Apoptosis Resistance in Endometriosis

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ABSTRACT

Introduction: In a cytological analysis of endometriotic lesions neither granulocytes nor cytotoxic T-cells appear in an appreciable number. Based on this observation we aimed to know, whether programmed cell death plays an essential role in the destruction of dystopic endometrium. Disturbances of the physiological mechanisms of apoptosis, a persistence of endometrial tissue could explain the disease. Another aspect of this consideration is the proliferation competence of the dystopic mucous membrane. *Methods:* Endometriotic lesions of 15 patients were examined through a combined measurement of apoptosis activity with the TUNEL technique (terminal deoxyribosyltransferase mediated dUTP Nick End Labeling) and the proliferation activity (with the help of the Ki-67-Antigens using the monoclonal antibody Ki-S5). *Results:* Twelve out of 15 women studied showed a positive apoptotic activity of 3-47% with a proliferation activity of 2-25% of epithelial cells. Therefore we concluded that the persistence of dystopic endometrium requires proliferative epithelial cells from middle to lower endometrial layers. *Conclusion:* A dystopia misalignment of the epithelia of the upper layers of the functionalism can be rapidly eliminated by apoptotic procedures.

Introduction

Endometriosis is a multifactorial complex disease characterized by the ectopic presence of endometrial glands and stroma. It can be presented as peritoneal disease, endometriotic ovarian cysts, and/or deeply infiltrating rectovaginal endometriosis and is associated with pelvic pain, adhesion formation, and infertility. Endometriosis occurs in 30%–40% of women with infertility and is a progressive disease in 40%–50% of reproductive-aged women (Sampson 1921; Halme *et al*. 1984; Agic *et al*. 2009).

The theory that has gained most supportive evidence for the pathogenesis of endometriosis is Sampson's theory (Sampson 1921) of retrograde menstruation. Retrograde menstruation has been reported in 83% of baboons and in 70%–90% of women with spontaneous endometriosis (Blumenkrantz *et al*. 1981; D`Hooghe *et al*. 1996). The existence of endometrial cells in the peritoneal fluid has been reported in 59%–79% of women during menses or during the early follicular phase (Koninckx *et al*. 1980; Kruitwagen *et al*. 1991). According to Sampson's hypothesis (Sampson 1921), menstrual debris, refluxed

into the peritoneal cavity, contains viable endometrial cells that can implant and develop into endometriotic lesions (Cleophas *et al*. 2006).

Halme (1989) and other investigators have shown that macrophages present in the peritoneal cavity are potent producers of cytokines, such as tumor necrosis factor-alpha, interleukin-6 and macrophage colony-stimulating factor (Bauer *et al*. 1989; Cleophas *et al*. 2006; Harada *et al*. 1997; Mettler *et al*. 2004; Riese *et al*. 2004; Salmassi *et al*. 2008; Surrey and Halme 1990). They also have suggested that growth factors are involved in the control of implantation and the growth of endometrial cells outside the uterus.

Recent studies have suggested that abnormalities in the regulation of specific genes are involved in the development and in the pathogenesis of endometriosis (Kao *et al*. 2003; Mettler *et al*. 2007; Ota *et al*. 2000; Sharpe- Timms *et al*. 1998; Tsudo *et al*. 2000).

Endometrium is divided into the superficialis or functionalis layer, which undergoes cyclic shedding, and the basalis layer, which is permanent. Endometrial tissue

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from the functionalis is subjected to a proliferative process highly regulated by hormones throughout the menstrual cycle. At the time of menstruation, it becomes necrotic and hypoxic and is shed. In addition, apoptosis seems to be an important biologic process involved in the cyclic remodelling of the endometrium (Be`liard *et al*. 2004; Hopwood and Levison 1976). It is well known that menstrual fragments are composed of both necrotic and living cells (Keetel and Stein 1951; Bartosik *et al*. 1986)

