Effect of aromatase inhibitors on ectopic endometrial growth and peritoneal environment in a mouse model of endometriosis

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Objective: To evaluate the effect of aromatase inhibitors on ectopic endometrial growth and on the release of proangiogenic and proinflammatory factors in peritoneal fluid (PF).
Design: Prospective experimental study.
Setting: Animal research and laboratory facility.
Animal(s): Female Balb/c mice 2 months of age.
Intervention(s): Mice had surgery performed to induce endometriosis-like lesions. Treatment with anastrozole or letrozole was started on either postoperative day 1 or 28 and continued for 4 weeks.
Main Outcome Measure(s): Endometriotic lesions were counted and measured and aromatase expression, cell proliferation, and apoptosis were assessed. Vascular endothelial growth factor (VEGF) and prostaglandin E (PGE) levels were evaluated in the PF.
Result(s): Endometriosis-like lesions express aromatase P-450. Treatment with either anastrozole or letrozole did not prevent lesion establishment; however, it significantly decreased the size of the endometriotic lesion. When treatment was initiated on postoperative day 1, letrozole and anastrozole decreased cell proliferation and increased apoptosis. When treatment was started on postoperative day 28, both aromatase inhibitors decreased cell proliferation, but only anastrozole augmented apoptosis levels. In addition, letrozole reduced VEGF and PGE levels in PF. Anastrozole diminished VEGF content but did not cause any significant change in PGE levels.
Conclusion(s): These findings support the further investigation of aromatase inhibition as a treatment option for endometriosis. (Fertil Steril® 2010;93:2513–8. ©2010 by American Society for Reproductive Medicine.)

Key Words: Endometriosis, aromatase inhibitors, PGE, VEGF

Endometriosis is a common gynecological disorder characterized by the presence of endometrial tissue outside the uterine cavity and associated with pelvic pain and infertility (1).

One of the most recent significant findings has been the discovery that ectopic endometrium expresses high levels of the aromatase P-450 (2, 3). This enzyme is critical for estrogen (E) biosynthesis as it catalyzes the rate-limiting step, the conversion of androstenedione (A) to estrone (E_1) and T to E_2 (4). In addition, it has been observed that aromatase expression is higher in eutopic endometrium from women with endometriosis compared with control women (2, 3, 5–7).

Bulun et al. (4) demonstrated that a molecular link exists between inflammation and E production in endometriosis. Prostaglandin E_2 (PGE_2) is the most potent inducer of aromatase expression and activity in endometriosis-derived stromal cells in vitro (8). In addition, it has been shown that E_2 induces Cliooxygenase-2 (COX-2) expression and PGE_2 production (9). These mechanisms comprise a positive feedback cycle that favors the continuous local production of E_2 and PGE_2 in endometriotic lesions (10).

In addition, it is known that both E_2 and PGE_2 induce the expression of vascular endothelial growth factor (VEGF) and, in turn, VEGF induces the expression of COX-2 (11–13). Vascular endothelial growth factor is one of the most important angiogenic factors involved in the pathogenesis of endometriosis (14, 15). It has been observed that VEGF expression is increased in eutopic and ectopic endometrium from patients with endometriosis (16, 17). Furthermore, VEGF levels are significantly higher in the peritoneal fluid (PF) of these patients (18).

Aromatase inhibitors are currently used as adjuvant and first-line treatment for breast cancer (19–21). Takayama et al. (22) were the first to report on the successful use of the aromatase inhibitor anastrozole in a postmenopausal woman who presented recurrent severe endometriosis and who previously failed to respond to conventional treatments. Recently, other investigators have used aromatase inhibitors to treat cases of endometriosis (23–28). In a previous work we observed that the aromatase inhibitors letrozole and anastrozole induce apoptosis and reduce cell proliferation in epithelial endometrial cells from women with endometriosis (29).

Based on the data reviewed, the aim of this study was to evaluate the effect of the aromatase inhibitors letrozole and anastrozole on lesion establishment, cell proliferation, and apoptosis of ectopic endometrial tissue and on the release of proangiogenic and proinflammatory factors in the PF of mice with experimentally induced endometriosis.

