

Spontaneous apoptosis of endometrial tissue is impaired in women with endometriosis

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Objective: To evaluate spontaneous apoptosis in single-cell suspensions of eutopic and ectopic endometrium from women with endometriosis and in eutopic endometrium from fertile controls without endometriosis.

Design: Paired specimens of eutopic and ectopic endometrial tissue from patients with endometriosis and eutopic endometrium from controls were assessed for spontaneous apoptosis.

Setting: Institute for the Study and Treatment of Endometriosis and university-based research laboratories.

Patient(s): Fertile controls (n = 10) and women with untreated endometriosis (n = 16).

Intervention(s): None.

Main Outcome Measure(s): Spontaneous apoptosis assessed with an ELISA-based cell death detection kit.

Result(s): Spontaneous apoptosis (monitored by absorbance) of eutopic endometrium from patients with endometriosis and fertile controls was 0.63 ± 0.1 and 1.43 ± 0.11 , respectively. Among patients with endometriosis, spontaneous apoptosis of ectopic endometrium was 0.26 ± 0.06 . Decreased apoptosis of ectopic versus eutopic endometrium was observed independent of cycle phase.

Conclusion(s): The susceptibility of endometrial tissue to spontaneous apoptosis is significantly lower in women with endometriosis than in fertile controls. We suggest that decreased susceptibility of endometrial tissue to apoptosis contributes to the etiology or pathogenesis of endometriosis. (Fertil Steril® 1998;69:1042-7. ©1998 by American Society for Reproductive Medicine.)

Key Words: Apoptosis, endometriosis, eutopic and ectopic endometrium

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Programmed cell death, commonly referred to as apoptosis, is a fundamental process responsible for maintaining homeostasis in multicellular organisms (1). In contrast with necrotic cell death, which is usually a result of trauma, programmed cell death is a physiologic process. The ordered progression of cell death occurring by apoptosis minimizes the leakage of cellular contents such as proteases from dying cells, thereby reducing the likelihood of an inflammatory response (2).

Apoptosis results from a series of related morphologic and biochemical processes. Morphologically, apoptotic cells present with condensed chromatin, multiple membrane-bound organelles (apoptotic bodies), and a shrunken appearance. Biochemically, apoptosis is characterized by monomeric or multimeric 180-base pair (bp) nucleosomal fragments resulting

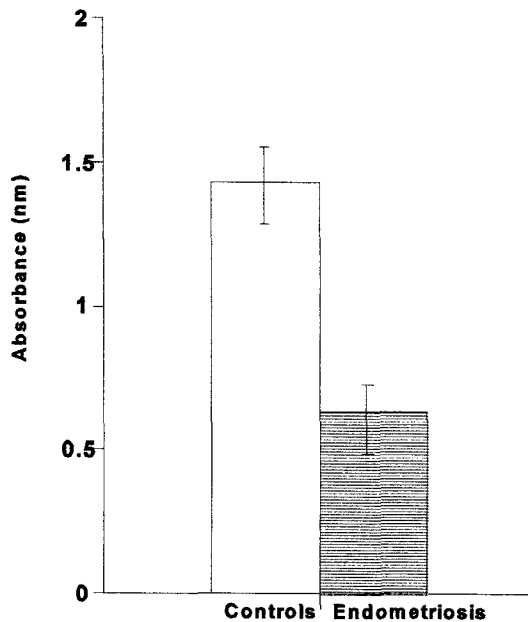
from the cleavage of double-stranded nuclear DNA (3, 4).

Studies of apoptosis have demonstrated its role in both physiologic and pathologic conditions. Thus, although apoptosis is important for normal development (e.g., morphogenesis) and health (e.g., elimination of virally infected cells), its aberrant activation may contribute to the pathogenesis of diseases ranging from neurodegenerative disorders to AIDS. Similarly, impaired apoptosis may contribute to the etiology or pathogenesis of cancer and autoimmune disorders (5-7).

