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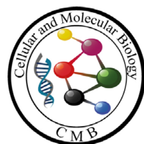

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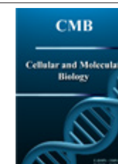

Original Article

Morphology of mitochondrial network in disseminated endometriosis cells in spontaneous pneumothorax diagnostic process

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Abstract

Circulating endometrial cells (CECs) have emerged as a new biomarker of advanced disease in women with endometriosis. The identification of several subtypes of CECs (e.g., stem cell-like, epithelial, glandular, stromal) has opened the way for characterization of endometriosis-associated CECs. This study focused on the isolation and characterization of CECs and disseminated endometrial cells (DECs) in patients with spontaneous pneumothorax (SP). The primary objective was to differentiate between cancer and non-cancer cells in patients with no previous cancer diagnosis. The MetaCell® size-based separation protocol was used to enrich CECs/DECs. Evaluation of the captured cells by 3D microscopy was performed using a NANOLIVE™ microscope using a holographic approach. Based on gene expression analysis (GEA), we can conclude that mitochondria are much more active in primary tumors compared to endometriosis tissue (e.g. MT-ND1, MT-ATP6 genes). The culture of DECs is made of stromal, stem and immune cells. *In vitro* culture of DECs is characterized by an increase in the epithelial marker KRT18. Similarly, NFE2L2, a proerythroid factor, is also elevated. Further, a significant decrease in the amount of stem and immune cells was observed in the cell culture of DECs. The data presented here show how morphologically plastic the changes in the mitochondrial network can be and how cells can reflect them at the level of gene expression. The markers identified could help in the accompanying diagnostic process of the spontaneous pneumothorax in women of reproductive age.

Keywords: Endometriosis, Liquid biopsy, Pneumothorax, MetaCell, Mitochondria.

1. Introduction

Endometriosis, endometrial tissues growing outside of the uterine cavity, has a significant impact on the physical, psychological, and social life of women. It is causing pelvic pain and infertility in 10–15% of women [1–3]. Its physiology still remains an unsolved question.

Circulating endometrial cells (CECs) became a new marker for advanced disease in women with endometriosis [4, 5]. Identifying several CECs subtypes (e.g. stem cell-like, epithelial, glandular, stromal), the path for their characterization in endometriosis has been opened [6].

Previously, the proof-of-concept study by our group demonstrated successful isolation and characterization of CECs in patients with spontaneous pneumothorax (SP). Identification of CECs in SP could alert endometriosis involvement and help with early referral to gynecologic consultation for further examination and treatment [6].

Recently, the idea of excessive stem cells presence in the endometriosis tissue [6] and their metabolic changes were assigned to be one of the initiating factors for endometriosis [7–9]. Markers of oxidative stress in peritoneal fluid, follicular fluid, and peripheral circulating blood are significantly elevated in patients with endometriosis [10–12], but nobody has reported oxidative stress changes on a cellular level so far.

This study focused on the isolation and characterization of epithelial cells from body fluids in patients with SP. Peripheral blood and thoracic effusion were used to enrich epithelial cells. Cells present in the enriched fractions may contain circulating tumor cells (CTCs) and disseminated endometrial cells (DECs) and/or disseminated tumor cells (DTCs) in addition to CECs. The aim was to differentiate cancer cells from non-cancer cells in patients without a previous cancer diagnosis using morphological characteristics of mitochondria.

High amounts of reactive oxygen species (ROS) can

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damage peritoneal mesothelial cells and promote ectopic endometrium implantation [13, 14]] via changes in morphology and function of vascular endothelial cells, in addition a persistent inflammation is present [6, 15–17]. When ROS generation and detoxification are imbalanced, the relative excess of ROS causes significant oxidative stress. Mitochondria are major sources for reactive oxygen species (ROS) generation, but what happens to mitochondria in patients with endometriosis is less clear.

Relatively dense mitochondrial network is typical for cells of non- blood origin (CECs, CTCs) circulating in the peripheral blood. Similarly, markedly dense and branched mitochondrion network has been described in DECs found outside uterus.

As we have shown, the difference between CEC/DEC and CTC can be easily demonstrated between groups of cells if fluorescently labeled glucose (2-NBDG) is used in parallel with visualization of mitochondria, as evidenced by the use of chemicals affecting mitochondrial metabolism (e.g., piracetam, tamoxifen).

Gene expression profiling of primary endometrial tissue, endometriosis and disseminated endometrial cells (DEC), was provided for more detailed molecular analysis. A total of 25 genes closely associated with mitochondrial metabolism were tested.

