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IN SEARCH OF FACTORS IN ENDOMETRIOSIS PERITONEAL FLUID THAT DECREASED DECIDUALIZATION PROCESS

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Abstract

Endometriosis is determined by local and systemic proinflammatory dysregulation and this reason directs our attention to search the specific marker molecules in endometriosis perithoneal fluid (ePF). This disease affects up to 10% of the women of reproductive age and accompanies infertility. In this study, we analyzed the influence of ePF onto decidualization process by measuring the secretion of Prolactin. Since we have found that ePF inhibits decidualization of human endometrial stromal cells (HESCs), we used 2D-PAGE separation to compare the protein profiles of two types of PFs – endometriosis and control non-endometriosis fluid, and to identify the most significant for endometriosis protein spots. Using a specific software for semi-quantitative analysis and bioinformatics, we identified possible key gene ontology classified pathways – toll-like receptor pathway, NFκB cascade pathway, cell surface receptor linked signal transduction pathways, in which these proteins are involved, and might elucidate our knowledge on the pathogenesis of this disease.

Key words: endometriosis, decidualization, 2D-PAGE, geneontology

Introduction. Endometriosis is a common, benign, estrogen-dependent, chronic gynecological disorder associated with pelvic pain and infertility. It is defined as the presence of endometrial glands and stroma outside the uterus, mainly on the pelvic peritoneum, on the ovaries, and in the rectovaginal septum [1]. The disease affects up to 10% of women of reproductive age [2]. Although endometriosis and normal eutopic endometrium might be similar in histology,

many differences in gene and protein level expression, steroid pathway, cytokine signalling were found to differ [3]. So far, the precise etiology of endometriosis remains unknown, with the most widely accepted pathogenesis theory being the Sampson's one [4, 5] suggesting that the disorder might originate from retrograde menstruation of viable endometrial cells through the fallopian tubes into the peritoneal cavity where it implants on the peritoneal surface or pelvic organs.

Numerous investigative efforts provide evidence that endometriosis develops as a result of specific molecular characteristics of the peritoneal environment and in particular of peritoneal fluid (PF) [6–9]. Since the peritoneal fluid reaches the site of human oocytes fertilization, it could contribute to this process outcome [10, 11]. Logically, therefore, changes in the characteristics might affect natural conception.

The mammalian decidua is a highly-specialized structure and develops after trophoblast invasion triggered differentiation of the endometrial stromal cells, having the major function to guarantee the best conditions for embryo implantation and placentation [12].

The present study investigates whether endometriosis peritoneal fluid might violate normal endometrial stromal cell decidualization in an in vitro model, and seeks for target factors playing a role in it.

Materials and methods. All biological materials (PFs, tissues) were collected after receipt of written informed consent from the patient, according to the requirements of the Ethics Committee of the Second Municipal Hospital for Obst/Gyn "Sheinovo" PLS, Sofia, Bulgaria and Ob/Gyn Hospital "Dr. Shterev".

Peritoneal fluid collection. Peritoneal fluid samples were collected from women with endometriosis and control subjects who underwent surgery laparoscopy for infertility or tubal sterilization. Briefly, all visible PFs were aspirated from the cul-de-sac and the vesico-uterine fold at the beginning of laparoscopy and the samples were mixed before centrifuging at 600 g for 10 min; the supernatant was collected and stored at $-80\,^{\circ}$C until assayed.

Human endometrial stromal cells (HESCs) – isolation procedure, cell culture conditions and in vitro decidualization. Samples of human endometrial stromal cells were collected from healthy women aged 27–32 years who had undergone hysterectomies. Each sample was kept in sterile phosphate-buffered saline (PBS, pH = 7.4) supplemented with 0.1% bovine serum albumin (BSA, Austria). In the laboratory, HESCs were isolated from endometriotic tissues by enzymatic digestion (0.5% collagenase) and the dispersed cells were nylon mesh filtered, washed, and seeded in culture dishes. The non-adherent cells were washed out after 24 h of incubation at 37 °C, as the culture medium was refreshed every second day.

