Circulating endometrial cells in peripheral blood

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A B S T R A C T

Objectives: Endometriosis is a common disorder amongst women of reproductive age. Despite extensive research, no reliable blood tests currently exist for the diagnosis of endometriosis.

Study design: We report several new approaches enabling study of cell specific characteristic of endometrial cells, introducing enrichment and culturing of viable circulating endometrial cells (CECs) isolated from peripheral blood (PB) and peritoneal endometrial cells (PECs) from peritoneal washing (PW). Size-based enrichment method (MetaCell® , Czech Republic) has been used for the filtration of PB and PW in patients with diagnosed endometriosis.

Results: The CECs were found in the PW in all of the tested patients (n = 17), but CECs only in 23.5% (4/17) cases. Their endometrial origin has been proved by immunohistochemistry. PECs were successfully cultured in vitro directly on the separating membrane (9/17) exhibiting both endometrial cell phenotypes: stromal and glandular within the culture. CECs were successfully cultured in the two of the four positive cases, but in none of them confluence has been reached. The occurrence in CECs in PB is clear and very specific evidence of an active endometrial disease.

Conclusions: We demonstrated efficient, quick and user friendly endometrial cells capture platform based on a cell size. Furthermore, we demonstrated an ability to culture the captured cells, a critical requirement for post-isolation cellular analysis directed to better understanding of endometriosis pathogenesis.

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Introduction

Endometriosis is a common disorder amongst women of reproductive age. Endometriosis, the presence of endometrial-like tissue outside the uterus, is a disease associated with pelvic pain and infertility. Endometriosis afflicts about 10% of women worldwide [1]. In women with pain, infertility or both, the frequency increases to 35–60% [2]. It is one of the most frequent benign gynecological diseases.

Relatively little is known about the pathophysiological mechanisms of endometriosis and so far endometriosis continues to remain a significantly under diagnosed and under-treated disease.

The mainstay of diagnosis is still the direct visualization of the lesions by invasive procedure such as a laparoscopy [3].

One of the major challenges facing gynecologists is the inability to diagnose endometriosis without the need of laparoscopy or laparotomy. At present we are not able to identify reliable biomarkers of the disease.

Many studies have focused on identifying biomarkers in blood or urine because of the ease of sampling. Despite extensive research, no reliable blood tests currently exist for the diagnosis of endometriosis. And a noninvasive test would be particularly welcome because early diagnosis is important in endometriosis, and its need in both asymptomatic and symptomatic disease [4–7].

Critical information to understand the pathogenesis of endometriosis may come from studies of controlled in vitro models. Such cellular systems could be then used for investigation of therapy targeted to endometriotic lesions [8–12]. However, establishment of human endometriosis derived permanent cell lines were exceptionally successful. The two recently reported in
vitro models consist of immortalized endometrial cells. These cells usually exhibit undifferentiated phenotype what does not represent situation in vivo \cite{13,14}.

We report a new testing method for detection and culturing of viable circulating endometrial cells. The aim of the presented study was to identify endometrial cells in peritoneal washing (PW) and in peripheral blood (PB) in patients with a diagnosed or prediagnosed endometriosis. There are recently no standardly introduced methods to identify viable endometrial cells in PW and PB, so the challenge would be to have more of them keeping then in vitro culture. The cells could then further be used not only for diagnosis support, but could also improve the treatment strategy in the direction of personalizing the hormonal endometrial treatment.

**Materials and methods**

**Patients**

Seventeen patients with diagnosed endometriosis or prediagnosed endometriosis undergoing planned laparoscopy were enrolled into the study. The final diagnoses were made by histopathology. Based on the informed consent the clinical data were collected from all participating patients. PB was collected prior the laparoscopy. For each patient, approximately 8 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. During the laparoscopy PW was collected. The samples were processed at room temperature using an isolation procedure completed within 24 hours after the blood draw. The ethics committee both participated University and

![viable usually the sent arrow.](image1)

![Figure 1](image2)

