeding and verified by ultrasound examination of the endometrium.

To enrich CECs approximately 2x8 mL of PB was drawn from the antecubital veins and placed into S-Monovette tubes (Sarstedt AG & Co., Numbrecht, Germany) containing 1.6 mg EDTA/mL blood as an anticoagulant. The samples were processed at room temperature using an isolation procedure completed within 36 hours after the blood draw. The ethics committees of the participating

universities and hospitals approved the study protocol according to the Declaration of Helsinki. Size-based filtration and *in vitro* culture method (MetaCell®, Czech Republic) were used to enrich CECs. [14]. The captured cells grew in the FBS enriched RPMI medium (10%) for the period of a minimum of 7-14 days on the membrane. The cultured cells were analyzed by vital fluorescent microscopy using unspecific nuclear (NucBlue™) and cytoplasmatic (Celltracker™) stain. Cells on the membrane were later put into RLT buffer lysis (Qiagen) and kept in the freezer for further analysis.

As mentioned earlier, further molecular analysis was initiated in the single site sample group with the highest number of CECs (2x13 samples in total). To confirm the origin of the cells on the separation membrane, CECs gene expression analysis can be performed. Gene expression analysis (GEA) allows up to 20 endometriosis-associated markers in RNA from different cell fractions to be tested within a single quantitative polymerase chain reaction (qPCR) run. Differential diagnostics markers for qPCR test are chosen in concordance with the expected diagnosis. The key purpose of GEA is to compare gene expression of endometriosis-associated markers in the CEC enriched fractions to that in the whole blood.

Soon after, RNA is isolated from the whole blood's white blood cell fraction (WBC) and CECs enriched fraction on the membrane. Finally, the CEC gene expression analysis allows identification of the relative amount of endometriosis-associated markers in the whole blood and in CECs enriched fractions. The RNA from the whole blood is isolated with modified procedure and the quality/concentration of RNA is measured by NanoDrop (ThermoScientific). As there are only a few hundred cells on the membrane, the median concentration of RNA is quite low (5-10 ng/µl). High Capacity cDNA Reverse Transcription Kit (Life Technologies) was used for cDNA production. qPCR analysis was performed using Taqman chemistry with hydrolysis probes for all the tested genes (Life Technologies). The tested genes which thought to be endometrial-associated are: CD68, EpCAM, KRT7, KRT18, KRT19, MUC1, MUC16, VIM, VEGFA, WT1, ESR1, PGR, HER2, CD10, FLT1, MMP1, MMP9, TP63, ESSRA, ESSRB, HIF1A, NANOG.

To analyze ectopically growing endometrial cells in tissues we obtained several layers of histologically proven endometrioma (n=11) from two patients during gynecological surgeries. Both patients had procedures planned because of pelvic pain and cystic adnexal tumor diagnosed during ultrasound examination. The perioperative findings in the first patient were bilateral massive endometriomas of the ovaries with no peritoneal or other lesions. The second patient had one sided endometrioma forming a convolute consisting of the ovary and fallopian tube, severe peritoneal lesions of the urinary bladder, sacrouterine ligaments and Douglas' pouch. Eutopic endometrial tissue during menstruation bleeding was acquired from a healthy control. All the tissues were further analyzed using the same qPCR protocol as for the CECs samples.

Gene expression analysis was conducted using Genex v. 6 (MultiD, SE) software to enable normalization and statistical analysis (cluster analysis, Mann Whitney tests) for qPCR-generated data. The relative RNA amounts are reported for tested groups in comparisons to white blood cells fractions (WBC) or endometriosis tissue.

RESULTS

CECs presence in endometriosis

CECs were detected by cytomorphological evaluation in 78.3 % (331/423) of the tested samples. Four main CECs-subtypes can be found in blood sample of patients with endometriosis: epithelial, stem cell – like, stromal, glandular. CEC positivity did not vary significantly in different patient cohorts from Italy (n=20), Poland (n= 82) or Czech Republic (n=321) (75%, vs. 66% vs. 81%) (see FIGURE 1. A).

In line with the rAFS classification CECs presence was confirmed for all of the acknowledged endometriosis stages: minimal, mild, moderate and severe. Surprisingly, the highest CEC negativity rate was reported for severe disease (21.1%) (see FIGURE 1. B).

