

Relationship between apoptosis and the number of macrophages in eutopic endometrium from women with and without endometriosis

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Objective: To investigate the relationship between apoptotic cells and macrophages in the eutopic endometrium of women with and without endometriosis.

Design: Retrospective analysis of archival uterine endometrial biopsy specimens.

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

Patient(s): Fifty-one women with endometriosis and 24 healthy control subjects without endometriosis.

Intervention(s): None.

Main Outcome Measure(s): The number of TUNEL+ (terminal deoxynucleotide transferase [TdT]-mediated deoxyuridine triphosphate [dUTP] nick end-labeling-positive) (apoptotic) cells and CD68+ (CD68 positive) (macrophages).

Result(s): Apoptotic cells and macrophage numbers were positively correlated in the eutopic endometrium of women with and without endometriosis. However, the number of apoptotic cells and the macrophage content in the endometrium of women with endometriosis was significantly reduced compared with that of healthy control subjects without endometriosis. Differences between apoptosis and macrophage numbers between the two populations were observed predominantly during the early proliferative phase of the menstrual cycle.

Conclusion(s): The reduction in apoptosis described for endometrial cells in women with endometriosis may be related to reduced macrophage trafficking into the eutopic endometrium during the early-proliferative phase of the menstrual cycle. (*Fertil Steril*® 2002;78:830–5. ©2002 by American Society for Reproductive Medicine.)

Key Words: Apoptosis, macrophages, endometrium, endometriosis

Endometriosis is becoming recognized as a condition in which endometrial cells exhibit abnormal proliferative and apoptotic regulation. Utilizing a cell death enzyme-linked immunosorbent assay (ELISA), we demonstrated that spontaneous apoptosis of uterine endometrium from women with endometriosis is significantly reduced compared with control subjects without endometriosis, and the apoptosis of ectopic endometrium from women with endometriosis is reduced even further (1). The reduced apoptosis observed in that early study was confirmed by using the TUNEL (terminal deoxynucleotide transferase [TdT]-mediated deoxyuridine triphosphate [dUTP] nick end-la-

beling) assay with endometrial biopsy specimens, which also showed that the relative deficiency in apoptosis observed in uterine endometria from women with endometriosis is confined largely to the early proliferative and late-secretory phases of the menstrual cycle (2).

We have also demonstrated that the proliferative activity of endometrial cells from women with endometriosis is enhanced by coculture with autologous monocytes, whereas the proliferation of endometrial cells from controls is suppressed by autologous monocytes (3). Furthermore, we reported that endometrial cells from women with endometriosis are re-

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sistant to *in vitro* cytolysis by autologous peritoneal macrophages, suggesting that such cells are able to survive a principal homeostatic mechanism within the peritoneal cavity (4). Recently, we extended these observations to show that enhanced endometrial cell proliferation is also observed when endometrial cells are cultured with autologous or heterologous peritoneal fluid from women with endometriosis, or with recombinant tumor necrosis factor (TNF)-alpha (5). Such effects were not observed with endometrial cells from control subjects.

Taken together, the picture that emerges from these studies is of a condition in which the regulation of cell birth and cell death in endometrial cells from women with endometriosis is disturbed, leading to the selection of cells that can survive normal homeostatic mechanisms to establish ectopic sites of endometriotic cell growth. What remains to be determined is whether abnormal growth control in these cells is intrinsic (i.e., a property of the endometrium itself); extrinsic (i.e., a reflection of disturbed physiology and homeostasis in the eutopic and/or ectopic environments); or a combination of these problems.

In the present study, we have addressed one aspect of this issue by investigating the relationship between cellular apoptosis and macrophage trafficking in the eutopic endometrium of women with and without endometriosis. The results show that there is a direct correlation between the apoptotic cell number and the macrophage content in the eutopic endometrium of both groups of subjects. Moreover, the reduced apoptotic activity observed in women with endometriosis during the early proliferative and late-secretory phases of the menstrual cycle was correlated with reduced macrophage numbers during these phases.