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MATERIALS AND METHODS

Animals

Balb/c female mice were used. All animals were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Surgery

At the time of surgery, all mice were 2 months of age. We induced endometriosis-like lesions through transplantation of one of the uterine horns to the bowel mesentery, as described previously (30). Briefly, equal pieces of right uterine tissue measuring approximately 4 mm^2 were sutured with the serosal layer in direct apposition to the peritoneum close to a vessel with the endometrial layer of the uterine square facing the serosa. A single suture (supralong 6-0) was used for each piece of tissue.

Animal Treatment

Animals were randomly assigned to experimental groups of 12 animals each. Initially, mice were divided into three groups: anastrozole, letrozole, and control. All mice receive a daily SC injection of anastrozole 10 μg (0.5 mg/kg), letrozole 10 μg (0.5 mg/kg), or saline, respectively. All treatment periods were 4 weeks in duration and the mice were monitored daily. No evidence of toxicity was noted at the doses administered based on body weight, food consumption, grooming behavior, or activity levels compared with controls. Treatment was initiated on postoperative day 1 to determine the effects of aromatase inhibitors on disease establishment and progression. In the studies to determine the effects on established lesions, treatment with aromatase inhibitors was delayed to postoperative day 28 and continued for 4 weeks.

Evaluation of Ectopic Uterine Tissue

After 4 weeks of treatment, animals were sacrificed by cervical dislocation. Lesions were identified, counted, and measured in two perpendicular diameters using a caliper. The volume of each ectopic uterine tissue was calculated by the following formula: $V = (4/3)\pi\, r_1^{\,2}\, r_2$, where r_1 and r_2 are the radiuses and $r_1 < r_2$ (31). The lesions, the left uterine horn, and bilateral ovaries were then dissected away and fixed in 10% buffered formaldehyde. Formalin-fixed specimens of ectopic tissue were paraffin-embedded, cut into 5-μm sections, and stained with hematoxylin and eosin (H & E). Sections were examined microscopically for the presence of histologic hallmarks of endometriosis.

Immunohistochemistry

Immunohistochemical staining was performed as described previously (32). The following primary antibodies were used: an antimouse proliferating cell nuclear antigen (PCNA) rabbit polyclonal antibody (1:300, FL-261; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and an antimouse aromatase rabbit polyclonal antibody (diluted 1:2,000, produced by Dr. I. Azcoitia, Madrid, Spain) generated from a 15-amino-acid peptide corresponding to residues 488–502 of mouse aromatase (33). As a negative control, immunoglobulin of the same immunoglobulin class and concentration as the primary antibody was used.

The number of cells expressing immunoreactivity for PCNA per 100 cells (PCNA index) was established using a standard light microscope by two independent observers. The total number of epithelial cells in 10 representative fields was counted. Any nuclear staining was regarded as positive.

Apoptosis Detection System

For apoptosis quantification, ectopic tissue sections were processed for in situ immunocytochemical localization of nuclei exhibiting DNA fragmentation, by the technique of terminal deoxynucleotidyl transferase-mediated dUTP digoxygenin nick-end labeling (TUNEL), using an apoptosis detection kit (Chemicon International, Temecula, CA). Sections were treated according to the manufacturer's instructions and as previously described (32). Slices of female rodent mammary gland obtained 3–5 days after weaning of pups were used as a positive control. As a negative control, a number of tissue samples were subjected to treatment without terminal deoxynucleotidyl transferase. The percentage of apoptotic cells were determined by counting labeled cells at $\times$400 in 30 randomly selected and homogeneous fields.

Also, apoptotic cells were identified by their characteristic morphological features in H & E-stained endometrial sections (32).

Quantification of VEGF

After 4 weeks of treatment, animals were sacrificed by cervical dislocation. Peritoneal fluid was collected by rinsing the abdominal cavity with 1 mL of saline and assayed for VEGF using a commercial ELISA kit (MMV00, R&D Systems Inc., Minneapolis, MN). The sensitivity level for the VEGF ELISA was 3 pg/mL. The intra-assay variability for VEGF was $\pm$4.3%, whereas the interassay variability was $\pm$5.7%. All samples were assessed in triplicate.