The endometrial cycle in regularly menstruating women consists of three distinct phases (proliferative, secretory, and menstrual). Accumulating evidence suggests that apoptosis helps to maintain cellular homeostasis during

FIGURE 1

Spontaneous apoptosis in endometria from women with and without endometriosis. Single-cell suspensions of eutopic endometrium from patients (n = 16) and controls (n = 10) were analyzed photometrically for spontaneous apoptosis as described in Materials and Methods. Values, reported in units of absorbance, are means \pm SEM.



the menstrual cycle, through the elimination of senescent cells from the functional layer of the uterine endometrium during the late secretory and menstrual phases of the cycle (8–10). This is followed by proliferation of new cells from the basal layer during the proliferative phase of the cycle.

Endometriosis, a disease with an estimated incidence of 10%–15% among menstruating women, occurs when endometrial tissue normally restricted to the uterine lining implants and grows at ectopic sites. Why some women develop endometriosis whereas others do not is unknown and is an area of active investigation. Previous studies suggest that the development of endometriosis is associated with anomalies of the immune system. Thus, women with endometriosis exhibit diminished cell-mediated immunity, frequently present with numerous autoantibodies, and display a disproportionate number of immune system disorders ranging from allergies to lupus (11–13).

Recently, we observed that, compared with eutopic endometrium, ectopic endometrial tissue is relatively resistant to monocyte- or macrophage-mediated cytotoxicity. This led us to speculate that there could be a loss of homeostatic control in the endometrial tissue of women with endometriosis compared with healthy controls.

In this study, we evaluated the degree of spontaneous apoptosis of eutopic and ectopic endometria from women

with endometriosis compared with eutopic endometrial tissue from fertile controls. Our data suggest that decreased susceptibility of uterine endometrium to apoptosis may contribute to the etiology or pathogenesis of endometriosis.

MATERIALS AND METHODS

Study Population

The subjects in this study were women of reproductive age undergoing laparoscopy for suspected endometriosis. At the time of laparoscopy, pelvic organs were examined carefully for the presence and extent of endometriosis. Staging of the disease was performed according to the revised American Fertility Society classification (14). Only subjects with fleshy exophytic endometriotic implants without connective tissue reaction were included in this study. Control subjects were fertile, healthy women without endometriosis who were undergoing laparoscopic tubal sterilization.

Sixteen women with endometriosis and 10 controls were entered into the study. Biopsy specimens of eutopic endometrium were obtained from all subjects with the Novak's curette. Ectopic endometriotic lesions were removed from all patients with laparoscopic biopsy forceps. Both eutopic and ectopic endometrial samples were placed in Ringer's lactate solution immediately after retrieval. Each sample was divided so that one portion was sent for routine histologic examination whereas a second portion was used for endometrial cell preparation.

Cycle phase (secretory or proliferative) for each subject was assigned based on histologic evaluation. Nine of the 16 patients were in the proliferative phase and 7 were in the secretory phase, whereas 4 of the 10 controls were in the proliferative phase and 6 were in the secretory phase. This study was approved by our institution's institutional review board, and all subjects signed informed consent forms.

Endometrial Cell Preparation

Single-cell suspensions of eutopic and ectopic endometrial samples were prepared by enzymatic digestion with a mixture of collagenase (0.014%) and deoxyribonuclease (DNase I; 0.01%) contained in Hanks' balanced salt solution (HBSS, Whittaker, Walkersville, MD) at

TABLE 1

Susceptibility of eutopic endometrium to spontaneous apoptosis among patients and controls.