MATERIALS AND METHODS

Study Population

Paraffin blocks of archived uterine endometrial specimens from 51 women with endometriosis and 24 healthy control subjects without endometriosis were retrieved from the pathology laboratory repository. Specimens were from women of reproductive age, who had exhibited regular menstrual cycles, and who had undergone laparoscopy as part of a comprehensive, infertility evaluation between 1996 and 1998. The subjects participated in a clinical study, approved by the institutional review board, in which portions of the eutopic and ectopic endometrial specimens were evaluated using functional immune assays. No hormonal medications were used during the cycle.

At the time of laparoscopy, pelvic organs were examined for the presence and extent of endometriosis. If no evidence of endometriosis was present, the subject was included in the control group. If endometriosis was present, staging of the disease was performed according to the revised American Fertility Society (AFS) classification (6). The study group

consisted of 18 patients with stage 1; 19 with stage 2; 8 with stage 3; and 6 with stage 4 of the disease. The control group without endometriosis excluded women with adhesions or pelvic diseases other than endometriosis. During the laparoscopic procedure, samples of the uterine endometrium were obtained with the Novak's curet from the uterine fundus. Part of each specimen was fixed immediately in 4% formaldehyde and transferred to the pathology laboratory.

Identification of Endometrial Phases and Apoptosis Analysis

The methodologies employed in this investigation were described in our previous study (2). Thus, paraffin blocks were retrieved, sectioned (5 μ m), mounted on glass slides, coded and sent to the pathology laboratory at another institution for a blind analysis. One set of slides was stained with hematoxylin-eosin and examined for the endometrial phase of the cycle. Endometrium was classified as early-proliferative (EP), mid-proliferative (MP), late-proliferative (LP), early-secretory (ES), mid-secretory (MS), late-secretory (LS), and menstrual (M) according to its histological appearance (7).

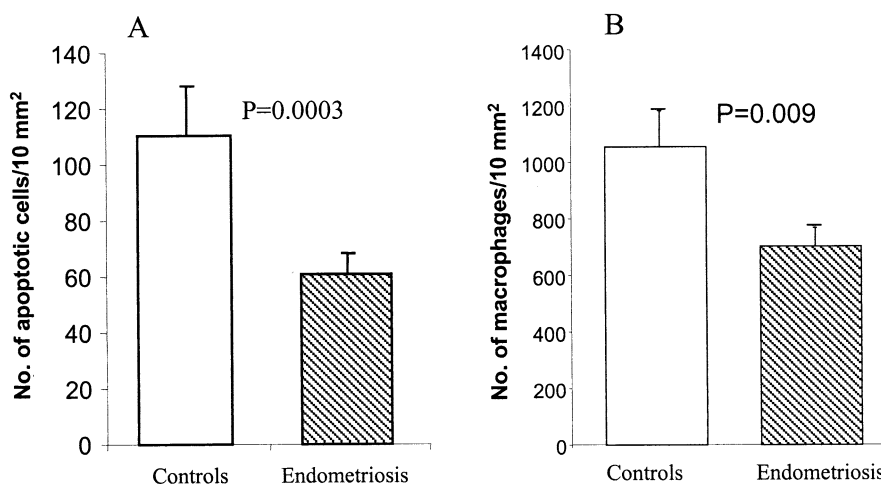
Another set of slides was stained using the TUNEL method as described by Gavrieli et al. (8), with minimal modification to identify the apoptotic cells. Briefly, one set of sections was deparaffinized and digested with 20 μ g/mL protease K (Sigma, St. Louis, MO) for 15 minutes at room temperature. The endogenous peroxidase was blocked with 2% H₂O₂ [diluted from 30% H₂O₂ in water (w/w), Sigma, St. Louis, MO] for 5 minutes. After briefly immersing in TdT buffer (30 mM Trizma, 140 mM sodium cacodylate, and 1 mM cobalt chloride, pH 7.4), the slides were incubated in TdT reaction solution containing 0.3 unit/ μ L TdT (Boehringer Mannheim, Indianapolis, IN) and 0.005 mM biotin-dUTP (Boehringer Mannheim) in a TdT buffer for 90 minutes at 37°C.