CECs load in patients with different endometriosis types

If accessible, CECs numbers were counted and then ascribed to the following categories: CEC negative (≤ 1 cell detected) and CEC positive: 1-10 cells (*), 10-100 cells (**), >100 cells (***) (see FIGURE 1C, 1D). The distribution of CECs load in the tested samples reflected normal distribution in the tested cohorts. The conclusion is that in 20% cases there are patients with very high CECs numbers and in 10-20% patients with endometriosis there are no CECs in PB.

The highest CECs numbers were detected in the after-ovulation periods (day 14-17 i.e. secretory phase), which corresponds with estrogen decrease and slow subsequent progesterone increase associated with uterine lining decidualization. We succeeded in setting up *in vitro* cultures of isolated cells.

For the ovarian, peritoneal, rectovaginal and extragenital endometriosis CECs were found in 90-95% of samples and mostly in numbers of 1-10 cells (*) for 8 mL of PB (see FIGURE 1D). It was confirmed that CECs presence is most probably independent of the different extrauterine (ectopic) locations of endometriosis tissue. Finally, it was shown that there is a subgroup of patients in all of the mentioned endometriosis subgroups that has very high numbers of CECs (up to 20% of patients) in the blood.

Gene expression profiling of endometriosis tissue and related CECs-samples

To confirm the origin of CECs in PB evaluated by cytomorphology, an additional molecular testing was provided analyzing gene expression of endometriosis tissue samples (TS) and blood samples from the same patient (WBC, CECs). Up to 20 samples were analyzed in total. The cluster analysis of normalized qPCR-results enabled to identify a group of "endometriosis" genes that could be used as confirmatory for CECs.

There are several genes strongly expressed in endometriosis tissue: EPCAM, KRT18, WT1, MUC16, MUC1 and ESR1 if compared to the endometrial cells from healthy controls. In some tissue samples also MMP1 was present. In the CECs fraction of these patients only KRT18, KRT19, VIM and NANOG were detected in a relatively high amount if compared to the WBC. Interestingly, in CECs samples ESR1 is expressed very rarely. The elevated genes (KRT18, KRT19, VIM and NANOG) could be used in the next analysis as endometriosis confirmatory genes. The cluster analysis of qPCR- results for one patient samples collection is shown on FIGURE 2A.

Gene expression analysis of super- positive CECs samples

The genes confirmed to be expressed in the endometriosis tissue and CECs as presented in the first part of the results (FIGURE 2A) were then analyzed in the group of patients (n=13) in whom CECs were detected in relatively high numbers (up to 1000 CECs per 8 mL PB). In this CEC cohort the following genes were found to be elevated: **VIM (elevated in 13 out of 13 CECs samples - 13/13), FLT1 (12/13), KRT18 (8/13), KRT19 (8/13), MMP9 (12/13), NANOG (8/13), ESR1 (7/13). The statistical significance of different expression was confirmed for VIM, MMP9, FLT-1, KRT19**. As expected, CD68 was elevated in all of the tested samples (13/13), which suggests that some of the frequently observed genes could be found because of the presence of the captured and *in vitro* cultured macrophages which have a very similar cytomorphology to the endometrial cells. However, correlation analysis revealed that KRT18, FLT1 and NANOG expression is CD68 independent. On the other hand, in this specific patients cohort there is a correlation between CD68 and VIM.

The cluster analysis of super-positive CEC samples revealed that there are at least two different sample types of super positive CECs. The first one is represented with the cluster showing high expression of FLT1, MMP1 and ESRRB (see FIGURE 2B – cluster on the right). The second group of samples shows elevation of NANOG, KRT18 and VIM expression. KRT19 was relatively high expressed in both clusters (not shown). Significantly elevated FLT1 does not correlate with any other of the tested genes. Interestingly, ESR1 expression was present in parallel with ESSRA and ESSRB in two of super-positive samples only. Very high expression of ESSRB was observed in samples with elevated VIM expression.

Gene expression profiling data of all CEC – samples tested

The analysis of CECs samples (n=52) showed that mRNA-expression of the following genes is significantly elevated in the CECs fractions when compared to WBC fraction: **KRT18, VIM, NANOG and MMP9. KRT19 gene is at the limit of significance** (see FIGURE 3A). The following genes are significantly decreased in CECs samples: HIF1A, CD10 and MUC1. There was a significant difference between all CECs samples and superpositive samples found for FLT1 expression (see FIGURE 3B – white arrow)

CECs prevalence and character during menstrual cycle (MC)

Based on a simple presumption CECs should be present in PB during all phases of MC if they are to be used as a biomarker of the disease. Blood samples from 11 patients were collected over the MC -phases. The highest CEC numbers were detected in the after-ovulatory periods (mid-secretory phase) of MC. CECs are present all over the MC-phases but their character varies. The character of CECs during MC reflects the physiological cycle of endometrium decidualization. The cytomorphology of CECs captured during MC is changing between the epithelial, stromal and stem cell-like.

