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Anti-apoptotic activity in deep pelvic endometriosis

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Summary. Since endometriosis is a proliferative disease we evaluated the presence of anti-apoptotic factor (Bcl-2) and pro-apoptotic factor (Bax) in deep pelvic endometriosis. A Cross-sectional observational study was performed at Santa Casa de Misericórdia de São Paulo, São Paulo, Brazil. Forty women aged 26 to 46 years with deep endometriosis were selected. They had not been clinically treated for at least 3 months prior to surgery and then underwent surgical laparoscopy to treat the disease. During the surgery, tissue was collected from the uterosacral ligaments and the rectosigmoid; an endometrial biopsy was also performed as a control. All interventions were performed by the same surgeon. The specimens were sent for pathological and immunohistochemical analyses; endometriosis was confirmed in all patients. After the immunohistochemical reaction a semi-quantitative evaluation of the staining intensity (relative optical density-ROD) was conducted, applying the digital densitometric analysis system. In the uterosacral ligaments 97.5% of the specimens were positive for Bcl2 whereas in the rectosigmoid 100% were positive. In the endometrium we observed that 87.5% were positive for Bcl2. BAX expression was null in the rectosigmoid and in the endometrium. In the uterosacral ligaments 2.5% of the specimens expressed BAX. The relative optical density of Bcl2 was higher in the rectosigmoid and in the uterosacral ligament when compared to the endometrium, 0.141±0.002; 0.129±0.001, respectively ($p<0.01$). We concluded that the anti-apoptotic factor Bcl-2 was expressed in all studied specimens, but in a higher staining intensity in the rectosigmoid and in the uterossacral ligaments in comparison to the endometrium. The pro-apoptotic factor Bax had virtually no expression in the studied tissues.

Key words: Tissue microarray, Bcl-2, Immunohistochemistry, Statistic

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Introduction

Endometriosis affects approximately 10% to 15% of women during their reproductive life (Ballweg, 2004). The lesions often infiltrate the uterosacral ligaments (Chapron et al., 2006; Ribeiro et al., 2006) and invade the retrocervical tissue (Koninckx et al., 1991) and the rectosigmoid in more than 50% of patients in advanced stages of the disease (Cornillie et al., 1990; Chapron et al., 2003). Deep lesions may be treated by resecting the affected segment (Redwine et al., 1996; Ribeiro et al., 2006).

Apoptosis is a physiological process that maintains the balance between cellular proliferation and programmed cellular death (Kerr et al., 1972) and it allows for cell death without inducing an immune response or inflammatory reaction (Garcia-Velasco et al., 2002) It is characterized by the control of cellular activity through induced cell death after a specific stimulus.

When endometrial cells penetrate the pelvic cavity, they are programmed for cell death (apoptosis) and may trigger an intense inflammatory reaction and attract a large number of cells from the immune system to the pelvic region (Harada et al., 1996). Nevertheless, the expression of apoptotic proteins varies according to the location of the endometriosis, which suggests the involvement of different apoptotic pathways (Matsumoto et al., 1999). The hypothesis that the reduction of apoptosis in both the endometrial cells and the immune cells of the reproductive system contribute to the pathogenesis of endometriosis, has been studied with different results (Meresman et al., 2000; Harada et al., 2004).

In order to evaluate partially the apoptotic activity in endometriotic tissue the objective of this study was to evaluate the presence of anti-apoptotic factor Bcl2 and pro-apoptotic factor Bax in deep endometriosis involving the uterosacral ligament and the rectossigmoid using as a control these women's endometrium.

Materials and methods

Patients

A prospective cross-sectional study was performed with the approval of the Research Ethics Committee of the ISCMSP (IRB#392/07), Brazil. During recruitment, 170 patients with a diagnosis of pelvic endometriosis were diagnosed and treated. Forty patients with suspected deep pelvic endometriosis were selected. The patients were treated at the Gynecologic Endoscopy and Endometriosis Clinic of the Department of Gynecology and Obstetrics at the Central Hospital and Santa Isabel Hospital of the ISCMSP, from 1st October, 2007 to 31st October, 2008.

