Quantitative expression of apoptosis-regulating genes in endometrium from women with and without endometriosis

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Objective: To quantitate antiapoptotic and proapoptotic gene expression in endometrial cells (ECs) of women with and without endometriosis.

Design: Determination of transcript abundance (TA) of apoptosis-regulating genes in eutopic and ectopic endometrial cells.

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

Patient(s): Women with (n = 10) and without (n = 6) endometriosis.

Intervention(s): None.

Main Outcome Measure(s): Quantitative virtually multiplexed transcript abundance measurement (VMTA) of the BCL2, BCLxL, defender against cell death-1 (DAD-1), BCLxS, P53, Caspase-1, and proliferating cell nuclear antigen (PCNA) genes.

Result(s): The TA ratio of antiapoptotic to proapoptotic isoforms of the BCL-X gene favors survival in eutopic and ectopic ECs from women with endometriosis, but not control ECs. This was found throughout the menstrual cycle for ectopic ECs. Eutopic but not ectopic ECs also expressed increased TA of the antiapoptotic DAD-1 gene in endometriosis. Eutopic and ectopic ECs from women with endometriosis expressed decreased TA of p53 and Caspase-1 compared to ECs from women without endometriosis. Expression of these genes was not correlated with the proliferative state of ECs based on TA of the PCNA gene.

Conclusion(s): Dysregulation in expression of pro- and antiapoptotic regulatory genes characterizes eutopic and ectopic ECs from women with endometriosis. These results are consistent with apoptotic resistance and enhanced survival of ECs in endometriosis. (Fertil Steril® 2007;87:263–8. ©2007 by American Society for Reproductive Medicine.)

Key Words: Apoptosis, mRNA, endometrium, endometriosis

Previous studies demonstrated that endometrial cells (ECs) from women with endometriosis exhibit reduced apoptosis compared to ECs from women without endometriosis (1, 2). Endometrial cells obtained from ectopic sites of disease are even more resistant to apoptosis, and are also resistant to peritoneal macrophage-mediated cytolysis (1, 3). Taken together, these observations provide a plausible explanation for the persistence of ECs in ectopic environments.

There is also evidence to suggest that the normal proliferative and apoptotic cyclicity of the endometrium is disturbed in women with endometriosis, with reduced apoptosis observed primarily in the early proliferative and late secretory phases of the menstrual cycle (4). Because the endometrium exists in a dynamic environment with multiple interacting cell types and factors, it is unclear whether reduced apoptotic sensitivity is intrinsic to the ECs themselves, or results from disturbances in the cellular and molecular interactions responsible for regulating proliferation and apoptosis in the eutopic and ectopic environments. In the present study, we began to explore this question by investigating the expression of various proapoptotic and antiapoptotic genes in both eutopic and ectopic ECs obtained from women with endometriosis.

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MATERIALS AND METHODS

Study Population

The subjects in this study were women of reproductive age who were undergoing laparoscopic tubal ligation or laparoscopic surgery for evaluation of suspected endometriosis. Endometrial biopsies were obtained at the time of laparoscopy with a Novak's curette. Endometriosis was staged

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according to the revised American Fertility Society classification (5). Exophytic endometriotic implants were removed from peritoneal surfaces with the use of laparoscopic biopsy forceps. Endometriotic lesions were then ablated with a neodymium-yttrium aluminum garnet laser, or excised. Hemostasis was secured with bipolar electrocautery.

Endometrial biopsies from 10 patients with endometriosis (3 stage I, 5 stage II, and 2 stage III), as well as ectopic ECs from exophytic implants in six individuals (3 stage II, 2 stage III, and 1 stage IV (4 patients who also provided eutopic tissues, and 2 who only provided ectopic tissues), were subjected to virtually multiplexed transcript abundance (VMTA) analysis. For comparison, endometrial biopsies from six healthy women without endometriosis also were analyzed. Tissues were obtained at various times during the menstrual cycle, and examined histologically to confirm the presence of endometrial glands and stroma and to classify the menstrual phase of the tissue. This study was approved by our institutional review board, and subjects gave written informed consent.