Cytologically endometriosis lesions show few granulocytes and cytotoxic T cells. Due to this observation, we posed the question of whether programmed cell death processes with a regular destruction of the dystopian endometrium plays an essential role in this disease. Disturbances of these physiological apoptosis mechanisms could induce the persistence of the endometrial tissue and support endometriosis. Another aspect of this consideration is the proliferation competence of the dystopic mucous membrane. While the upper functional layers have no significant proliferative activity, epithelial cells from deeper endometrial layers show a considerable ability to proliferate. In order to analyze quantitatively these relationships in 15 endoscopic excised endometriosis nodules, the frequency of the apoptotic mucosa membrane epithelia cells were determined with the help of the terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) technology in this study.

The proliferation activity was determined with help of the Ki-67-Antigens under employment of the monoclonal antibody Ki-S5. This protein is expressed in all cell cycle phases S, G2, M and G1, but not in G0 (Kreipe *et al*. 1993; Rudolph *et al*. 1993; Salmassi *et al*. 2000).

Materials and methods

Tissue samples

Samples of ectopic endometrium (endometriosis, n = 15) and eutopic endometrium (n=3) were obtained from patients undergoing laparoscopy for the treatment of endometrioma and diagnostic hysteroscopy, respectively.

The patients ranged in age from 25-40 years and none of them received hormonal treatment prior to their surgery. For the investigation of apoptosis and proliferation, these samples were processed as follows:

•   Cryostat sections were prepared and stained with hematoxilin-eosin.

Eectopicendometrium were subjected to histopathological assessment which confirmed their site of origin, i.e. proliferative endometrium and endometrioma cyst wall, respectively.

•   The tissue samples were placed in a fresh 4% paraformaldehyde solution in phosphate-buffered saline, pH 7.4. To provoke a homogenous coloring, larger tissue samples were cut into smaller pieces with a sterile scalpel. The specimens were shaken on ice for 20 hours, then embedded in paraffin and stored at –20°C until further examination.

Apoptosis (TUNEL technique)

Apoptosis was assessed with the principle of terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) to detect fragmented DNA in apoptotic cells.

Sections were treated according to the manufacturer's recommendations (OncorApop Tag, Heidelberg) by the methods of Gavrieli *et al*. (1992), Labat-Moleur *et al*. (1998) and Negoescu *et al*. (1996). Briefly, sections were deparaffinized and rehydrated, permeabilized in proteinase K, and treated with terminal deoxynucleotidyltransferase incubation buffer at 37°C for 60 minutes. Sections were then treated with peroxidase-labeledantidigoxigenin antibody, followed by 3`-3 diaminobenzidinecolor reaction. Sections were counterstained with hematoxylin.

Immunohistochemical detection of Ki-67 antigen

Paraffin cuts of the endometriosis samples were deparaffined with xylene and rehydrated with decreasing ethanol concentrations of 100-70%. The unmasking of the epitopes was carried out by boiling the sections in a steam pressure pot for 2.5 min in 0.01 M citrate buffer (pH 6) according to the methods from Shi *et al*. 1991 and Kreipe *et al*. 1993.

The deparaffined sections were incubated 45 min at RT, incubated in a moist chamber with the monoclonal antibody Ki-S5. After washing four times with a Tris buffer (pH 7.5), the sections incubated with 1:20 diluted rabbit anti-mouse antibody (Z259, DAKO, Hamburg) in 10% human serum albumin in Tris buffer (TBS) for 30 min. They were then washed 4x in TBS and incubated with alkaline phosphatise anti alkaline phosphatise (APAAP) complex at a dilution of 1:50 in TBS (DAKO, Hamburg, D0651) for 30 min. After washing four times with TBS, the development of the Ki-67 positive nuclei was occurred, by a new fuchsin-diazo-color reaction. Then, a nuclear counterstaining was performed with hematoxylin.