Radioimmunoassay for PGE

The PGE levels were evaluated by RIA as described previously (34). Briefly, the peritoneal liquid was acidified to pH 3.0 with 1 M of HCl and extracted for PGE determination three times with 1 volume of ethyl acetate. Pooled ethyl acetate extracts were dried under an atmosphere of N_2 and stored at -20°C until prostaglandin RIA was performed. The PGE levels was quantified by using rabbit antiserum from Sigma Chemical Co. (St. Louis, MO) Sensitivity was 10 pg/tube and cross-reactivity was <0.1% with other prostaglandins. Results were expressed as picograms per milliliter.

Statistics

Statistical comparisons were performed by Kruskal-Wallis nonparametric analysis of variance (ANOVA) test, followed by Dunn's multiple comparison test. Regardless of the statistical test, only a P value $\leq$.05 was considered significant.

RESULTS

Aromatase Expression in Mouse Ectopic Endometrium

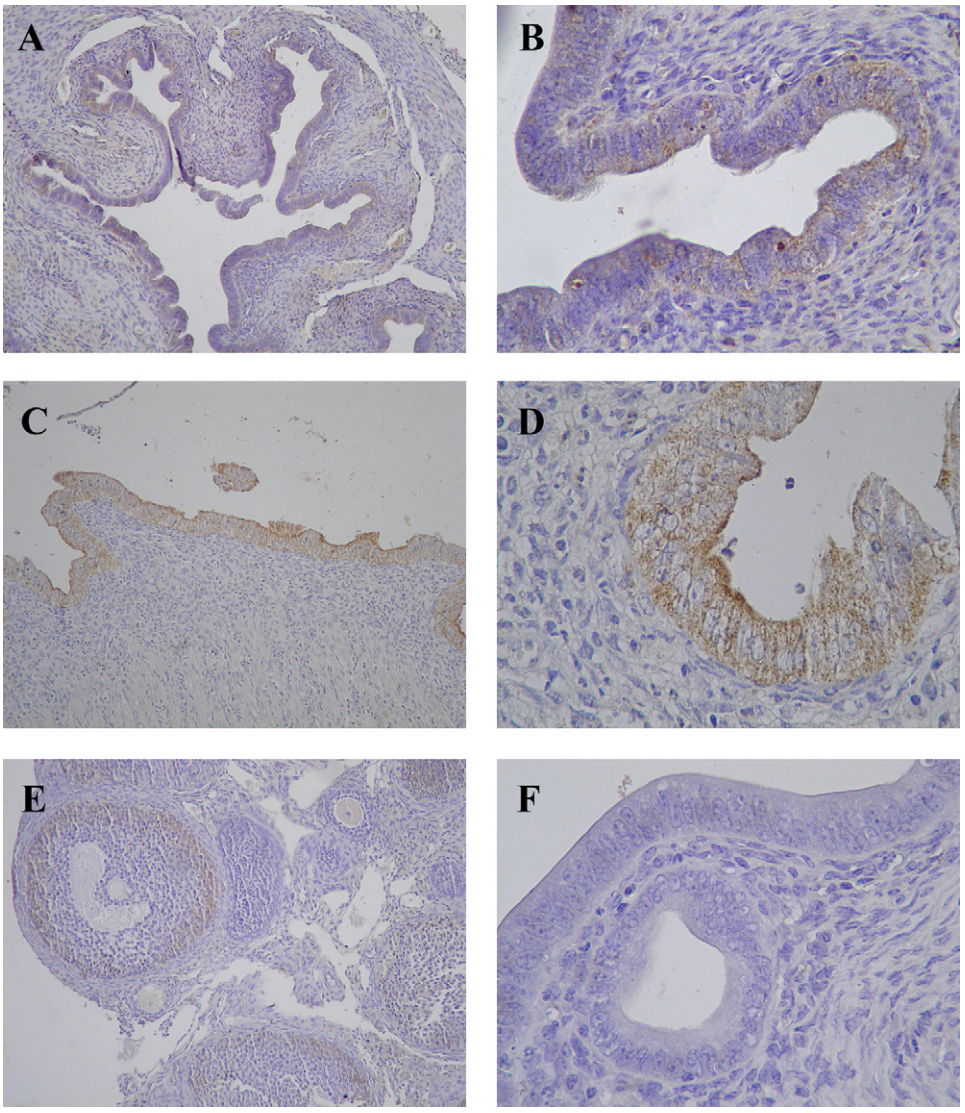
Aromatase expression was evaluated by immunohistochemistry in endometriosis-like lesions from control mice. Aromatase was detected in 64% of lesions analyzed (n = 22). Aromatase was immunolocalized in the cytoplasm of glandular cells (Fig. 1A–D). Immunoreactivity was not detected in stroma. Different levels of aromatase expression were observed: low aromatase expression was detected in 57% of positive lesions (Fig. 1A, B) and high aromatase expression was seen in 43% of positive lesions (Fig. 1C, D).