The reaction was terminated by incubating slides in a TB buffer (300 mM sodium chloride and 30 mM sodium citrate) for 15 minutes. Afterward, slides were incubated in 2% bovine serum albumin (BSA; Sigma) for 10 minutes and then in 0.5% HRP-streptavidin (Zymed, South San Francisco, CA) for 30 minutes. The TUNEL was developed with 0.05% 3'3'-diaminobenzidine (DAB) and counterstained with Mayer's hematoxylin (Sigma). For each batch of TUNEL staining, positive and negative controls were run in parallel.

Positive controls consisted of 2 cases of normal spleen, thymus, bowel, and embryonic kidney. Negative controls were processed by omitting the TdT from the TdT reaction solution of the same TUNEL procedure. The criteria and scoring of apoptotic cells were the same as we have described previously (2). The apoptotic index was defined as the number of apoptotic cells per 10 mm² unit area.

FIGURE 1

Apoptotic cells (A) and macrophage numbers (B) in uterine endometrium in women with and without endometriosis. Simple means (mean \pm SE) were calculated with a *t*-test. Number of samples for apoptosis: 24 and 51 for controls and endometriosis, respectively. Number of samples for macrophages: 19 and 37 for controls and endometriosis, respectively.



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Immunohistochemistry for Identification of Endometrial Macrophages

Standard avidin-biotin-peroxidase complex (ABC) method was applied to identify macrophages. Briefly, paraffin sections were deparaffinized with xylene and rehydrated with gradual alcohol till distilled water. Antigen retrieval was applied prior to the initiation of the ABC method.

Sections were then treated with 0.075% H₂O₂ to remove the endogenous peroxidase effect, followed with incubation in 5% normal goat serum to block Fc-receptor and nonspecific binding. Afterward, sections were incubated with the CD68 monoclonal antibody (1:50 dilution, clone No. PG/M1; DAKO, Carpinteria, CA) to identify endometrial macrophages. Sections were then incubated in biotin conjugated goat-antimouse IgG (1:200 dilution; PharMingen, San Diego, CA) and peroxidase conjugated streptavidin (1:200 dilution; Zymed), sequentially. Immunostaining was developed with 0.05% 3'3'-diaminobenzadine (DAB) and counterstained with Mayer's hematoxylin.

Quantitative analysis of endometrial macrophages was performed by counting CD68-positive (CD68+) cells from every high-power field (HPF, \times 400) of the whole tissue section, with the aid of a cytometer. The number of macrophages per 10 mm² for each sample was used for statistical analysis.

Statistical Analysis

The data were subjected to a *t*-test for comparison of means between control and endometriosis. Analysis of variance (ANOVA) and multiple comparisons (LSD) were used

to compare the means of endometrial cycle phases within and between the groups (e.g., control and endometriosis). The variables included in the ANOVA model were endometriosis (controls vs. endometriosis), cycle phase, and endometriosis-by-cycle phase interaction. The relationships between the apoptosis and macrophage numbers were tested by linear regression and correlation analysis.

Statistically significant difference was declared when the *P* value was \leq .05. The data were presented as mean \pm standard error (SE) (for *t*-test) or the least squares mean (LSM) \pm SE of LSM (for ANOVA). The *t*-test as well as the linear regression and simple correlation analysis were performed with Exile software; ANOVA was carried out using the general lineal model procedures of the Statistical Analysis System (SAS, Release 6.12; SAS Institute, Cary, NC).