The patients were made aware verbally and by a written document of the details of the diagnosis of deep pelvic endometriosis. They were then invited to participate in the study, information about complying with the study protocol was reviewed, and consent forms were signed.

All the selected patients underwent primary surgical laparoscopy for treating endometriosis. Specimens from the uterosacral ligament and the rectosigmoid (Study Group) were obtained for pathological analysis and confirmation of endometriosis. An endometrial biopsy was performed to obtain specimens from the anterior wall of the endometrium (Endometrium Group). The endometriosis staging was determined using the revised classification of the *American Fertility Society* (1997)

The inclusion criteria were as follows: no previous surgery for endometriosis, clinical and radiological signs suggestive of deep pelvic endometriosis infiltrating the uterosacral ligaments and the rectosigmoid (Ribeiro et al., 2006), of reproductive age, i.e., between 18 and 50 years of age, with (Ribeiro et al., 2008) eumenorrheic cycles and without hormonal drug treatment or anti-inflammatory treatment for at least three months prior to inclusion in the study. Patients with a chronic disease or malignant tumors were excluded from the study. Using rigid radiological criteria we managed to find uterosacral and rectosigmoid endometriosis compromising at least the muscular layer (or submucosa/mucosa but not only the serosa) in all the selected patients.

Surgical procedure

The surgical procedure was scheduled to be done always in the post ovulatory phase of the menstrual cycle, controlling either with echography or clinical data. The main objective of the surgical treatment was to remove most of the endometriotic tissue and preserve fertility when desired (Ribeiro et al., 2006). The following criteria were used to define the specimens to be sent for immunohistochemistry and further analysis: 1) Uterosacral Ligament: the most significant endometriotic nodule (fibrotic area) closed to the uterus, either left or right side. 2) Rectosigmoid: a segmental resection of the affected area including the endometriotic nodule was performed and a 1.0 centimeter long specimen including the mucosa and the muscular layer was sent to histological analysis.

Histopathological analysis

After fixation in 10% buffered formaldehyde, the specimens were sent to the Service of Pathological Anatomy of ISCMSP. Endometriosis was confirmed in all patients by two investigators, and the tissue samples were selected for preparation of a matrix tissue arrangement, also known as tissue microarray (TMA). Negative and positive controls were included in the slides for further analisys. In all specimens from the rectosigmoid the depth of the disease matched the inclusion criteria.

Immunohistochemistry study

Immunohistochemistry was used to evaluate expression of the monoclonal antibody clones of Bcl-2 (DAKO®) and the polyclonal antibody to Bax (a synthetic protein that corresponds to amino acids 43-61 of Bax, Dako®). These antibodies were diluted at 1/200, and 1/650, respectively. The immunohistochemistry technique used in this study was performed at the Department of Anatomic Pathology of ISCMSP in compliance with the standard protocols recommended by the manufacturers for each reaction (Fig. 1).

For the qualitative analysis of the expression of Bcl-2 and Bax in the different anatomical locations evaluated in this study (endometrium, uterosacral ligament and intestine), scores 0 and 1 were both considered clinically negative, and scores 2 and 3 were considered positive, the HercepTest-Dako (Kelm Junior et al., 2008) was the used criteria .

Semi-quantitative evaluation of immunostaining using optical density

A semi-quantitative analysis measuring the relative optical density (ROD) was conducted. We applied the digital densitometric analysis system (InterFocus Imaging Ltd., Linton, England).

Statistical analysis

The results were collected and stored using the software Statistical Package for Social Sciences (SPSS®, version 16.0 for Windows). Then, using SPSS for statistical analysis, the Student t test was performed to evaluate the optical density in the diferent locations.