2. Materials and Methods

2.1. Cell enrichment and culture

To enrich CECs/DECs/CTCs/ a size-based separation protocol and tube MetaCell® was used [18–21]. CECs/CTC presence is evaluated by single-cell cytomorphology, which could be followed by molecular testing (e.g., qPCR analysis) or standard immunohistochemistry. A total of 32 patients admitted to the hospital because of endometriosis/or/and spontaneous pneumothorax have been enrolled in the study in accordance with the Declaration of Helsinki. The study obtained approval from Multicentric Ethic Committee Faculty Hospital Kralovske Vinohrady, Prague and Masaryk´sHospital Usti and Labem. All participants signed informed consent before participating in the study CECs/DECs/CTCs samples from involved patients were collected.

Enriched CECs/DECs and CTCs were used for *in vitro* cultures and detailed morphology description. For each CECs/CTCs patient, approximately 2 × 8 mL of venous blood was drawn from the cubital 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 24 hours after the blood draw. Out of the tested 32- endometriosis suspicious blood samples with spontaneous pneumothorax, all were CECs positive. If applicable, in 12 patients peritoneal and/or pleural effusion samples were taken to detect DECs presence after size-based separation. The DECs were present in all tested samples (12/12). In some patients (n= 23) also primary endometriosis tissue was accessible.

The complex of separation membrane filter, which is kept in a plastic ring, was transferred directly with enriched cells into a 6-well culture plate. A total of 4 mL RPMI media is added to the filter top and enriched cells are cultured on the membrane *in vitro* under standard cell culture conditions (37 °C, 5% CO2 atmosphere) and observed using an inverted microscope. CTCs are grown in FBS-enriched RPMI medium (10%) for a period of minimum 3–5 days. A microscopic slide was placed under the separation membrane and CECs/DECs/CTCs may naturally grow invasively and set up new cell colonies on the microscopic slide.

2.2. Microscopy analysis of CECs/DECs/CTCs

CECs/ DECs/ CTCs grown *in vitro* on the separation membrane were stained by vital fluorescent stains (NucBlue™, CellTracker™, or MitoTracker H_2 ROS™, 2—NBDG glucose, Thermo Fisher Scientific). Tumor cells and cells of endometrial origin were discriminated by morphological features and fluorescent stainings reporting metabolic activity of the captured cells, as first active mitochondrial network was visualized by MitoTracker H_2 ROS, as second fluorescent 2—NBDG glucose uptake rate (2-NBDG - fluorescent d-glucose analog 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose) was used to discriminate cell origin.

Cells were evaluated by means of vital fluorescence microscopy (Olympus X10; Olympus) in the following two steps: (1) screening at 10x and 20x magnification to locate viable cells; and (2) observation at 40x and 60x magnification for detailed cytomorphologic analysis of the cytoplasm, nucleus, and mitochondria. Enriched cells and/or cell clusters of interest were scanned and digitized, and the images were subsequently examined by an experienced researcher and/or pathologist. Each sample was evaluated by two different specialists. After completing vital fluorescence microscopy analysis of the cells, the separation membrane was fixed by drying, used later for immunohistochemistry, and/or stored in the RLT buffer for planned RNA gene expression analysis (GEA).

The 3D microscopy of tumor cells and cells of endometrial origin evaluation process was established by NANOLIVE™microscope, using the holographic approach.

2.3. Gene expression profiling

As next, gene expression profiles of the 25 genes (ACTB, KRT18, VIM, VEGF, HIF1A, MRP1, POUSF, SOX2, SOD2, MT-ND1, CD274, NANOG, BAX, BCL2, TXN, CASP3, CASP8, CAT, SIRT3, IL1B, MT-ATP6, NFKB1, NFE2L2, PRDX3, NRP1) were compared by quantitative PCR [22]. Samples of endometriosis tissue and DECs from pelvic and pleural cavities and CECs enriched out of the blood were tested and compared. The tested genes were chosen to reflect mitochondria metabolism, hypoxia stages, apoptosis activation and inflammatory signals. Gene expression analysis was measured by COBAS®480 (Roche s.r.o., Czech Republic). Temperature conditions were set according to manufacturing instructions of TaqMan® Fast Advanced Master Mix (Thermo Fisher Scientific, USA).

2.4. Statistical analysis

The gene expression data was analyzed and compared using GENex v.s 6 software (MultiD, Sweden).