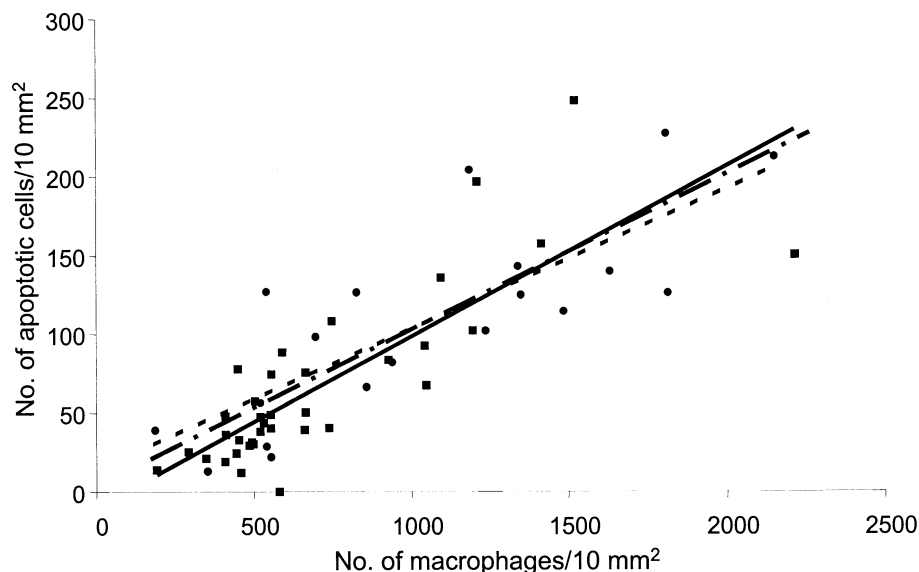
RESULTS

Apoptosis and Macrophage Number in Uterine Endometria From Women With and Without Endometriosis

The number of TUNEL-positive (TUNEL+) (apoptotic) cells and CD68+ macrophages in uterine endometria was compared for women with and without endometriosis (Fig. 1). The results demonstrate a statistically significant reduction in the levels of apoptotic cells (mean = 60.87 and 110.2, *P* = .0003) and macrophages (mean = 700.0 and 1053.5, *P* < .009) in specimens from women with endometriosis compared with controls. When the relationship between apoptosis and the macrophage number for individual

FIGURE 2

Plot of apoptotic indices vs. macrophage indices in women with (n = 37) and without (n = 19) endometriosis, and trend (regression) lines for control, endometriosis, and all subjects (pooled).



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specimens was tested by linear regression analysis, a highly significant positive relationship was found for the entire study population (correlation coefficient = 0.83, $P < .0001$) and for the control group (correlation coefficient = 0.78, $P < .0001$), as well as for the endometriosis group (correlation coefficient = 0.82, $P < .0001$) when these populations were analyzed separately (Fig. 2). A significant positive correlation between apoptosis and macrophage numbers was also found when the samples were grouped according to the stage of the endometriosis (data not shown).

Apoptosis and Macrophage Number in Uterine Endometria at Different Phases of the Menstrual Cycle

As we reported recently (2), endometria from controls without endometriosis exhibit a cyclicity of apoptotic activity with the greatest levels being observed during the early-proliferative and late-secretory phases of the menstrual cycle. This pattern is lost or disturbed in the endometria from women with endometriosis due to abnormally low levels of apoptotic cell numbers in the uterine endometria of women with endometriosis compared with controls during the early-proliferative and late-secretory phases of the menstrual cycle. In the current study, a significantly reduced number of macrophages was also evident during the early-proliferative phase of the menstrual cycle for specimens from women with endometriosis compared with controls (mean = 702.9 and 1625.8, $P = .0005$; $n = 6$ for endometriosis and control specimens, respectively).

In contrast, no significant difference in macrophage numbers was found for specimens obtained during the late-secretory/menstrual phase ($n = 7$ for endometriosis and control specimens, respectively), or for any other phase of the menstrual cycle ($n = 23$ and 8 for mid- to late-proliferative, and 15 and 3 for early- to mid-secretory phases of the menstrual cycle for endometriosis and control specimens, respectively) (Fig. 3).