Effects of Aromatase Inhibitors on Ectopic Uterine Tissue Growth

The effect of anastrozole and letrozole on endometriotic lesions was evaluated in two stages of lesions growth: [1] on lesion establishment (treatment was started on postoperative day 1) and [2] on established lesions (treatment was started on postoperative day 28). The percentage of mice that developed lesions and the number of lesions developed per mouse were similar in all groups (data not shown). However, both anastrozole and letrozole diminished the size of endometriosis-like lesions compared with the control group when treatment was initiated on postoperative day 1 (P<.05 and P<.001, respectively) (Fig. 2A) and when treatment was initiated in postoperative day 28 (P<.05 vs. control) (Fig. 2B).

FIGURE 1

Aromatase expression in endometriosis-like lesions. Endometriosis-like lesions from control mice were dissected away, formalin fixed, paraffin embedded, and cut into sections. Aromatase expression was evaluated by immunohistochemistry using a polyclonal antibody. Low (**A** and **B**) and high (**C** and **D**) aromatase expression was seen in mouse ectopic endometrium. Mouse ovary sections were used as positive control (**E**). In negative control (**F**) immunoglobulin of the same immunoglobulin class and concentration as the primary antibody was used. Magnification, ×100 (**A, C,** and **E**); ×400 (**B, D,** and **F**).



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Effect of Aromatase Inhibitors on Epithelial Cell Proliferation and Apoptosis of Ectopic Uterine Tissue

Treatment with both aromatase inhibitors caused a decrease in epithelial cell proliferation and an increased in apoptotic index compared with the control group (Fig. 3). When treatment was started on postoperative day 1, the percentage of PCNA-positive cells decreased after treatment with anastrozole ($P<.001$ vs. control) and after treatment with letrozole ($P<.01$ vs. control) (Fig. 3A). Complementary to these results the percentage of apoptotic epithelial cells increased in the lesions from mice treated with anastrozole ($P<.05$ vs. control) and in the lesions from mice treated with letrozole ($P<.01$ vs. control) (Fig. 3B).

Similar results were observed when treatment was initiated on postoperative day 28. The percentage of PCNA-positive cells decreased in anastrozole and letrozole groups compared with controls ($P<.01$) (Fig. 3A). In addition, the percentage of apoptotic epithelial cells increased after treatment with anastrozole ($P<.05$ vs. control) (Fig. 3B). Treatment with letrozole caused an increase in the percentage of apoptotic cells, but it was not statistically significant ($P>.05$ vs. control) (Fig. 3B).

FIGURE 2

Effect of aromatase inhibitors on endometriotic lesion size. Mice with surgically induced endometriosis were treated either with anastrozole, letrozole, or saline (control) starting on postoperative day 1 (**A**) or 28 (**B**). After 4 weeks of treatment mice were sacrificed and endometriotic lesions were measured. *$P<.05$ vs. control; ***$P<.001$ vs. control.