3. Results

3.1. Tumor cells and cells of endometrial origin discrimination using the 3D microscopy

For endometrial cells of epithelial origin, the process of

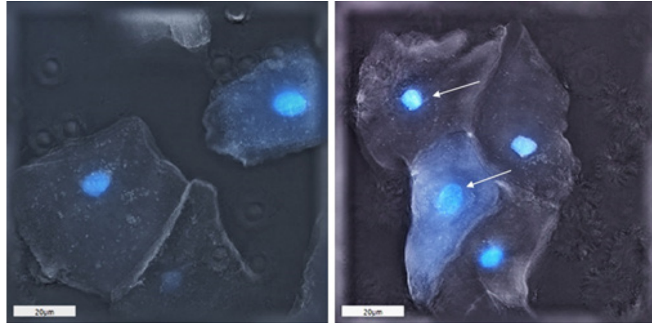

Fig. 1. Endometrial cells 3D morphology during the decidualization process visualized by NANOLIVE®. The cells on the figure are displaying features of an epithelial and endometrial origin. For the epithelial endometrial cells, a process of decidualization is typical. It is characterized by decreasing nuclear size (see arrows).

decidualization is one of the main distinguishing epithelial features. This process is accompanied by the loss of large nuclei. During *in vitro* culture, epithelial nuclei shrink over time, as shown in Figure 1. The process of shrinking nuclei size is not present in epithelial tumor cells; on the contrary, tumor epithelial cells are characterized by enormously large nuclei with increasing size and number of nuclei. Analysis by 3D microscopy allowed to show some hidden details of the morphology of endometrial epithelial cells (Fig. 1).

3.2. Tumor cells and cells of endometrial origin discrimination using fluorescent 2—NBDG glucose uptake rate.

2-NBDG is a fluorescent glucose analog that has been used to monitor glucose uptake in viable cells. 2-NBDG was uptaken into the cancerous cells during short incubation period (< 15 minutes), whereby the endometriosis-associated cells were not significantly stained even after 2–24 hours incubation period (Fig 2). 2-NBDG fluorescence typically displays excitation/emission maxima of ~ 465/540 nm and can be visualized using optical filters designed for fluorescein.

3.3. Tumor cells and cells of endometrial origin discrimination using mitochondria visualization by Mitotracker H_2ROS

The mitochondria found in endometriosis cells are specific to individual cell subtypes, and we show for the first time how the mitochondrial network can help distinguish between epithelial, stromal, stem and glandular cells of ectopic endometrial tissue. Stromal endometrial cells and their mitochondria are shown in detail in Figure 3 (Figure 3 A, B, C). These cells are characterized by a dense and branched mitochondrial network; the mitochondria are fused and form a "spaghetti-like" network accompanied by peroxisomes. If mitochondrial modifiers (e.g. piracetam) are added to the culture, the mitochondrial network becomes even more pronounced. A single standing (fission) mitochondrion is typical of endometrial stem cells.

3.4. Gene expression analysis data characterizing metabolism of disseminated endometriosis cells

The enriched DECs were cultured *in vitro*. Four main morphological cell types were observed in culture: most commonly stromal endometrial cells (Fig. 3A), epithelial cells, stem cells and blood cells (leukocytes). For each of these cell groups, we expected different markers of gene expression. During the culturing of DEC over time (from day 0 to day 3, Fig. 4), the subset of IL1B-producing cells appeared to be rapidly reduced, so that after 3 days of culturing, only a minimum of IL1B-expressing cells are among the surviving cells. The IL1B gene can be thought

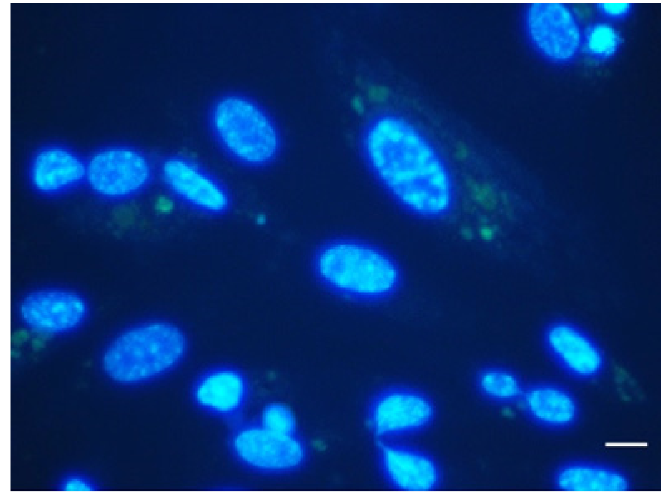

Fig. 2. Staining by fluorescent 2-NBDG in disseminated endometrial cell *in vitro* cultures (DECs) is shown. The fluorescent staining reflecting glucose uptake (shown in green) is very weak even after prolonged incubation period (2 hours), if compared to the cancer cells (staining period < 15 min). Bar represents 10 µm.