Decidualization of HESCs. The cells were seeded in 24-well plates (Orange Scientific, Belgium) at a concentration of 1×10^4 cells/cm^2. When the cells reached 80%–90% confluence, an induced decidualization was initiated using

steroid hormone supplemented media (Sigma): 17-β-estradiol (10^{-8} M), Medroxyprogesterone (10^{-6} M) and 8-bromo-cyclic adenosine monophosphate (8-bromo-cAMP) (0.5 mM) in 2% FBS–DMEM, and it was maintained for 10 days, with culture medium being collected every 48 h. Successful decidualization was validated by monitoring the prolactin secretion and endometrial cell morphology. Prolactin concentrations were measured with the Access Prolactin Kit and Access Immunoassay System (Beckman Coulter, CA). The sensitivity of the test is 0.25 ng/ml and precision $< 10\%$ coefficient of variation. In the test group, decidualized cells were supplemented with PFs from non-endometriotic and from endometriosis patients. The control group of endometrial cells was cultured with no hormone supplement media, and control decidual cells were cultured in hormone supplemented media, without PFs added.

Two-dimensional gel electrophoresis. The PF samples (ePF, $n = 6$ and nPF, $n = 6$) proteins having an isoelectric point (pI) ranging from 4–7 and molecular weights (Mr) ranging from 20 to 193.5 kDa were separated using 2D-PAGE approach, combining isoelectric focusing as 1st dimension and SDS–PAGE as 2nd dimension. All protein samples standardized to 65 µg (pH = 8.5) were loaded into Precast IPG Immobiline Dry Strips (pH = 4–7, 13 cm, GE Healthcare). Following the manufacturer's procedure, the strips were subjected to isoelectric focusing using Ettan IPGphore 3 (GE Healthcare). The focused strips were buffer equilibrated, prepared and subjected to 2nd dimension separation by SDS–PAGE, where the proteins were transferred to a 12% polyacrylamide gel in standard dual cooled vertical unit (SE 600 Ruby; GE Healthcare), without using stacking gel. Analytical gels were silver-stained using ProteoSilverPlus Stain Kit (Sigma) and digitalized at 300 dpi resolution.

Analysis of protein expression. Protein gel spots differential analysis was done using ImageMaster 2D Platinum 7.0 (GE, Healthcare) software. Gels were compared for differences in protein expression by using the normalized percentage volume of the spots (%Vo) = (spot volume/volume of all spots in the gell)*100. The MW and pI of each spot cluster were estimated using MW marker and isoelectric focusing strip pI values.

Bio-informatics statistical analysis. The protein expression variations among the gels were evaluated using gel matching. The analytical methods used included scatter plots, descriptive statistics, histograms and factor analysis.

Database searching. The putative protein targets corresponding to the individual protein spots (defined by their pI, MW and %Vo) on the gel were identified using the ExPASy tool Tagident. The putative protein IDs were filtered and Gene Ontology (GO) network enriched using the Functional Annotation Tool of DAVID system.

Results and discussion. Using Medroxyprogesterone (10^{-6} M) in combination with 17-β-estradiol (10^{-8} M) and 8-bromo-cAMP (0.5 mM), we were able to induce decidualization of human endometrial stromal cells in vitro. Ten

days after the initiation of decidualization process, morphological changes in our endometrial stromal cell cultures were easily detected, with demonstrated multilayering, and increased vacuolization, mimicking the in vivo decidual transformation (Fig. 1*A*, *B*). During the decidualization phase, endometrial stromal fibroblasts undergo mesenchymal-to-epithelial transformation, differentiating to rounded, large epithelioid-like or polygonal, secretory decidual cells [13–15]. Decidualization has recently been found to attenuate the contractility of HESCs [16], and the attachment to extracellular matrices to be enhanced in human endometriosis stromal cells [17]. The functional modulation of the decidualized stromal cells was characterized by their Prolactin (PRL) secretion. To trace the influence of ePF onto the decidualization process, 3 different concentrations of hormone-supplemented media-added ePF and nPF were used: 1%, 5% and 25%. As shown in Fig. 1*C*, there was a decrease in the secretion of Prolactin during treatment with ePF compared with nPF and the other two control groups – control endometrial stromal cells (treated only with 2% DMEM), decidual control cells (treated with steroid hormones and cAMP in 2% DMEM). These results clearly demonstrate that the decidualization process in HESCs was inhibited in the presence of ePF. On the other hand, the level of secreted Prolactin was highest at 25% ePF. This fact explains the presence of a large amount of growth factors normally presented in the peritoneal environment.