Endometrial Cell Preparation and Culture

Endometrial tissues were subjected to a single 30–40-minute cycle of digestion with a mixture of collagenase (0.14% w/v) and DNAse (0.1% w/v) in Hanks' balanced salt solution (HBSS; Whittaker, Walkersville, MD) at 37°C to prepare single cells. Following digestion, ECs were filtered through sterile PeCap polyester mesh (Tetko, Inc., Briarcliff Manor, NY) and collected by centrifugation. Eutopic ECs were separated from other cell types by centrifugation over 50% Percoll (this was not done with the ectopic cells, which came from exophytic lesions that were devoid of inflammatory cells). Thereafter, eutopic and ectopic ECs were resuspended in RPMI-1640 medium containing 5% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from GIBCO, Grand Island, NY), and $5 \times$ to 10×10^6 cells were cultured in 5 mL medium ($1 \times$ to 2×10^6/mL) in a 60-mm Falcon tissue culture-treated dish (BD Biosciences, San Jose, CA) and incubated at 37°C for 24 hours to facilitate isolation of viable, nonnecrotic cells for analysis.

Cultures were harvested by sequential pipetting and washing, after which cells were lysed in 1–2 mL of RNAzol-B (Tel-Test, Inc., Friendswood, TX), and samples were frozen at −80°C until RNA extraction. Attached cells were >90% ECs, and contained both stromal cells and glandular epithelial cells based on morphology. No attempt was made to separate stromal cells from epithelial cells, and thus the results represent the responses obtained with a mixture of both EC types.

RNA Extraction, cDNA Preparation, and VMTA Measurement

Total RNA was extracted according to the protocol provided by the manufacturer of RNAzol-B (Tel-Test, Inc.), and RNA was reverse-transcribed into complementary DNA (cDNA) with random Oligo$(dT)_{15}$ primers and AMV reverse transcriptase (Promega Corp., Madison, WI). The cDNA samples were subjected to VMTA measurement of expression of the proapoptotic genes BCLxS, Caspase-1 (apoptosis-related cysteine protease), and P53, as well as expression of the antiapoptotic genes BCL-2, BCLxL, and DAD-1 (defender against cell death-1). In addition, VMTA measurement of the proliferating cell nuclear antigen (PCNA) gene was performed to assess the proliferative state of the ECs coincident with the expression of the apoptosis-regulating genes.

Virtually multiplexed transcript abundance measurement of gene expression was performed using the standardized reverse transcriptase polymerase chain reaction (StaRT-PCR) method (6–8) (Gene Express, Inc., Toledo, OH). For each gene, TA measurement was made relative to an internal standard within a standardized mixture of internal standards (SMIS). After reverse transcription, each cDNA sample was diluted so that the native template (NT)/competitive template (CT) β-actin ratio was close to 1.0 when 6×10^4 or 6×10^3 β-actin CT molecules were included in the PCR reaction.

A master mix was prepared containing RNAse-free water, $MgCl_2$ buffer, deoxyribose nucleotide triphosphates (dNTPs), the amount of complementary deoxyribonucleic acid (cDNA) in balance with 6×10^3 or 6×10^4 β-actin molecules, the amount of CT mixture D that contained 6×10^3 or 6×10^4 β-actin CT molecules, and Taq polymerase. The master mix was placed into tubes containing primers for individual genes, and cycled in a Rapidcycler (Idaho Technology, Inc., Idaho Falls, ID). The denaturing temperature was 94°C, the annealing temperature was 58°C, and the elongation temperature was 72°C for each cycle. After amplification, each PCR product was analyzed by microfluidic capillary electrophoresis on an Agilent 2100 Bioanalyzer machine (Agilent Technologies, Wilmington, DE). The area under the curve for the PCR product of each NT was compared to that of its respective internal standard (CT) to determine gene expression values. The unit for each expression value was molecules/per 10^6 β-actin molecules.

Experience with the StaRT-PCR method has demonstrated that the coefficient of variation in the test is on the order of 10% with the automated method, and can detect changes in messenger RNA (mRNA) level ≥2-fold. Due to variations in menstrual phase of the donors, and the limited sample size, the data were not normally distributed. Therefore, the data were analyzed statistically by the nonparametric Wilcoxon rank sum test.

RESULTS

Measurment of PCNA TA in ECs From Women With and Without Endometriosis

The PCNA TA level was used as a basis for comparing the proliferative state of ECs from the different populations. Each population exhibited comparable PCNA TA levels

when the data were grouped into the following categories: eutopic controls (n = 6; mean ± SD = 8.2 ± 5 × $10^3/10^6$ molecules β-actin mRNA); eutopic endometriosis (n = 10; 5.1 ± 5 × $10^3/10^6$ β-actin molecules, not significant compared to group 1); and ectopic endometriosis (n = 6; 4.2 ± 2 × $10^3/10^6$ β-actin molecules, not significant compared to group 1 or 2). The data also demonstrate that matched eutopic and ectopic endometrial cells (n = 4) exhibited a similar PCNA TA level as a group, with eutopic values greater than ectopic values for two donors, and less than ectopic values for the other two donors. Thus, EC preparations from the donor populations were shown to have comparable proliferative activities.

