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 uterosacral 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

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 (Köninckx 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 (Mercesman 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 rectosigmoid 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.

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, 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 different 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 menstrual cycle.

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

<table>
<thead>
<tr>
<th>Biopsy site</th>
<th>Relative Optical Density Bcl-2 (Mean± S.E. (95%CI))</th>
<th>BAX (Mean± S.E. (95%CI))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endometrium</td>
<td>0.129±0.001 (0.127-0.132)</td>
<td>0.116±0.002 (0.113-0.119)</td>
</tr>
<tr>
<td>Uterosacral ligament</td>
<td>0.138±0.001 (0.134-0.141)*</td>
<td>0.117±0.001 (0.114-0.119)</td>
</tr>
<tr>
<td>Rectosigmoid</td>
<td>0.141±0.002 (0.136-0.146)*</td>
<td>0.114±0.001 (0.112-0.116)</td>
</tr>
</tbody>
</table>

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

Fig. 1. Rectosigmoid strongly positive for Bcl2 (A) and weak staining for BAX (B). Red arrows in the glands. x 400
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
reason 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 bel-
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 epiopathologies (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 uterosacral ligament was almost
negative and there was no difference in the
immunostaining 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 analysis 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 sort 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 inverted
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 rectosigmoid 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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