**Fig. 1.** Scheme of separating membrane culture system. The blue arrows appoints the flow of the filtrated liquid (PW and PB). The PECs and CECs-cells are captured on the membrane kept in a plastic ring and cultured directly after separation in vitro under the standard culturing conditions. The specific diffusion flow is shown by red arrow. Cells exhibiting high potential of plasticism can grow over the membrane to the culture flask bottom. Finally, two cell populations can be identified after several days of in vitro culture. The first one on growing the membrane and the second one growing under the membrane. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

![viable usually the sent arrow.](image3)

![Figure 2](image4)

**Fig. 2.** Viable endometrial gland cells (PECs) isolated from PW, grown in vitro. The presented PECs were growing under the membrane. There they start to proliferate and to form a kind of cell-islets of epithelioid character (A), following they start to scallope the borders of the cell-islets and to detach from the bottom (B and C) resulting finally in the floating clumps (D). A bar represents 10 μm.
Hospital approved the study protocol according to the Declaration of Helsinki.

**Endometriosis-related cells enrichment and in vitro culture**

Size-based enrichment method (MetaCell®, Czech Republic) has been used for the filtration of PB and PW through porous polycarbonate membrane (pores with 8 μm diameter). The minimum and maximum volume of the filtered liquid may be adjusted up to 50 mL of fluid. Standardly, 8 mL PB from patients suffering with endometriosis can be transferred into the filtration tube, and approximately 25 mL of PW is filtered. Based on the consistence of PW, the PW could be before filtering centrifuged and the cell pellet released in PBS and subsequently transferred into the filtration tube. The PB or PW flow is supported by capillary action of the absorbent touching the membrane filter. Afterwards the membrane filter kept in a plastic ring is transferred into the 6-well cultivation plate, 4 mL RPMI media is added to the filter top and captured cells are cultured on the membrane in vitro under the conditions of standard cell cultures (37°C, 5% atmosphere of CO₂) and observed by inverted microscope. The cells grow in the FBS enriched RPMI medium (10%) for the period of minimum 7–14 days on the membrane (see Fig. 1). The grown cells are analyzed by means of histochemistry (May-Grünwald staining, MGG) and immunohistochemistry (IHC). Antibodies against pan-cytokeratin, CD10, vimentin (DAKO, Denmark) were used for cell origin identification.

Alternatively the enriched cell fraction can be transferred from the membrane and cultured directly on any plastic surface or a microscopic slide. Microscopic slide culture is preferred if the IHC/immunofluorescence analysis is planned. If an intermediate cell analysis is awaited, the cell fraction is transferred in PBS (1.5 mL) to the cytospin slide. The slide is then dried for 24 h and analyzed by IHC.

**Cytological analysis**

The fixed and stained cells on the membranes were examined using light microscopy in two steps: (1) screening to locate cells, (2) observation at higher magnification for detailed cytomorphological analysis. Isolated cells and/or clusters of cells of interest were selected, digitized, and examined by an experienced researcher and pathologist. We compared the cells enriched from PB with the cells captured from PW, to identify the endometrial gland cell morphology and stromal cell morphology.

**Results**

In total, 17 patients have been enrolled into the study in the years 2012–2013 (age mean 39.3 years). Following the aims of the study, we have identified viable endometrial gland and stromal cells in PW (see Figs. 2 and 3) and endometrial stromal cells in PB (see Fig. 4). This is the first time to our knowledge reporting the

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**Fig. 3.** Endometrial gland cells isolated from PW, grown in vitro (PECs). The presented PECs are growing on the separating membrane. There they start to proliferate and to form cell-bulks (PECs-bulks) (A), sometimes floating as a group of cells (B). Following they start to adhere to the membrane creating cell-islets of an epitheloid character (C), exhibiting also an abundance of the stromal-like PECs in the culture—see arrow (D). A bar represents 10 μm.
endometrial cells have been isolated from PB. We call them circulating endometrial cells (CECs), the endometrial cells found disseminated in peritoneal washing (PW) could be assigned as peritoneal endometrial cells (PECs).