Results

Figure 1 shows a histological (HE staining) image of an endometriosis lesion from a 32 years old patient who was on her 8th cycle day. The subepithelialstroma tissue contains hardly neutrophil granulocytes or lymphocytes.
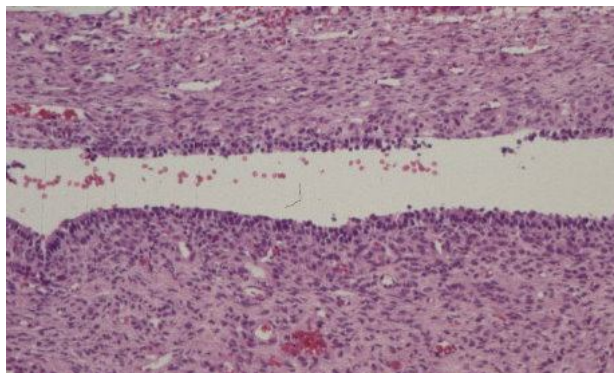

Fig. 1. Histological representation of an endometriosis lesion (HE staining).

Detection of apoptosis in paraffin sections by the TUNEL technique:

With TUNEL technology apoptotic cells can be observed. The brown staining in Figure 2A and B shows apoptosis in epithelial cell nuclei of endometriosis lesions from 27 and 32 years old patients.

Immunohistochemical detection of the proliferation marker Ki-S5-antigen:

With proliferation markers, which recognize the Ki-67-Antigen by Ki-S5-Antibody, proliferating cells which are in the cycle could be selectively represented. The red staining of epithelial cell nuclei in Figure 3a and b shows the proliferation of endometrial lesions of 27 and 32 years old patients.

Table 1 shows the frequency of apoptotic and proliferated epithelial cells in endometriosis and normal Endometrium.

Table 1. The frequency of apoptotic and proliferated epithelial cells in endometriosis and normal Endometrium

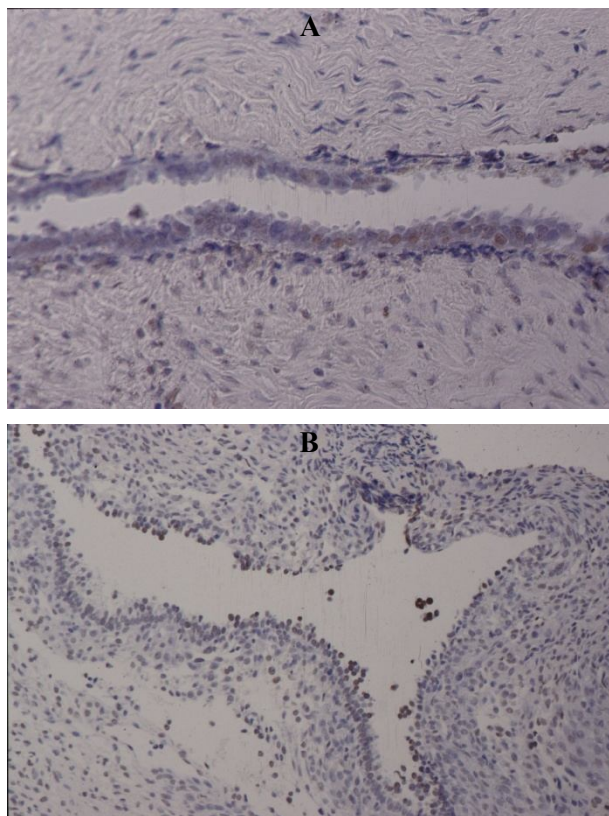|	Apoptosis	Proliferation
Endometriosis (n=15)	12/15 (+)	13/15 (+)
Frequency	3-47%	2-25%
Endometrium (n=3)		
Frequency	1%	26%

Fig. 2. Detection of fragmented DNA in apoptotic cells by using terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling technique. The brown coloration of cell nuclei characterized the apoptotic cells in ectopic endometrium from 27 (A) and 32 (B) years old patients.