Expression of BCL2, BCLxL, and BCLxS in ECs From Women With and Without Endometriosis

Transcript abundance levels of the antiapoptotic BCL2 gene (9, 10) in each study population varied with the menstrual cycle, and this variation was greater than the variation among groups. Thus, BCL2 TA level did not distinguish control subjects from women with endometriosis in this study.

This was not the case, however, when results for the proapoptotic and antiapoptotic isoforms of the BCL-X gene were considered (Fig. 1). The results are presented as the ratio of TA levels for BCL-xL:BCL-xS (antiapoptotic:proapoptotic), because BCLxL opposes the action of BCLxS, and the ratio of these two isoforms is thought to play a major role in setting the "apoptotic threshold" of various cell types (11–13). The BCLxL:BCLxS ratio was substantially higher in eutopic endometria from women with endometriosis compared to endometria from women without endometriosis ($P<.05$). This difference was most pronounced in the midproliferative through midsecretory phases of the menstrual cycle, with minimal differences found with samples collected toward the end of the cycle.

The results with ectopic ECs from women with endometriosis were even more striking, revealing that the BCL-xL: BCL-xS mRNA ratio greatly favors the BCL-xL gene, regardless of the menstrual phase of the cells. Thus, the amount of BCL-xL mRNA exceeded the amount of BCL-xS gene by 4–6.5-fold among the six ectopic EC specimens tested ($P<.01$ for ectopic ECs versus eutopic ECs from endometriosis patients or controls, respectively; Fig. 1).

FIGURE 1

Quantitative TA measurement of the antiapoptotic and proapoptotic isoforms of the BCLX gene, designated BCLxL and BCLxS, respectively, in ECs from women with or without endometriosis. Data are plotted as the ratio of BCLxL:BCLxS for individual samples against the time of collection relative to the menstrual cycle.

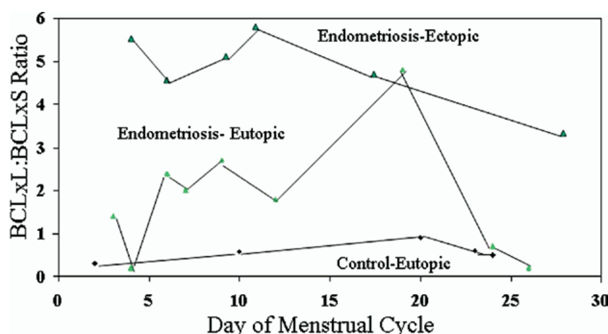

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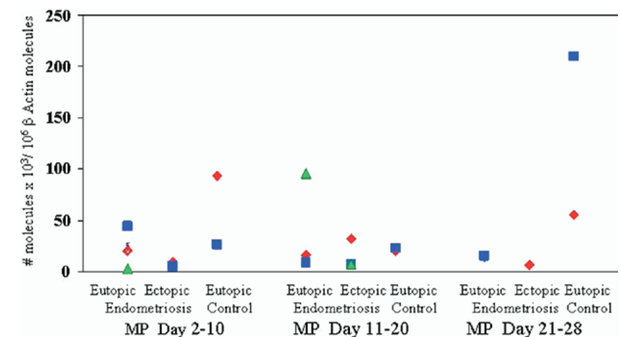
FIGURE 2

Quantitative TA measurement of the proapoptotic gene Caspase-1 in ECs from women with or without endometriosis. Individual values and times of collection relative to the menstrual cycle are shown for eutopic endometrium from women with (n = 10) or without (n = 6) endometriosis, and for ectopic endometrium from women with endometriosis (n = 6).



Braun. Apoptosis genes in endometrium. Fertil Steril 2007.

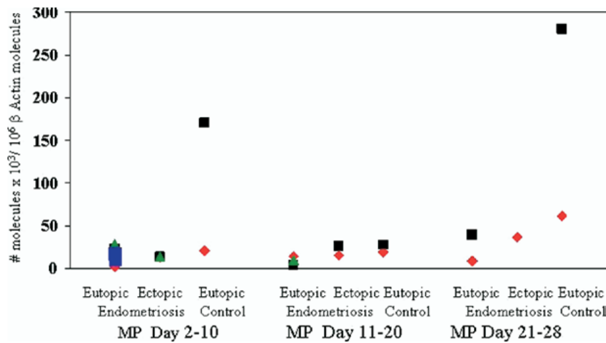
Expression of Additional Proapoptotic Genes in ECs From Women With and Without Endometriosis

With respect to the apoptosis-related cysteine protease, Caspase-1, there was an apparent cyclicity of expression, with increased values being seen in the early and late phases of the cycle in eutopic ECs from control subjects (Fig. 2). This cyclic pattern of expression was not seen with eutopic endometrium from endometriosis patients, which also demonstrated reduced Caspase-1 TA levels compared to controls ($P<.05$), primarily in late-phase samples. Similarly, there was no apparent cyclicity in Caspase-1 TA levels in ectopic samples, with levels of expression substantially lower than in control specimens ($P<.05$).