Generally, the PECs were found in the PW in all of the tested patients using the size-based filtration method, but CECs only in 23.5% (4/17) cases in PB. CECs were kept viable in the two of the four positive cases (Fig. 4) without reaching confluence in 14 days, but enabling to have enough cell material for introduction of subsequent histochemistry testing. The origin of endometrial cells (PECs and CECs) has been proven by IHC using antibodies against cytokeratin, vimentin and CD10 (see Fig. 5).

In the 9 of the 17 cases we have successfully cultured the PECs in vitro on the separating membrane (Fig. 6A and B). Some of the PECs grow through the membrane and set up culture under the membrane on the culture flask- bottom (Fig. 6C and D). Analyzing
the cytomorphology of the PECs captured on the membrane, we were able to identify endometrial stromal cells growing on the membrane and under the membrane (Figs. 6 and 7) within the first 5 days of culture. Later on much smaller endometrial glandular cells started to be visible (Fig. 8). The presence of the small endometrial glandular cells (up to 8 μm) on the membrane with relatively big pores (8 μM) could be explained most probably by the fact, that the endometrial glandular cells are usually found in PW in an organized cell clusters (e.g. gland-like structures, honeycomb, polarized-sheets, spheroids). Some of the small endometrial gland cells can be found later growing under the membrane as well reflecting the dynamics of endometrial glandular cells folding (Fig. 8).

The PECs cultures were introduced for the purpose of the real-time cell proliferation monitoring by means of bioimpedance-based real-time testing, but as observed in the culture, the endometrial glandular cells do not grow adherently. According to our observations, we may conclude following: After the filtration of PW through the membrane we have identified several cell types on the membrane, some of them were PECs. We assume that the PECs-cells are of both endometrial cell types: glandular and stromal.

After the 7 days in vitro culture, some of the PECs exhibited heavy proliferation, which yielded in the generation of numerous PECs glandular-bulks, with a typical morphology-consisting out of the cells with nuclei without detectable nucleoli (Fig. 6A and B). Within the 7-days, some of the PECs, have grown through the porous membrane to the culture-plate bottom. There a new cell propagation started, yielding into the typical proliferation-islets, which demonstrated an endometrial gland-like cell character (Fig. 2A). After reaching a specific time point (we assume this could be a kind of internal islet-confluency point), the cells on the borders of the islets start to scallop (and to detach from the bottom (see Fig. 2B and C).

Next, the cell population starts to behave as a suspension culture, with floating cell-clumps with a typically scalloped borders (Fig. 2D). This is mainly true for the PECs-cells of the endometrial gland character. If the cell islet does not detach, it starts to develop into the—PECs-bulk, similar to them seen previously on the membrane (Fig. 3A). But still some of the relatively big stromal cells can be found growing on the plate-bottom and their heavily proliferation can be observed (see Fig. 7). We could assume that the big endometrial stromal cells possess a specific character of plasticity or invasiveness, which enables them to grow over the membrane pores and behave according the conditions in the medium. Typically, the stromal cells exhibit big rounded nucleus (sometimes bigger than 30 μM) with several very big nucleoli. The bi- or tri-nucleated stages of stromal cells are also very typical (Fig. 8). We observe several very interesting cell differentiation points of the in vitro culture, but to study the differentiation dynamics it will be necessary to separate

**Fig. 6.** Endometrial cells isolated from PW, grown in vitro (PECs) after May–Gruenwald staining. The presented PECs growing on the membrane do show both phenotypes: stromal and glandular (see arrows) (A and B). Some of the cells captured on the membrane grow through the pores in the direction to the bottom of the culture flask. There they start to proliferate and form cell-islets of epitheloid character (C). Big nuclei of endometrial stromal cells exhibit also several big nucleoli, glandular cells in the middle (D). A bar represents 20 μm.
endometrial stromal cells from the endometrial glandular cells in future experiments.

