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DFF45 expression in ovarian endometriomas

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ABSTRACT

Objective: Endometriosis, defined as a spread of endometrium outside the uterus cavity, affects up to 30% women of reproductive age, with the ovaries being its most common localization. In the ectopic lesions, endometrial cells show abnormal proliferation and impaired apoptosis. The DNA destruction during apoptosis is a direct result of activation of the DFF40/DFF45 complex. DFF40 (DNA fragmentation factor of 40 kDa) is responsible for direct DNA fragmentation while DFF45 (DNA fragmentation factor of 45 kDa) acts not only as a DFF40 inhibitor, but also as its chaperone. Therefore, the presence of DFF45 is required for proper DFF40 synthesis. The aim of this study was to determine the DFF45 level in human ovarian endometriosis.

Study design: The endometriosis samples were collected from 43 affected women, while the 81 normal endometrial specimens were obtained from the control group. Western blot and immunohistochemistry tests were used to determine the DFF45 level in examined tissues.

Results: The expression of DFF45 in normal human endometrium and ovarian endometriosis was confirmed using both the Western blot and the immunohistochemistry tests. In normal eutopic proliferatory endometrium, a lower DFF45 expression was observed compared with secretory endometrium, while no cyclic changes in DFF45 expression were observed in the ovarian endometriomas. In the normal eutopic endometrium, stronger DFF45 staining was noted in the endometrial glands in comparison to the stroma, irrespective of menstrual cycle phase. However, in the ovarian endometriosis no difference between the glandular layer and stroma in DFF45 immunoreactivity was appreciated. The lowest level of DFF45 was observed in ovarian endometriosis when compared with both normal eutopic proliferatory and secretory endometria using the Western blot and immunohistochemistry analysis.

Conclusions: A decreased level of DFF45 observed in ovarian endometriosis may be a part of an apoptosis-resistant mechanism enhancing the disease progression.

1. Introduction

Endometriosis is defined as the spread of endometrial tissue beyond the uterus cavity. The disease affects up to 30% women in their reproductive years and it is most commonly localized in the ovaries [1]. Moreover, progression from endometriosis through the atypical endometriosis to ovarian cancer is possible [2]. The first hypothesis of endometriosis was presented in 1909 by Meyer who suggested that peritoneal mesothelial cells have the ability to differentiate into Muellerian-type epithelium under the influence of nonspecific stimuli [3]. However, the most well-known theory was presented by Sampson in 1925 and implied that the cause of endometriosis is a reverse regurgitation of exfoliated endometrial tissue via the Fallopian tubes during menstruation. Godal and then Blumenkrnz confirmed that during menses endometrial cells can be found in the peritoneal cavity in 50–80% of women, however, only a few number of those women eventually developed endometriosis [4,5]. In women with endometriosis, Dmowski observed an impaired natural killers (NK) activity and decreased ability of cytotoxic T lymphocytes (CTL) to induce apoptosis in the endometrial lesions [6].

During the menstrual cycle, changes are present in the apoptotic index of endometrial cells and the apoptotic peak is observed at a late secretory phase [7]. In the endometrial lesions, cells show abnormal proliferation and impaired apoptotic regulation. Spontaneous apoptosis in endometriomas is significantly reduced and the cyclic pattern of tissue apoptotic activity is lost [8] as compared with the eutopic endometrium.

In normal eutopic endometrium, apoptosis is induced by both the receptor pathways, which involves the main pro-apoptotic factors such as Fas ligand (FasL) and Tumor Necrosis Factor α (TNFα), and the mitochondrial cytochrome c release, that together lead to an activation of caspases, finally resulting in DNA fragmentation lead by DNA Fragmentation Factor (DFF) [9].

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DFF is a dimer consisting of 40 kDa subunit (DFF40/CAD) with nuclease activity and 45 kDa subunit (DFF45/ICAD), which acts as a DFF40 chaperone and is a substrate for caspase-3. After DFF45 cleavage, the DFF40 is released and joins H1 histone, causing DNA fragmentation [10]. In viable cells there is a stoichiometric 1:1 ratio of DFF40:DFF45 and DFF45 is required for proper DFF40 synthesis. Thus, DFF45 acts not only as an inhibitor but also as a chaperone allowing for the DFF40 to properly fold and gain nuclease activity [11]. In-vitro studies revealed the crucial role of DFF45 in apoptotic process of mice's DFF45-deficient tymocytes which were resistant to apoptosis [12]. Similarly DFF45-deficient human fibroblasts were resistant to TNFα induced apoptosis [13].

2. Materials and methods

2.1. Patient enrollment and samples collection

The study group consisted of 43 women with diagnosed endometriosis confirmed by the histopathological examination. Endometriosis was graded according to the guidelines of the revised American Society for Reproductive Medicine (rASRM) Classification [14]. The control group consisted of 81 endometriosis-free, premenopausal, multigravid women treated for uterine fibroids and ovarian simple cysts. Inclusion criteria were as follows: women, age 18 years or above, and without hormonal treatment within the past 6 months. Pregnant, postmenopausal and women with a suspicion of neoplasm were excluded from the study. During surgical procedures 27 samples of ovarian endometriosis and 44 samples of normal eutopic endometrium were collected for the Western blot analysis. Laparoscopy was performed in 48 cases, laparotomy in 15 cases and eight patients underwent diagnostic hysteroscopy. Additionally, 16 archival paraffin-embedded endometriotic specimens and 37 endometrial specimens were obtained from the Department of Pathomorphology of the Jagiellonian University for immunochistochemical analysis. The study was approved by University Review Board and informed consents were obtained from all the participants.