Comparable results were seen for the apoptosis-mediating gene, p53 (Fig. 3). The p53 TA levels were cyclical in eutopic ECs from controls, with greater levels in the early and late phases of the cycle compared to levels in the middle of the cycle. Furthermore, p53 TA levels did not fluctuate

FIGURE 3

Quantitative TA measurement of the proapoptotic gene p53 in ECs from women with or without endometriosis. Individual values and times of collection relative to the menstrual cycle are shown for eutopic endometrium from women with (n = 10) or without (n = 6) endometriosis, and for ectopic endometrium from women with endometriosis (n = 6).



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relative to the menstrual cycle in either eutopic or ectopic ECs from women with endometriosis, with TA levels reduced significantly compared to control specimens ($P<.01$ and .05, respectively).

Expression of the Antiapoptotic Gene DAD-1 in ECs From Women With and Without Endometriosis

Transcript abundance levels for the antiapoptotic gene DAD-1 were also quantified in ECs from the different study populations (Fig. 4). The results demonstrate cyclicity of expression for this gene, with increased levels in early and late phases of the cycle, for eutopic endometrium from both control subjects and women with endometriosis. In the majority of specimens, levels expressed in endometriosis patients were greater than levels expressed during the same phase of the menstrual cycle by control subjects, but this difference was not statistically significant. In contrast, expression of the DAD-1 gene in ectopic endometrial samples from women with endometriosis was not cyclical, and was reduced considerably compared to the expression in eutopic endometrial samples from women with or without endometriosis ($P<.001$ and .01, compared to eutopic ECs from women with and without endometriosis, respectively).

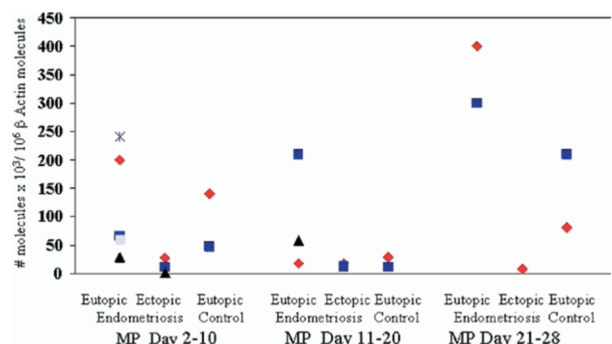
DISCUSSION

In this study, we observed that genes controlling cell survival and apoptosis were expressed at quantitatively different levels in endometrial tissues from endometriosis patients compared to those from women without endometriosis. These findings support previous studies from this and other laboratories that endometrium from women with endometriosis is less sensitive to apoptosis than normal. In most control samples, TA levels varied according to the cyclical eutopic endometrial differentiation pattern associated with the menstrual phase of the donor. This is consistent with results demonstrating that the balance between BCL-2 and related BCL-x genes, and the proapoptotic BAX gene, fluctuates to favor cell survival in the proliferative phase while preparing cells to undergo apoptosis in the secretory phase (10). In women with endometriosis, this cyclical gene expression pattern was not observed for either proapoptotic or antiapoptotic genes in eutopic or ectopic ECs. These results provide further evidence for an intrinsic abnormality in apoptotic sensitivity of the endometrium of women with endometriosis.

With respect to genes of the BCL2 family, we were unable to detect a statistically significant difference between women with and without endometriosis for the BCL2 gene with either eutopic or ectopic tissues. This may be attributable to the limited number of samples available for study, since others showed increased BCL2 expression and reduced apoptosis in proliferative-phase endometrium from women with endometriosis compared to controls (2). However, our results are consistent with those reported by Goumenou et al., who compared apoptosis with BCL-2 and Bax expression in glandular and stromal cells from women with ovarian endometriosis (11). In that study, stromal cells showed significantly increased apoptosis, but significantly reduced expression of BCL-2 and Bax proteins compared to the glandular cells, leading the investigators to suggest that there is no relationship between BCL-2 and Bax expression and apoptosis in endometriotic tissues.