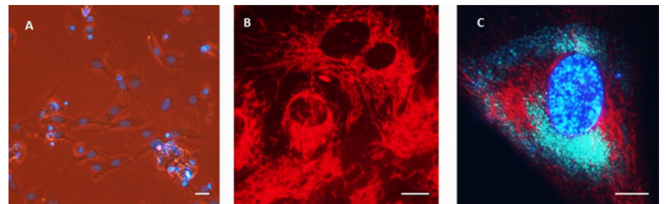

Fig. 3. Disseminated endometriosis cells *in vitro* culture (DECs)– A. Stromal cell subtype is present in majority of the *in vitro* cultured cells B. Stromal cells display a significant mitochondria activity, shown by red fluorescence in heavily fused mitochondria, which became more prevalent after piracetam addition C. Stromal endometrial cells are surviving in vitro culture conditions probably thanks to an enormous peroxisomal activity (light green organelles). Bar represents 10 µm.

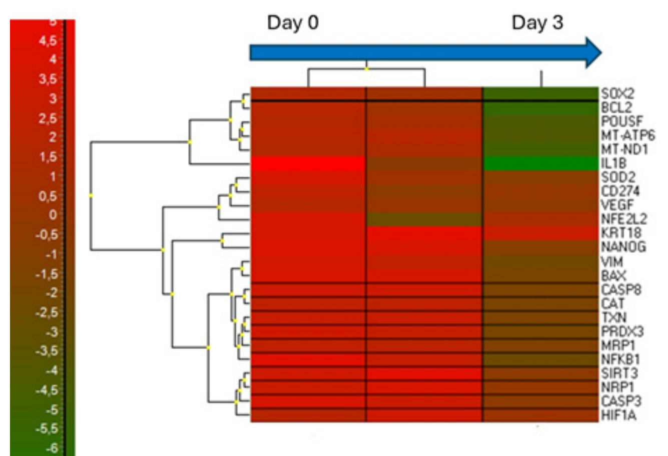

Fig. 4. Cluster analysis of gene expression profiling of disseminated endometrial cells (DECs) *in vitro* culture under different conditions in time (Day 0-Day 3) is shown. Please follow the blue arrow direction from the day 0 to the day 3.

of as a factor that assigns cells with a certain immune, proinflammatory potential. Cells that survive *in vitro* express much more keratin 18 on day 3 than on day 0, which shifts them more toward an epithelial-like character.

The surviving cells have less active mitochondria, which is shown by decreased MT-ATP6, MT- ND1 and BCL2 mRNA levels. There are also less stem cell-like cells found among the DECs survivors after the 3 days *in vitro* culture. The rapid drop of the stem cells is described by lowered expression of POUSF, SOX2 and NANOG.

Apoptosis is not activated in resting cells as CASP3 and CASP8 are relatively low. Interestingly, the level of NFE2L2 was significantly increased. NFE2L2 belongs to the erythroid transcription factor family and one would expect some of these factors to be elevated in endometriosis, which is principally caused by dysregulation of the uterine mucosal blood supply. SOD2 gene expression is also increased. The SOD2 gene product should be able to scavenge mitochondria of reactive oxygen species (ROS).

In summary, there is increased expression of genes associated with epithelial and erythroid cell character in *in vitro* culture of DECs, with a significant reduction in stem and immune cells.

3.5. Gene expression analysis data characterizing metabolism of primary endometriosis tissue (PT) and disseminated endometriosis cells

The main objective of gene expression profiling of endometriosis tissue was to identify some markers present in endometriosis cells. These markers could then be used in tests for CECs. As such, keratin 18 was significantly elevated in endometriosis tissue cultures and DEC when compared to primary tumor tissue (lung and kidney cancer). Keratin 18 mRNA was elevated in the endometriosis samples tested, so it could be used to identify endometriosis cells in the future.

A significant portion of IL1B and NANOG was expressed in the primary endometriosis tissue too, whereby NANOG was surprisingly decreased in the tested primary tumors of lung in general.