Results

We observed an overall positive result of Bcl2 expression in all the specimens. In the study group we observed that in the uterosacral ligaments 97,5% of the specimens were positive for Bcl2, whereas in the rectosigmoid 100% were positive. The controls in the endometrium showed positive results for Bcl2 in 87,5% of the patients. BAX expression was null in the rectosigmoid and in the uterosacral ligaments 2,5% of the specimens expressed BAX. All the controls in the endometrium were negative for BAX.

The relative optical density of Bcl2 was higher in the rectosigmoid (0.141±0.002) and in the uterosacral ligament (138±0.001) when compared to the endometrium (0.129± 0.001) (Table 1).

Discussion

Currently, a potential relationship between anti-apoptotic and pro-apoptotic factors in the pathogenesis of endometriosis is the focus of this research.

To better understand the pathophysiology of endometriosis, we studied the expression of the anti-apoptotic factor Bcl-2 and the pro-apoptotic factor BAX in tissues from women with deep endometrioses. Specific studied sites were those commonly affected by deep endometriosis: uterosacral ligament and rectosigmoid.

The immunohistochemical analysis detected the presence of the anti-apoptotic factor (Bcl-2) and the absence of pro-apoptotic (Bax) in endometriotic tissue, similar to what was previously described in the literature (Meresman et al., 2000, Braun et al., 2007). These findings allowed us to conclude that Bcl-2 expression and the absence of Bax could grant these cells decreased susceptibility to apoptosis, increased life expectancy, and therefore an ongoing disease process of endometriosis.

Both qualitative and semi-quantitative data related to the expression of apoptosis have been previously observed in cases of endometriosis involving the peritoneum, ovary and colorectum. It was found that expression of Bax protein does not change regardless of the phase of menstrual cycle or site of the disease (McLaren et al., 1997). Analysis of protein Bcl-2 expression in endometrial tissue confirmed that its expression accompanies the menstrual cycle and is increased in the proliferative phase and decreased to non-detectable levels during the second half of the

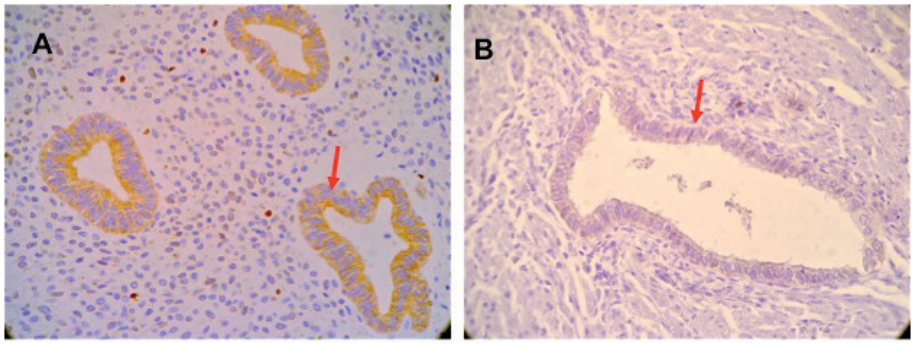

Fig. 1. Rectosigmoid strongly positive for Bcl2 **(A)** and weak staining for BAX **(B).** Red arrows in the glands. x 400

Table 1. Analysis of Bcl-2 and BAX expression (Relative Optical Density-ROD) in women with deep pelvic endometriosis.

Biopsy site	Relative Optical Density Bcl-2 (Mean± S.E. (95%CI))	Bax (Mean± S.E. (95%CI))
Endometrium	0.129±0.001 (0.127-0.132)	0.116±0.002 (0.113-0.119)
Uterosacral ligament	0.138±0.001 (0.134-0.141)*	0.117±0.001 (0.114-0.119
Rectosigmoid	0.141±0.002 (0.136-0.146)*	0.114±0.001 (0.112-0.116)

*: t test , p<0.001 in comparison with the endometrium.

secretory phase (Harada et al., 2004). In our study we confirmed a higher expression of the anti apoptotic protein Bcl-2 in the endometriotic disease when compared to the endometrium of the same patients. On the other hand, the absence of a control group including the endometrium of age/parity matched patients is a criticism to our data and is a real point of interest for further studies.