FIGURE 4

Quantitative TA measurement of the antiapoptotic gene DAD-1 in ECs from women with or without endometriosis. Individual values and times of collection relative to the menstrual cycle are shown for eutopic endometrium from women with (n = 10) or without (n = 6) endometriosis, and for ectopic endometrium from women with endometriosis (n = 6).



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The most striking finding in endometriosis samples seen in this study was the increased TA level of antiapoptotic BCLxL mRNA relative to proapoptotic BCLxS mRNA. Because the relative amounts of the long and short isoforms of the BCL-X gene are thought to reflect the overall apoptotic threshold in different tissues (12–14), the current results are consistent with an overall apoptotic resistance of ECs from women with endometriosis. This pattern was observed for the majority of eutopic samples collected at different times during the menstrual cycle, but notably, was not observed for samples collected toward the end of the cycle. In those samples, BCLxL and BCLxS mRNA expression levels were roughly equivalent and comparable to the values determined for eutopic samples from controls without endometriosis, suggesting the acquisition of a more "normal apoptotic threshold" toward the end of the cycle.

Given those considerations, the data for expression of BCL-X gene isoforms in ectopic tissues from women with endometriosis are striking. In those cells, expression of the BCLxL gene exceeded that of the BCLxS gene by >4-fold, regardless of the menstrual phase of the donor, and for each of the six samples tested. While it is important to appreciate that the number of samples analyzed is limited, the consistency of this result provides some of the most compelling molecular genetic evidence to support functional data demonstrating apoptotic resistance of ectopic ECs from women with endometriosis. Furthermore, the lack of cyclicity with the menstrual cycle for BCLxL:BCLxS expression suggests that the survival of these tissues is not subject to regulation by the ovarian hormones. This is consistent with results reported by Fauvet et al. for the expression of various apoptosis-regulating proteins, including BCLxL, in endometriomas (15).

The expression of p53 and caspase-1 mRNA was cyclical in control ECs, with the greatest levels exhibited during the early and late phases of the menstrual cycle. Cyclicity of expression for these genes was not observed with eutopic or ectopic ECs from women with endometriosis, leading to significantly decreased TA levels in each tissue. Because BCLxL expression is regulated by various proapoptotic genes such as p53 and Caspase-1 (16, 17), the decreased expression of these genes in ECs from endometriosis patients is consistent with the elevated BCLxL expression and BCLxL:BCLxS ratios that were documented in this study.

The DAD-1 gene was originally described as a BCL2 homologue that behaves similarly to BCL2 and BCLxL in regulating cellular apoptosis. The subcellular localization of the DAD-1 protein in mitochondrial and smooth endoplasmic reticulum membranes is critical to its antiapoptotic role (18). Other studies documented that the DAD-1 protein functions as part of the oligosaccharyl transferase complex responsible for n-glycosylation of mitochondrial proteins (19). In some tissues, DAD-1 binds to MCL-1, a BCL2-like molecule that also mediates protection against apoptosis (20).

In the present study, DAD-1 mRNA expression was cyclical in eutopic endometria from women with and without endometriosis, with greater levels seen during early and later phases of the cycle. Although DAD-1 gene expression was increased in eutopic cells of endometriosis patients relative to controls in some cases, this was not true for all specimens, and therefore significant differences between endometriosis and control specimens were not shown for eutopic ECs. In contrast, significantly decreased TA levels for the DAD-1 gene were shown for ectopic ECs compared to eutopic cells from women with or without endometriosis. Thus, we conclude that the DAD-1 protein does not play a significant role in protecting ectopic ECs from apoptosis in women with endometriosis.

The results of this study provide at least a partial molecular genetic basis for the reduced apoptotic sensitivity of ECs reported for women with endometriosis. These results also suggest that some of the genes that are critical for apoptotic resistance are upregulated in both eutopic and ectopic endometrium, while others may be more important for one tissue or the other. The substantially increased levels of BCLxL, in conjunction with the substantially reduced levels of Caspase-1 and p53 that were observed in ECs from women with endometriosis regardless of phase of the menstrual cycle, provide a molecular genetic foundation for both the resistance of these cells to apoptosis, and their apparent resistance to elimination by peritoneal macrophages and other homeostatic mechanisms within the peritoneal cavity.

The fact that a molecular genetic basis for apoptotic resistance can be detected in eutopic endometrium from patients suggests that there may be a predisposition to develop endometriosis in at least some women, which precedes the development of an inflammatory pelvic and peritoneal environment. Whether these molecular genetic capacities are inherited or acquired as a consequence of somatic events elicited by environmental factors cannot be elucidated from the results of the present study. Nevertheless, the molecular genetic results presented herein substantiate the notion that ECs from women with endometriosis have an intrinsic ability to survive and thrive in ectopic environments.

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