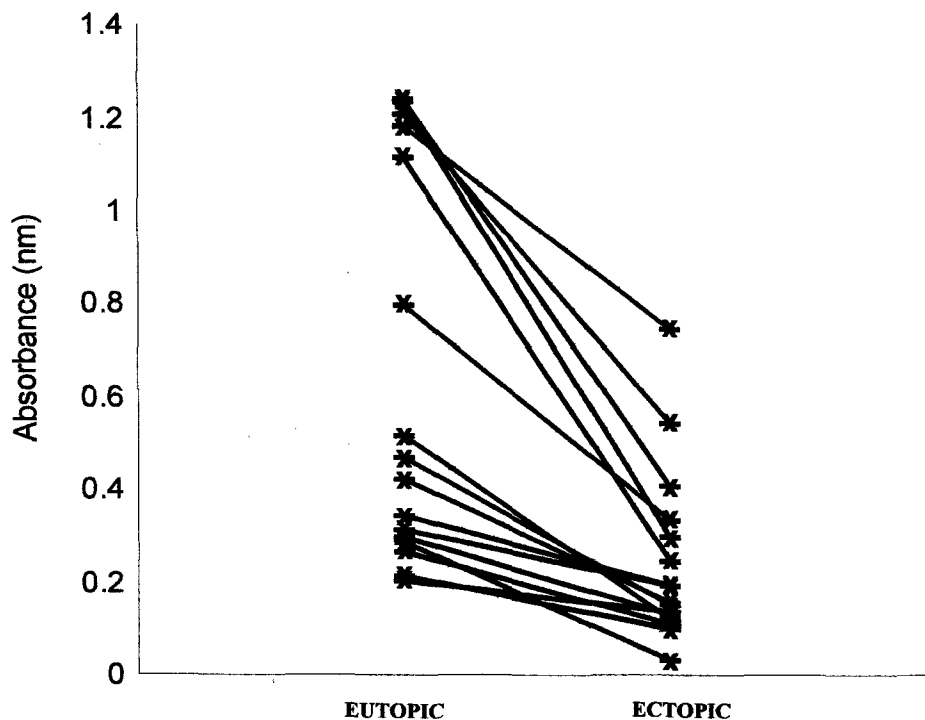
Group	Proliferative phase	Secretory phase	P value
Patients	0.68 \pm 0.14 (n = 9)*	0.56 \pm 0.16 (n = 7)*	NS
Controls	1.40 \pm 0.22 (n = 4)	1.45 \pm 0.41 (n = 6)	NS

Note: All values (generated as described in Materials and Methods) are means \pm SEM. NS = not significant.

* $P < .001$ (patients versus controls).

FIGURE 2

Spontaneous apoptosis of eutopic and ectopic endometrial cells from women with endometriosis. Single-cell suspensions of eutopic and ectopic endometrium were prepared, and paired samples from each patient were analyzed photometrically for spontaneous apoptosis as described in Materials and Methods. Values are presented in units of absorbance.



37°C for 20 minutes. The final product was a single-cell suspension consisting of stromal cells and glandular epithelial cells. After digestion, cells were filtered through sterile mesh (3-163T Nitex Mesh, Martin Supply, Baltimore, MD), collected by centrifugation, and resuspended to a concentration of 1×10^6 /mL in RPMI 1640 medium (Whittaker) supplemented with 10% fetal bovine serum (Whittaker), 100 μ of penicillin per mL, and 100 μ g of streptomycin per mL.

Apoptosis Assay

Single-cell suspensions of ectopic and eutopic endometrium were analyzed for spontaneous apoptosis using the cell death detection ELISA kit (Boehringer Mannheim Corporation, Indianapolis, IN), which, as recently reviewed by Allen et al. (15), is a highly sensitive and specific assay for apoptosis. Briefly, cells were resuspended in 500 μ L of a lysis buffer at 1×10^5 cells/mL and incubated for 30 minutes at 4°C. At the end of incubation, the cytoplasmic fraction (supernatant) containing fragmented DNA was removed after a 20,000 \times g centrifugation for 10 minutes. The supernatant was diluted 1:10 in isotonic buffer and overlaid in triplicate and incubated in microtiter plate modules coated with anti-histone antibody. All samples were treated with anti-DNA-peroxidase followed by development with ABTS® substrate. The

resultant photometric data (in which increasing units of absorption correlate with the level of apoptosis) were measured with an ELISA plate reader.