From the gene expression analysis reported in Figure 5 we may conclude, that the mitochondria are much more active in primary tumors if compared to the endometriosis tissue (see gene MT-ND1, MT-ATP6, SOX2). Primary tumor tissues are more hypoxic than endometriosis tissue as seen by HIF1A expression. There is significantly increased level of NFE2L2 and VEGF in primary endometriosis tissue.

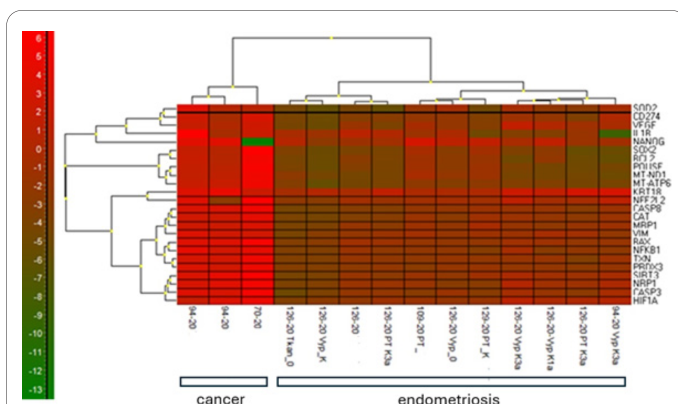

Fig. 5. Cluster analysis of gene expression data for primary endometriosis tissue (PT), and disseminated endometrial cells (DECs) in culture under different conditions is shown. The data is shown in comparison to the cancer cells of primary tumors of different origins (samples No. 70-20, 94-20). The amount of MT-ND1 is significantly higher in cancer tissue if compared to endometriosis samples. Similarly, elevated expression of KRT18 is elevated in the endometriosis samples after culture.

4. Discussion

The results presented here provide detailed information on mitochondria-related metabolic changes during cell proliferation, either from primary endometriosis tissue and/or primary tumors, based on qPCR and cytomorphological analysis. All migrating cell clones (CES/ DEC/ CTC) were shown to be energetically extremely well supported by a highly developed and branched mitochondrial network that is built from fused mitochondria. The data presented here show for the first time how plastic changes in the mitochondrial network can be and how cells can reflect them at the level of gene expression.

In cases where rapid diagnosis is needed, 2-NBDG glucose could be used to enrich cell populations based on size to distinguish between cancer and non-cancer cells. An experienced cytopathologist could define cancerous and noncancerous cell clones within 15 minutes using simple (2-channel) fluorescence microscopy assessing glucose uptake, nuclear morphology, and mitochondrial activity. The results are even more reliable when 2-NBDG glucose is used in combination with modulators of mitochondrial function, e.g. piracetam. Piracetam appears to significantly enhance mitochondrial function, so that glucose then reaches the cancer cells more quickly. Similar results are obtained using mitochondrial staining (e.g., Mitotracker H_2 ROS) to detect mitochondrial activity via real-time ROS production *in vitro*.

Based on the gene expression results, we need to discuss the changes in gene expression of stem and stromal cell markers in the culture of DECs in vitro. DECs were grown *in vitro*, and the results of gene expression analysis show that cells migrating from primary endometriosis tissue have a very interesting gene expression profile indicating a preferred survival similar to stromal cells in addition to special confluent mitochondria. The stromal cell character is manifested by increased KRT18 expression. The excessive amount of stem cells detected in primary endometriosis tissue decreases at the time of culture, and based on gene expression data, stem cell-like genes (POUSF, SOX2, NANOG) are not present at high levels in *in vitro* cultures of DEC.

In summary, there is increased expression of epithelial- and erythroid-associated genes in *in vitro* cultures of DECs, with a significant reduction in stem and immune cell-like cells. These facts could support the consideration of new drugs used to combat these processes.

All the data presented should support new approaches to differential diagnosis in trials when women of reproductive age are admitted to hospital for spontaneous pneumothorax due to unknown causes. A diagnosis of cancer could be excluded in the first instance if the nucleus of the captured cells is smaller than 15 μm and if the uptake of 2-NBDG of the isolated cells is too slow (more than 2 hours). However, much more is needed to confirm an endometrial cause of spontaneous pneumothorax. For this purpose, the increased expression of KRT18 and NFE2L2 should be analyzed as a discriminative marker in a con-

firmatory clinical study.

Acknowledgements

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Conflicts of interest

The authors confirm that there are no conflicts of interest.

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