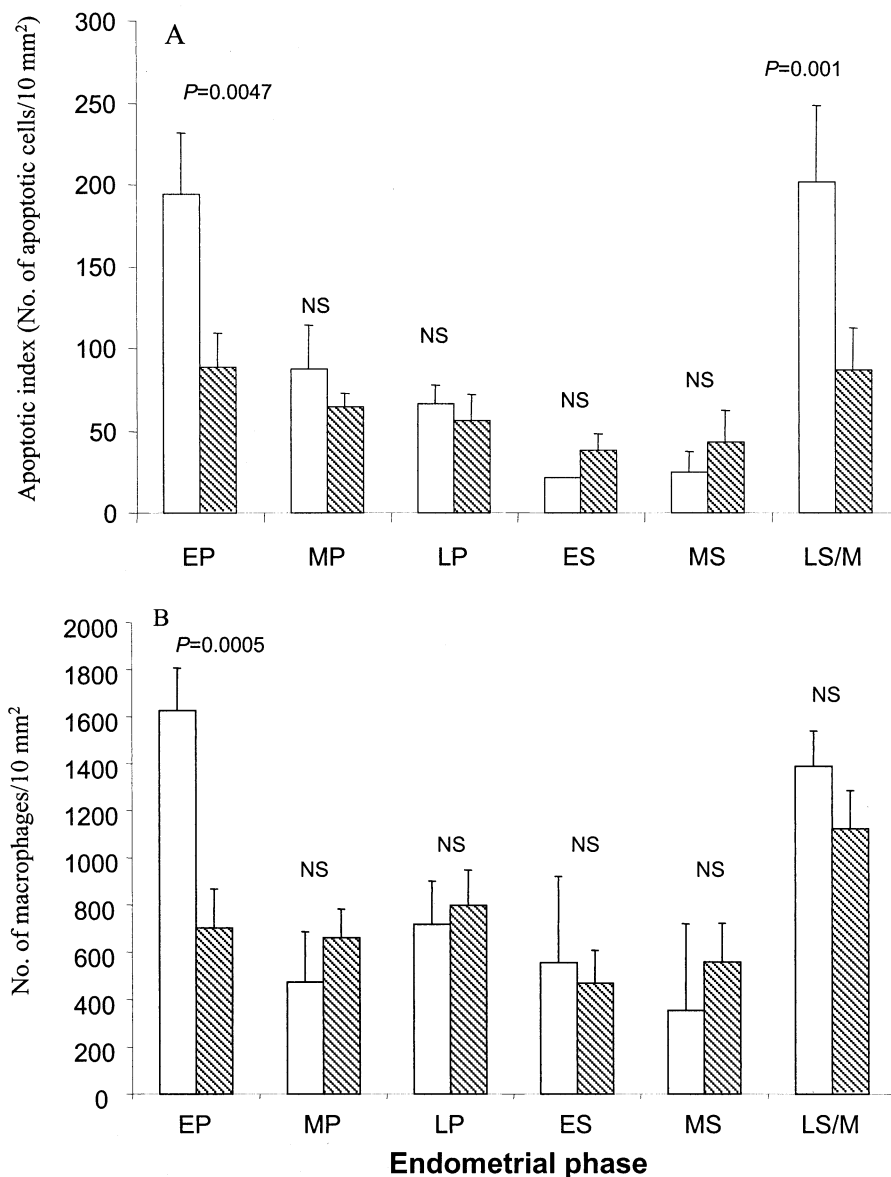
DISCUSSION

The results of this study show that there is a direct relationship between macrophage numbers and apoptotic cell numbers in the eutopic endometrium of women with and without endometriosis. Accordingly, the reduced apoptosis reported for uterine endometria in women with endometriosis is mirrored by a reduction in the macrophage numbers in these tissues. Reduced macrophage numbers were observed primarily with specimens collected during the early-proliferative and late-secretory phases of the menstrual cycle. This also parallels the relationship observed between apoptosis and the menstrual cycle in women with endometriosis (2).