Statistical Evaluation

Data in this study were analyzed by a paired or unpaired *t*-test and, for nonparametric data, by Wilcoxon's rank sum test. Data are expressed as means \pm SEM.

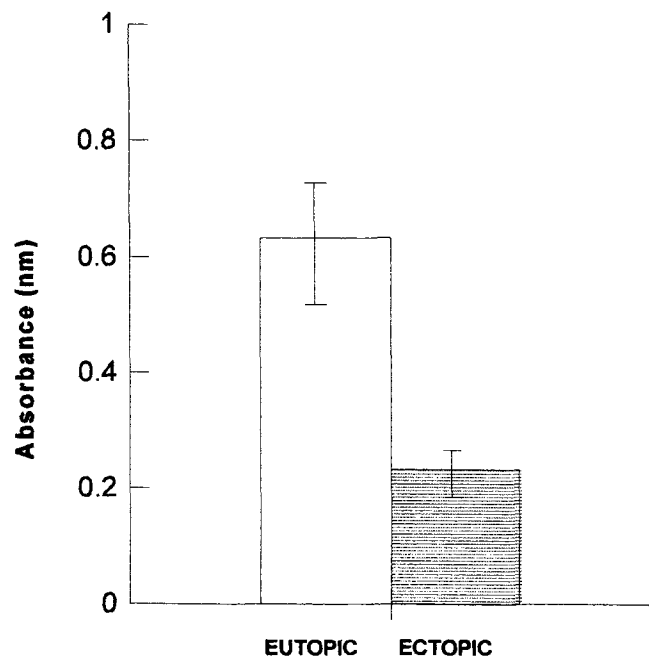
RESULTS

Spontaneous Apoptosis in Eutopic Endometrial Cells in Women With Endometriosis Compared With Healthy Controls

Spontaneous apoptosis, as reflected by absorbance, was significantly lower in the eutopic endometrial tissue obtained from patients with endometriosis compared with healthy, fertile controls (0.63 ± 0.01 versus 1.43 ± 0.11 ; $P = 2.8 \times 10^{-5}$) (Fig. 1). Comparison of patients ($n = 7$ secretory phase; $n = 9$ proliferative phase) with controls ($n = 6$ secretory phase; $n = 4$ proliferative phase) in the same phase of the cycle revealed that the decreased level of spontaneous apoptosis in patients was not attributable to cycle phase (Table 1).

FIGURE 3

Spontaneous apoptosis of eutopic and ectopic endometrial cells from women with endometriosis. Single-cell suspensions of eutopic and ectopic endometrium from 16 patients were analyzed photometrically for spontaneous apoptosis as described in Materials and Methods. Values, reported in units of absorbance, are means \pm SEM.



Spontaneous Apoptosis in Eutopic and Ectopic Endometria in Women With Endometriosis

Spontaneous apoptosis was decreased in the ectopic endometrium compared with eutopic endometrium in all paired samples (Fig. 2). The mean \pm SEM absorbency values were 0.26 ± 0.06 for ectopic and 0.63 ± 0.1 for eutopic endometrium ($P = 0.0001$) (Fig. 3). As shown in Table 2, the reduced susceptibility of ectopic endometrium compared with eutopic endometrium of patients was maintained independent of cycle phase (ectopic versus eutopic proliferative phase: 0.21 ± 0.03 versus 0.68 ± 0.14 ($P = 0.002$); ectopic versus eutopic secretory phase 0.34 ± 0.13 versus 0.56 ± 0.16 ($P = 0.001$). Among the 16 patients with endometriosis, 11 were classified as stage I/II (mild to moderate disease), and 5 were classified as stage III/IV (severe disease). As shown in Figure 4, spontaneous apoptosis was reduced significantly in the ectopic endometrium from both groups of patients compared with eutopic endometrium.

DISCUSSION

Apoptosis, or programmed cell death, is a process by which multiple cell types are eliminated during embryogenesis and in fully developed adult multicellular organisms.