These findings have directed our attention to seek for factors in the ePF that could impede normal decidualization, resulting in infertility of patients with endometriosis. For that purpose, 2D-PAGE approach was chosen to identify the specific proteins that could be differentially present only in PFs of endometriotic women, therefore the protein profiles of PFs from both groups of patients were compared (Fig. 2). The mean number of PF protein spots per gel was approximately equal (357 ± 30). No protein spot was consistently ($\geq 90\%$ of the case) present in PF of women with endometriosis and absent in PF of control subjects. Similarly, no protein spot was uniquely present in PF of control subjects. Totally, 74 protein spots were consistently present in PF of women with and without endometriosis. Scatter plot for matched spots was produced by ImageMaster Platinum to analyze for disparities in spot stain intensity on the 2 gel groups. The linear dependence relationship between the spot values on endometriosis gel vs reference non-endometriosis gel was assessed using best fit-line through the data points, with correlation coefficient $\mathrm{Corr} = 0.58$ (Fig. 2).

Endometriosis-specific sample spot population was distinguished using Factor Analysis (FA) applied on the densitometry and morphological parameters of the detected spots. The Factor Projection Plot displays the projection of each protein spot match ID (cross) and each gel (blue vector) on the two factorial axes, showing the relationships between endometriosis and non-endometriosis gel spot populations in terms of associations with specific spot patterns. The factors are ranked in order of importance, the first ones generally being the best for

characterizing the gels and matches that behave similarly. The most significant matches are displayed on the projection plot, as further away a spot is from the origin, the more important it is likely to be in terms of characterizing the gels. The endometriosis-specific protein expression profile was determined by the spots: 29, 36, 70 and 56, while the non-endometriosis determining spots were: 7, 6, 66, 65 (Fig. 2). The coordinates of each of the gels are projected on the axes and are listed in the table, with the first axis generally correlating with the protein abundance, and the second axis related to the ratio between the mean spot values in each gels population. The matches at the top of the table are those with the highest relative volumes (Fig. 2).

For the selected spot matches (IDs), the pI and MW of the putative proteins corresponding to the individual protein spots were subsequently identified using the ExPASy Tagident tool. The retrieved lists of proteins (match No 29 – 94 proteins, No 36 – 23 proteins, No 70 – 63 proteins, No 56 – 14 proteins) were further subjected to protein ID filtering (exclusion of protein ID ambiguities) and Gene Ontology Networks Enrichment Analysis using the Functional Annotation Tool of DAVID system. The GO terms membrane receptor, toll-like receptor pathway and NFκB pathway were significantly enriched in the FA-derived putative protein target set union.

We demonstrated that decidualization process of HESCs inhibits their Prolactin secretion in the presence of ePF suggesting that ePF could possibly impede the normal decidualization, eventually resulting in infertility in endometriosis patients. An integrated 2D-PAGE semi-quantitative densitometry, spot match Factor Analysis and Gene Ontology Network Enrichment, allowed for retrieval of series of differentially-regulated pathways in ePF that might have an impact on the development and establishment of endometriosis or might be a subject of differential regulation due to endometriosis itself.

In addition, the aberrant integrin expression [18] involved in adhesion interactions may trigger the cell surface receptor-linked signal transduction pathways, which we found to be enriched for subsequent cell proliferation, differentiation and invasion that are necessary for disease progression.

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