Although a causal relationship between apoptosis and macrophage content cannot be demonstrated in this study, the results are consistent with that hypothesis. Thus, a highly significant positive correlation between apoptosis and macrophage numbers was found with specimens from women with and without endometriosis, and the pattern of apoptotic activity and

FIGURE 3

Apoptotic index (A), LSM \pm SE/10 mm², and macrophage number (B), LSM \pm SE/10 mm², in eutopic endometrium according to the endometrial phase. EP = early-proliferative; MP = mid-proliferative; LP = late-proliferative; ES = early-secretory; MS = mid-secretory; LS/M = late-secretory/menses. *P* values at the top of the bars are comparisons between controls and women with endometriosis at the same endometrial phase. NS = not significant, *P* > .05.



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macrophage trafficking throughout the menstrual cycle was comparable for both groups of specimens as well. If, in fact, a causal relationship exists between macrophage infiltration into the endometrium and the induction of apoptosis, this would imply that the reduced endometrial cell apoptosis found in women with endometriosis may be attributable, in part, to a physiological disturbance in normal homeostatic mechanisms within the uterine cavities of these individuals.

The reduced macrophage numbers observed in the uterine endometria from women with endometriosis in the present study was unexpected because an increase in macrophage numbers in the pelvis and peritoneal cavities of these women is well documented (9). Furthermore, some studies suggest that macrophage numbers are not reduced in the eutopic endometrium of women with endometriosis (10). Upon reflection, however, increased macrophage numbers in the

ectopic environment coincident with decreased macrophage numbers in the eutopic environment in women with endometriosis may be at least physiologically possible. Such effects could reflect altered chemokine gradients that favor macrophage mobilization to the ectopic environment due to the presence of cyclical, inflammatory stimuli within that environment.

Evidence to support this contention was reported recently by Hornung et al. (11), who showed that stimulated ectopic endometrium induces RANTES messenger ribonucleic acid (mRNA), which could lead ultimately to recruitment of macrophages to the ectopic environment. The result could be a net loss of macrophages in the eutopic environment. This possibility is consistent with studies by others that have shown, for example, increased numbers of T cells and macrophages in ectopic endometrial tissues compared with the matched, eutopic endometrium in women with endometriosis (12).

Alternatively, disturbances in normal macrophage trafficking into the eutopic endometrium in women with endometriosis could result from the disturbed expression of regulatory molecules on the eutopic endometrium, which govern the synthesis and/or response to various chemoattractants as has been demonstrated for the interleukin-1 (IL-1) type II receptor (13). It was suggested, however, that this phenomenon leads to increased levels of macrophage chemotactic protein-1 (MCP-1) following IL-1 stimulation and, presumably, to increased macrophage numbers in inflamed eutopic endometrium. Nevertheless, such events do suggest that a physiologic disturbance occurs in women with endometriosis that may disrupt normal homeostatic mechanisms within the eutopic endometrium. Some of those disturbances may facilitate endometrial cell survival, especially in cells with an intrinsic capacity to resist programmed cell death.

The preponderance of evidence from previous studies also suggests that at least some endometrial cells from women with endometriosis demonstrate an intrinsic resistance to apoptosis. This has been reported for both glandular and stromal cells in the uterine endometrium (2), as well as for ectopic endometrium that is microscopically devoid of contaminating leukocytes and fibroblasts (1). As we have reported previously, this can also be inferred by the resistance of eutopic and ectopic endometrial cells to macrophage-mediated cytolysis *in vitro* in women with endometriosis (4). Intrinsic resistance to apoptosis, in conjunction

with a physiologic disturbance in macrophage trafficking in the eutopic endometrial environment, would be expected to favor the survival of endometrial cells, which could lead to establishment of ectopic sites of endometrium.

In conclusion, the results of the present study show that a physiologic disturbance in macrophage trafficking into the eutopic endometrium occurs in women with endometriosis. This disturbance is most apparent during the early proliferative phase of the menstrual cycle. Because this is associated with reduced apoptosis in these tissues, this may provide evidence of defective physiological responses that favor persistence of endometrial cells, which can establish ectopic sites of the disease. It will be important to elucidate the mechanisms responsible for regulating macrophage trafficking into the eutopic endometrium because this knowledge may offer direction for developing new approaches to patient management and treatment.

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