Comments

Endometriosis is highly unpredictable. Some women may have a few isolated implants that never spread or grow, while in others the disease may spread throughout the pelvis or extra-abdominopelvic localization. Establishing a correct diagnosis of endometriosis is often problematic, because the presenting symptoms can be non-specific and associated with a number of different conditions [1]. Imaging methods such as transvaginal ultrasound and magnetic resonance imaging may help to identify ovarian endometriomas or a rectovaginal endometriotic nodule, but they have no value in diagnosing peritoneal or generalized endometriosis [15,16]. Consequently, it is recommended that pelvic endometriosis should be diagnosed surgically [16].

Considerable effort has been invested in searching for non-invasive methods of diagnosis. A biomarker that is simple to measure could help clinicians to diagnose or exclude endometriosis; it might also allow the effects of treatment to be monitored. If effective, such a marker or panel of markers could prevent unnecessary diagnostic procedures and/or recognize treatment failure at an early stage [4–7].

A biomarker is a measurable “biologic marker” that correlates with a specific outcome or state of the disease [17]. The biomarker in peripheral blood may be not represented only by chemical or biological substances (indirect biomarker) but also by direct presence of endometrial cells (CECs) in circulation. The occurrence in CECs in peripheral blood is clear and very specific evidence of endometrial disease. However, CECs are probably extraordinarily rare, with only a few CECs circulating among billions of blood cells, making their isolation and characterization a tremendous technical challenge. Thus, high-efficiency and high-purity isolation of CECs from patient blood is urgently needed to obtain accurate information of CECs.

The MetaCell separation system was utilized to isolate CECs from endometriosis patient blood samples and peritoneal washings samples, with CECs detected in 4 of 17 samples and PECs detected in 100% of the diagnosed endometriosis. We demonstrated efficient, quick and user friendly CECs and PECs capture platform based on a cell size. Furthermore, we demonstrated the ability to culture the captured cells, a critical requirement for post-isolation cellular analysis. Although it is extremely challenging to culture the isolated CECs from patient blood and to develop a new cell line, our system shows the possibility to culture endometrial cells out of PW, after the sophisticated capture and release process, while maintaining their viability and proliferation capability. Therefore, our CECs capture system shows great potential for efficient CECs enrichment, isolation, and cellular/genetic analysis, leading to possible “blood biopsy” of generalized endometriosis.

It could be of interest for the next research design to compare the cell populations growing on the membrane and growing through the membrane. Most probably, the invasiveness of the endometriosis can be assigned to the endometrial stromal cells, which create a support for the endometrial glandular cells growth. Another point of interest could be to define the amount of the endometrial gland cells and stromal cells in the connection to the disease progress. So, that it could be of interest to develop more sophisticated strategies for in vitro PECs and CECs propagation. The CECs-cells captured on the membrane did not show as much proliferation potential in comparison to the PECs, but it could be definitely developed in future with a help of growing medium substitutes.

Various histological and molecular genetic studies have even indicated that endometriosis may transform into cancer or that it could be considered a precursor of cancer. Epidemiological studies have shown that women with endometriosis have an increased
risk of different types of malignancies, especially ovarian cancer and non-Hodgkin’s lymphoma \cite{18,19} and dysplastic nevi, and melanoma, and breast cancer \cite{20–22}.

Clinical studies have demonstrated that circulating tumor cells (CTCs) are correlated with disease progression for a wide range of cancers, such as breast, colorectal, and prostate cancer and gynecological cancers too \cite{16}. CTCs are tumor cells disseminated from primary tumors which subsequently travel through the blood circulation to distant organs. Therefore, CTCs hold the key to track metastasis, and they can be used for cancer diagnosis and monitoring of cancer status. On the above mentioned principle the presented MetaCell technology shows great possibility to detect and test not only CTCs \cite{23,24}, but CECs as well.

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Peripheral biomarkers show promise as diagnostic aids, but further research is necessary before they can be recommended in routine clinical care. Our future efforts include culturing the captured CECs from patient, cellular and genetic study of isolated CECs.

Condensation

The paper describes an isolation and cultivation of the circulating endometrial cells from peripheral blood in patients with endometriosis. It should be new biomarker.

Acknowledgements

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