Discussion

Endometriosis cases, examined in this study, concern female patients who suffer a chronic Endometrioses. I.e., here it concerned the women who cannot eliminate regularly and effectively the dystopia Endometrium mucosa. The investigations show that the proliferation activity was simultaneously accompanied by an apoptosis activity. Possibly the procedures of proliferation and Apoptosis go hand in hand, so that none of the mechanisms is able to win the prevailing. Endometriosis persists without increasing actively in size. Accordingly the apoptotic activity remains high, while the Endometrioses lesions cannot be eliminated completely.

Watanabe et al. (Watanabe et al. 1997) and some other authors (Agic et al. 2009; El-Ghobashy et al. 2007; Harada et al. 1996; Jones et al. 1998; McLaren et al. 1997; Meresman et al. 2000; Suganuma et al.1997) were consistent with our results and found the Bcl2 and Fas expression in eutopic and ectopic human endometrium. The results also allow as a conclusion that any significant disruption of the apoptotic ability, such as

point mutation, in the pro-apoptotic genes with 'loss of function' cannot be counted. In consideration of the relatively high frequency of apoptosis might the persistence of the dystopian mucous membrane led back often remarkable proliferative capacity of dystopian epithelia. On the other hand it is known that the upper functional layers of the endometrium do not exhibit proliferations' activity, because they have short telomeres and telomerase activity cannot develop. In comparison, epithelial cells of the deeper layers because of the high telomerase activity have a sufficiently extended telomere length in order to develop a higher proliferative activity (Kyo *et al*. 1999; Tanaka *et al*. 1998; Yokoyama *et al*. 1998).
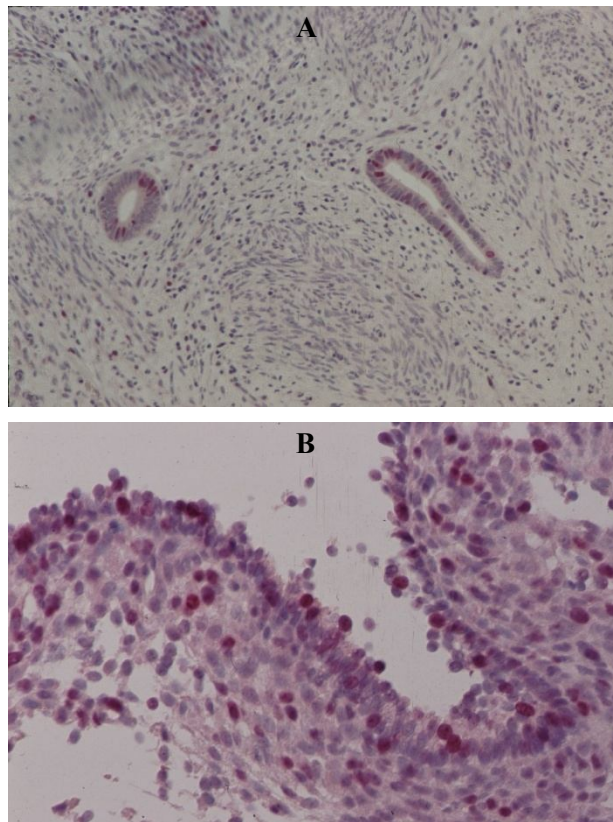

Fig. 3. Immunohistochemical presentation of proliferating epithelial cells with the monoclonal Ki-S5-Antibody (red staining of nuclei) using alkaline phosphatase anti-alkaline phosphatase (APAAP) reaction. 4µm cryostat section ectopic endometrium: (A) for a 27 years old female patient and (B) for a 32 years old patient.

Conclusion

The results suggest the following hypothesis inaugurate endometriosis development:

Under the dystopian endometrial mucous membrane particles found solely surface epithelium, prevail

apoptosis and dystopia tissue rapidly eliminated. Dystopian endometrium particles contain also epithelia from deeper layers, so proliferative processes of the apoptotic activity can counteract. The dystopia endometrium persisted and becomes the basis of Adenoma, proliferative endometriosis disease.

Ethical issues

None to be declared.

Conflict of interests

Authors declare no conflict of interests.

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