The literature supports that decreased expression of apoptotic factors in women with endometriosis, specifically in the late secretory and early proliferative phases, can lead to an increase in viable endometrial cells that are regurgitated into the pelvic cavity during menstruation, thus facilitating cell survival and ectopic implantation (Dmowski et al., 2001). It has been also discussed in the literature that apoptosis is likely to be one of the mechanisms that interfere in the development and progression of endometriosis (Hassa et al., 2009).

Our data demonstrated a high optical density of bcl-2 expression (ROD) in colorectal endometriosis compared to the endometrium of the same patient, which is in accordance with the literature and suggests a lower sensitivity of colorectal endometriosis to apoptosis (Beliard et al., 2004). Moreover, the difference in apoptosis- related protein expressions according to the locations of endometriosis could be explained by different etiopathologies (Bontis and Vavilis, 1997).

Our patients were evaluated in the secretory phase of the menstrual cycle and it was found that there was no expression of the pró-apoptotic proteins Bax.

Our data showed a high optical density of Bcl-2 expression in the fragments of uterossacral ligments when compared to the endometrium. On the other hand, BAX expression on the uterossacral ligament was almost negative and there was no difference in the imunostaining intensity (ROD) when comparing the uterosacral ligaments and the endometrium. It is important to emphasize that a relative optical density was measured in all cases, even those with *HercepTest*-Dako scores 0 and 1, allowing a semi-quantitative statistical analisys of our data in all situations.

In the literature we found that BAX expression tended to be lower in colorectal than in ovarian endometriosis. This lack of statistical significance could be explained by the small number of samples studied (Dufournet et al., 2006).

In another study an inverted bcl-2/bax ratio in ovarian endometriosis relative to peritoneal and colorectal endometriosis was found. These results agree partially with those showing a strong correlation between low bcl-2 and high bax expression in ovarian endometriosis (Goumenou et al., 2001). However, these latter authors did not analyze the expression of these proteins in other endometriotic locations. An inversed bcl-2/bax ratio in peritoneal and colorectal endometriosis compared to ovarian endometriosis was observed in the literature, suggesting that the apoptotic pathways may differ between these locations (Dufournet et al., 2006).

In our study the relative optical density analysis of Bcl-2 expression in the rectosigmoid and in the uterosacral ligament was significantly superior to its expression in the endometrium. The data support the hypothesis that, as endometriosis progresses and infiltrates deeper tissues, cells lose the capacity to control programmed cell death. The significant increase in Bcl-2 in the rectossigmoid not only reinforces this conclusion but also demonstrates the aggressive behavior of the disease in this place. Our data still lack information regarding the expression of those factors in endometrium of patients without endometriosis and also normal tissue without endometriosis from uterosacral ligament and rectum.

Endometriosis is a process that culminates with the implantation and survival of endometrial cells in the peritoneal cavity and regarding the genesis of the disease several hypotheses may be correct; therefore, more research is needed for this disease (Gualco et al., 2008). The endometrium of women with endometriosis may have fundamental differences when compared to the endometrium of women without endometriosis and further studies are needed to clarify the changes that occur in the endometrium of patients with endometriosis. These differences could contribute to the maintenance of endometrial cells that are regurgitated into the peritoneal cavity and subsequently develop into endometriosis (Hassa et al., 2009).

It was concluded that some women may have an increased susceptibility for maintaining anti-apoptotic factors and, therefore, persistence of the disease. Nonetheless, future studies are needed to better understand the apoptosis phenomenon involved in the pathogenesis of this complex disease.

Conflicts of interest: The authors declare that they have no conflicts of interest.

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