In line with this finding gene expression changes during MC phases in CECs were analyzed and it was observed that the structural genes like KRT18, VIM, NANOG are expressed in a relatively stable manner in all four MC phases but their expression is significantly elevated in the middle of MC (early/mid secretory phase) (see FIGURE 4). In this period the expression of KRT18 and VIM rises. Additionally, in the late secretory and early proliferative phase elevation of FLT1 and MMP1 expression was observed.

Cells shed into circulatinion during the decidualization process are mostly stromal-like endometrial cells as shown by their cytomorphology and gene expression profile. These cells are most probably estrogen or progesterone non-responsive but they do express ESRRB. The highest expression of ESSRB was found after ovulation in the secretory phase between day 20-26.

The ESR1 positive cells are regularly shed to the blood in proliferative phases of MC (day 1-14). Epithelial KRT19+ cells which are ESR1 positive are typically found in this part of MC.

Discussion

Our study confirms the presence of CECs in patients with histologically proven endometriosis independently of hormonal changes during the menstrual cycle. The different expression of genes in

the endometrial lesions and parallel CECs samples from PB identified a range of potential biomarkers which could be used to identify CECs in patients with undiagnosed endometriosis. Early detection of CECs in women with pelvic pain or other symptoms in addition to objective gynecological examination suspecting endometriosis could accelerate and help to make the correct diagnosis. The various CECs types isolated from patients with endometriosis in our cohort have different gene expression profiles represented by typically elevated gene expression of KRT18, KRT19, NANOG and VIM.

The shedding of CECs into PB could be ascribed to the well-known physiology of decidualization. The process of decidualization of the uterine lining denotes the transformation of endometrial stromal fibroblasts into specialized secretory decidual cells that provide a nutritive and immunoprivileged matrix essential for embryo implantation and placental development. Decidualization of the human endometrium is driven by the postovulatory rise in progesterone levels and increasing local cAMP production. In response to falling progesterone levels, spontaneous decidualization causes menstrual shedding and cyclic regeneration of the endometrium.

Under endometriosis conditions, the decidualizing cells tend to be progesterone non-responsive, which results in the need for a different energy supply (16). The CECs cells characterized in our cohort did not express PGR (progesterone receptor) and in at least half of the cases ESR1 was also not present. On the other hand, the CECs captured by size-based separation method express ESRRB in several specific cases.

The histological appearance of the endometrium was referred to as predecidua in several previous publications (16). In parallel with the predecidua changes, various CECs types isolated from patients with endometriosis in our cohort have shown different gene expression profiles represented by typically elevated gene expression of KRT18, NANOG and VIM group or KRT19, ESR1 group. KRT18, VIM, NANOG were typical for secretory phases, while KRT19, ESR1 were observed in the proliferative MC phases. Angiogenesis was driven by FLT1, MMP1 in the late secretory phases.

The results discussed in this paper offer a chance to properly identify CECs types circulating in PB and may facilitate preoperative and postoperative endometriosis therapy management. Further studies are necessary to fully understand the advantages of CECs isolation and its use in clinical practice.

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Author contribution:

I Kiss: Data collection or management , Data analysis, Manuscript writing/editing

E Pospisilova: Data collection or management, Manuscript writing/editing

H Souckova: Data collection or management

P Tomes: Data collection or management

J Spicka: Protocol/project development, Data analysis

R Matkowski: Data collection or management, Data analysis

M Jedryka: Data collection or management

S Ferrero: Data collection or management

K Kolostova: Protocol/project development, Data analysis, Manuscript writing/editing

V Bobek: Protocol/project development, Manuscript writing/editing

Disclosure of interests

We confirm that all authors declared no conflicts of interest with this manuscript.

Details of ethics approval

The protocol for this study was approved by Ethical Committee of University hospital Kralovske Vinohrady in Prague, Czech Republic (EK-VP/20/0/2015) and Ethical Committee of Medical University Wroclaw, Poland (Nr.KB-242/2015).

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