Apoptosis, which occurs under both physiologic and pathologic conditions, is involved in a diverse group of processes including morphogenesis, immunologic tolerance, malignant cell growth, autoimmunity, neurodegenerative diseases, and AIDS (2, 5–7, 16, 17).

There are numerous stimuli that trigger apoptosis, including withdrawal of essential growth factors or hormones or engagement of various receptor/ligands including Fas/Fas ligand and tumor necrosis factor (TNF)/TNF receptor (1, 18). Recent evidence indicates that internal triggering of cell death involves a common signaling motif, namely, intracytoplasmic proteins containing so-called “death domains” (reviewed in ref. 19) and the activation of acidic sphingomyelinase (20), which hydrolyzes sphingomyelin to ceramide. In turn, ceramide, serves as a second messenger capable not only of signaling apoptosis, but, paradoxically, cellular activation (21). Apoptosis is regulated by several additional genes, which potentiate (p53; Bax; c-myc) or inhibit (Bcl-2; Bcl-xL; sentrin) programmed cell death (19, 22).

Each month during menstruation, the uterine endometrium of healthy, fertile women is subject to cyclic changes (23, 24) resulting, at least in part, from necrotic injury (25). Apoptosis is also likely to play a role in the endometrial cell death occurring during the menstrual cycle (4, 10). Tabibzadeh et al. (26) reported that eutopic endometrium continuously expresses Fas and TNF-receptor throughout the menstrual cycle. He also reported that an inhibitor of apoptosis, Bcl-2, is expressed continuously on eutopic endometrium. Interestingly, the expression of Bcl-2 diminishes during the secretory phase of the cycle (27, 28). Furthermore, apoptotic bodies and DNA laddering become increasingly apparent during the late secretory and menstrual phases of the cycle (8).

The results of our studies are consistent with the notion that apoptosis is one of several factors involved in the cyclic shedding of uterine endometrium. It is well established that withdrawal of estrogen and progesterone at the end of the menstrual cycle activates a variety of local events resulting in the endometrial sloughing both in women with and with-

TABLE 2

Differences in the susceptibility of eutopic and ectopic endometrium to spontaneous apoptosis in women with endometriosis are not influenced by cycle phase.

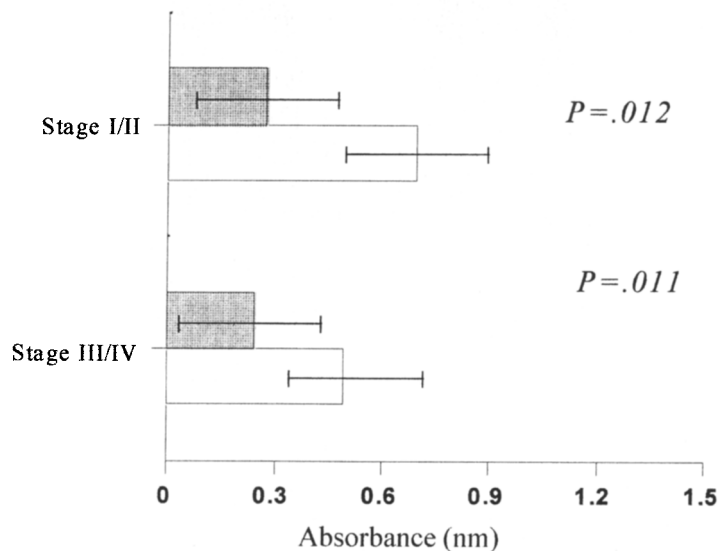
Group	Proliferative phase	Secretory phase	<i>P</i> value
Eutopic endometrium	$0.68 \pm 0.14^*$	$0.56 \pm 0.16^*$	NS
Ectopic endometrium	0.21 ± 0.03	0.34 ± 0.13	NS

Note: All values (generated as described in Materials and Methods) are means \pm SEM. NS = not significant.

* $P < .001$ (eutopic versus ectopic).