2.1.1. Ovarian endometriosis samples

Surgical treatment of ovarian endometrial cysts was performed via their enucleation by laparoscopic approach, or in case of very small foci, by their electrocoagulation after taking samples for Western blot analysis. Presence of endometriosis was confirmed during surgery and afterwards by histopathological examination.

2.1.2. Endometrial samples

Forty-four samples of eutopic endometrium were derived from fertile women without endometriosis. The indications for surgical intervention were the following: (1) uterine myomas, (2) ovarian dermoids, (3) pelvic pain syndrome, and 4) suspicion of abnormal uterine cavity shape (all excluded after hysteroscopy). Tissue samples were classified according to the menstrual cycle phases based on the result of hystopathologic examination as proliferatory and secretory endometrium.

2.2. Preparation of tissue extracts and Western blot methods

Tissue samples of 0.5 cm × 0.5 cm × 0.5 cm dimensions were frozen immediately after collection and stored at the temperature of minus 20 °C. For DFF45 detection, mouse monoclonal IgM-class antibodies were used initially, and then rabbit anti-IgM chain specific antibodies in IgG class were applied. Structural cell protein β-Actine was used as a control to confirm the equal protein load in the collected tissue samples. The membranes used for DFF45 detection were also used for β-Actine detection using anty-β-Actine mouse antibody following by anti-mouse-IgG-AP antibody according to the same protocol. The methodology of Western blot analysis was described precisely in our previous publications [15,16].

2.3. Immunohistochemistry

Five micrometer sections were stained to visualize DFF45 expression by applying Envision method using Dako Autostainer. 100 μg of primary monoclonal anti-DFF45 rabbit antibodies (Abcam Co, Cambridge, UK) were used in 1:75 dilution with Tris–HCl buffer (DakoCytomation) according the manufacturer's instructions. Subsequently, the slides were incubated at room temperature for 30 (±1) min. Visualization of reaction products was performed using AEC (3-amino-9-ethyl-carbazole) as a chromogen (AEC Substrate Chromogen) at room temperature for 8 (±1) min. DFF45-positive cells were counted independently by two histopathologists in 10 high power fields (hpf) of each slide. According to manufacturer's guidelines a colon carcinoma sample was used as a positive control. For the negative control, the same specimen and method were used as for the positive one, but without the primary antibody. Every staining was considered as a positive result of immunochemistry. According to the number of positive cells, the staining was semiquantitatively evaluated as follows: (0) expression in up to 10% of the cells, (l+) expression in 10–50% of the cells, (2+) expression in 51–80% of the cells and (3+) expression in more than 80% of the cells.

2.4. Statistical analysis

Using the Shapiro–Wilk test, the distributions of variables in the examined groups of women were analyzed. Clinical features of the study group and control group were compared using parametric Student t test and non-parametric Mann–Whitney *U* test as appropriate. Multivariate analysis of variance (MANOVA) was used to identify factors that might have an influence on DFF45 expression. Differences between tissue specimens were evaluated using Kruskal–Wallis analysis of variance, with post hoc test used when appropriate as distribution of analyzed variables differed from the normal ones. Clinical features were shown as mean values ± standard error (SD). Data from Western blot analysis were presented in arbitrary relative units (U) as medians ± standard error of the mean (SEM) and the results of immunohistochemistry tests were shown as the number of DFF45-positive cells at each level. A p value of 0.05 was accepted as statistically significant. All calculations were carried out with the use of STATISTICA software v. 8.0. (StatSoft, USA, 2007).

3. Results

3.1. Characteristics of the study subjects

In the study group, the early stage of endometriosis (stages I and II) was diagnosed in 15 women and advanced stage of the disease (stages III and IV) was recognized in 28 women. There were no significant differences between the study and control group concerning the mean age of the first menstrual period (±SD) [13.2 (±1.5) years vs. 14.7 (±2.4) years; $p = 0.074$], the mean length of the menstrual cycle (±SD) [28.1 (±2.2) days vs. 28.6 (±2.4) days; $p = 0.296$], and the parity [(nulliparous/primiparous/multiparous) 8(18%)/21(48%)/15(34%) vs. 11(14%)/24(30%)/46(56%); $p = 0.253$]. The women with endometriosis were significantly younger [the mean age (±SD): 34.5 (±7.0) years vs. 42.9 (±7.9) years; $p < 0.001$] than disease-free participants, had shorter periods [the mean length of menstrual bleeding (±SD): 4.3 (±0.6) days vs. 4.5 (±0.6) days; $p < 0.001$] which were often painful [painless/painful periods: 20(45%)/24(55%) vs. 58(72%)/23(28%); $p = 0.025$].