FIGURE 4

Spontaneous apoptosis of eutopic and ectopic endometria among patients with moderate (stages I/II) versus severe (stages III/IV) endometriosis. Single-cell suspensions of eutopic (□) and ectopic (■) endometrium obtained from patients with either stage I/II ($n = 11$) or stage III/IV ($n = 5$) disease were analyzed photometrically for spontaneous apoptosis as described in Materials and Methods. Values, reported in units of absorbance, are means \pm SEM.



out endometriosis. Our data suggest that the sloughed endometrial cells and tissue fragments differ in their degree of apoptotic changes among the two categories of subjects. We propose that in healthy women, the majority of sloughed cells undergo programmed cell death and do not survive whether they are expelled outside of the body or remain within the female organism. Among women with endometriosis, however, the percentage of sloughed endometrial cells undergoing apoptosis is reduced greatly, increasing the number of surviving cells that then may continue to exhibit physiologic activity if not expelled outside the organism.

Although concepts such as retrograde menstruation, lymphatic spread, metaplasia, and embryonic rests have been proposed as mechanisms to explain how endometrial tissue appear in the ectopic sites, they do not address why these misplaced cells survive in women with endometriosis but not in healthy controls. Our data suggest that resistance to apoptosis contributes to this phenomenon. In this study, we observed that endometrial tissue obtained from women with endometriosis is significantly less susceptible to spontaneous apoptosis than endometrial tissue from fertile controls (Fig. 1).

Results from 16 of 16 patients with endometriosis revealed that spontaneous apoptosis of ectopic tissue was always less than that occurring in eutopic tissue from the same patient, independent of their cycle phase (Figs. 2–4; Tables 1 and 2). Interestingly, endometria from patients with stage III/IV endometriosis tended to have less spontaneous apoptosis than endometria from patients with stage I/II dis-

ease (Fig. 4), suggesting a possible relationship between disease severity and susceptibility to spontaneous apoptosis. In this study, however, the differences among the two groups of patients did not reach statistical significance.

We initiated this study speculating that endometriosis could result from defective apoptosis and have shown, for the first time, that eutopic endometrium from patients with endometriosis displays significantly reduced apoptosis compared with eutopic endometrium from healthy controls. Collectively, these data support our hypothesis that the cause or pathogenesis of endometriosis is at least partially because of a reduced sensitivity of endometrial tissue to apoptosis.

Any of several mechanisms could explain these data. It is conceivable that eutopic and ectopic endometrium of patients with endometriosis fail to express cell surface receptors associated with triggering apoptosis (e.g., Bcl-2) that inhibit apoptosis. However, Watanabe et al. (28) recently reported that eutopic and ectopic endometrium of patients with endometriosis express comparable levels of Fas and Bcl-2. This would suggest that proteins other than Fas and Bcl-2 regulate apoptosis within endometrial tissue.

Another (and more appealing possibility) to explain our results is inappropriate transduction of an apoptotic signal. Thus, within the endometrium of patients, second messengers that trigger apoptosis in healthy controls may instead trigger cellular proliferation. A prime candidate to mediate these two apparently contradictory activities is ceramide, a metabolite of sphingomyelin (21). Previous reports from our

laboratory are consistent with data from the current study. Specifically, we reported that the proliferation of endometrium from normal healthy controls is suppressed by TNF, whereas the proliferation of endometrium from patients with endometriosis is enhanced (29).

Collectively, our data suggest that abnormal survival (i.e., lower apoptosis) of eutopic endometrial cells results in their continuing physiologic activity after displacement into ectopic sites, giving origin to endometriosis. Considering that only those cells that survived in the ectopic sites formed the endometriotic implants that we studied, it is not surprising that apoptosis was much lower in ectopic than eutopic endometrium. It would be of interest to study the different eutopic endometrial cellular components for their susceptibility to apoptosis. Theoretically, those cells with the lowest apoptosis would be major components of the ectopic implants. Because stromal cells were not separated from glandular cells in this study, the possibility that these distinct cell groups exhibit differential apoptosis and contribute preferentially to the ectopic endometrial tissue should be considered.

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