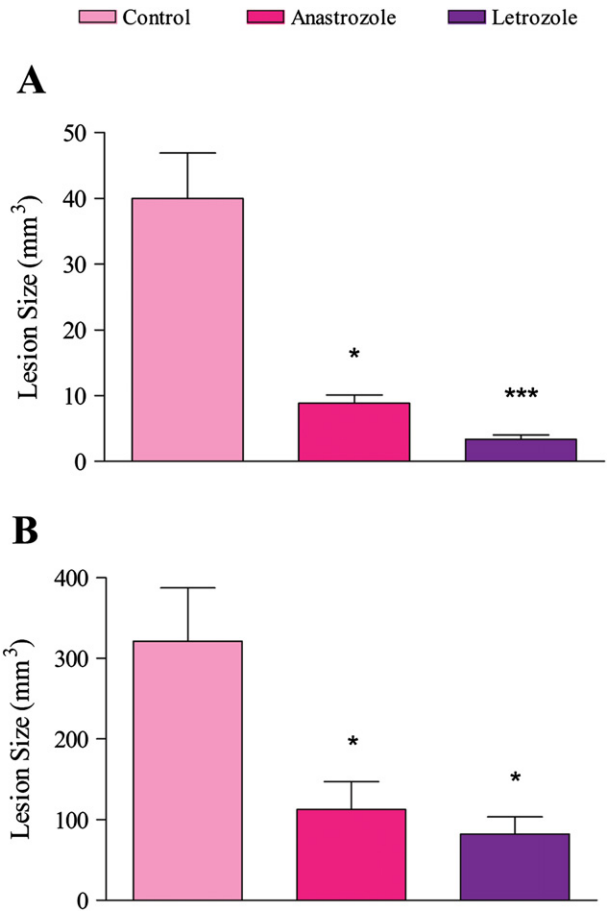

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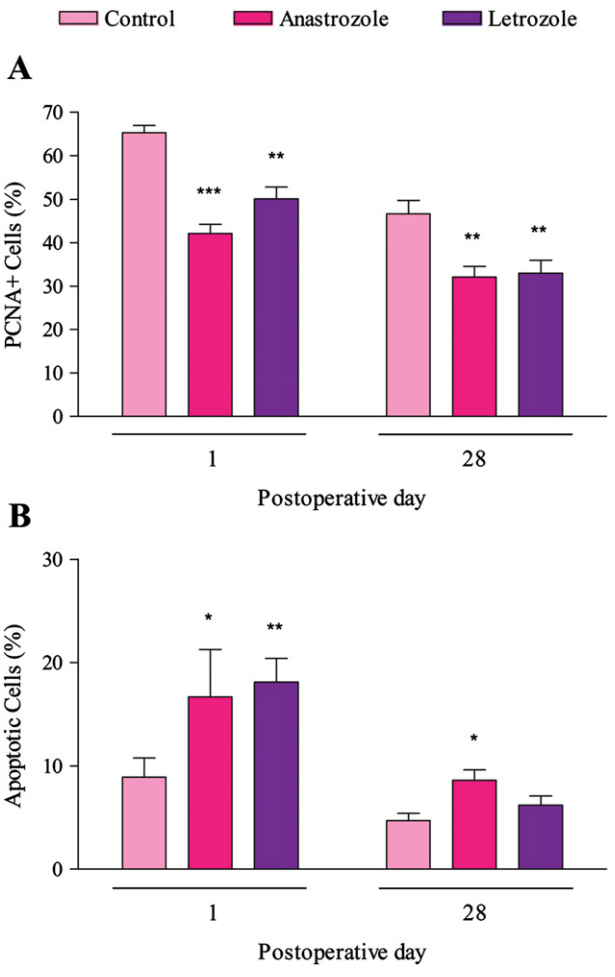
FIGURE 3

Effect of aromatase inhibitors on epithelial cell proliferation (PCNA+) (**A**) and apoptosis (**B**) of ectopic uterine tissue. Mice with surgically induced endometriosis were treated either with anastrozole, letrozole, or saline (control) starting on postoperative day 1 or 28. After 4 weeks of treatment mice were sacrificed and endometriosis-like lesions were dissected away, formalin fixed, paraffin embedded, and cut into sections. Cell proliferation (**A**) was evaluated by immunohistochemistry for PCNA and apoptosis (**B**) was evaluated by terminal deoxynucleotidyl transferase-mediated dUTP digoxygenin nick-end labeling (TUNEL). *$P<.05$ vs. control; **$P<.01$ vs. control; ***$P<.001$ vs. control.



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Effect of Aromatase Inhibitors on VEGF and PGE Levels in PF

Treatment with either anastrozole or letrozole started on postoperative day 28 decreased the levels of VEGF in the PF ($P<.05$ vs. control) (Fig. 4A). When treatment was initiated on postoperative day 28, the levels of PGE were decreased after letrozole administration compared with the control group ($P<.001$) (Fig. 4B). However, nonsignificant changes were observed in PGE levels after treatment with anastrozole ($P>.05$ vs. control) (Fig. 4B).

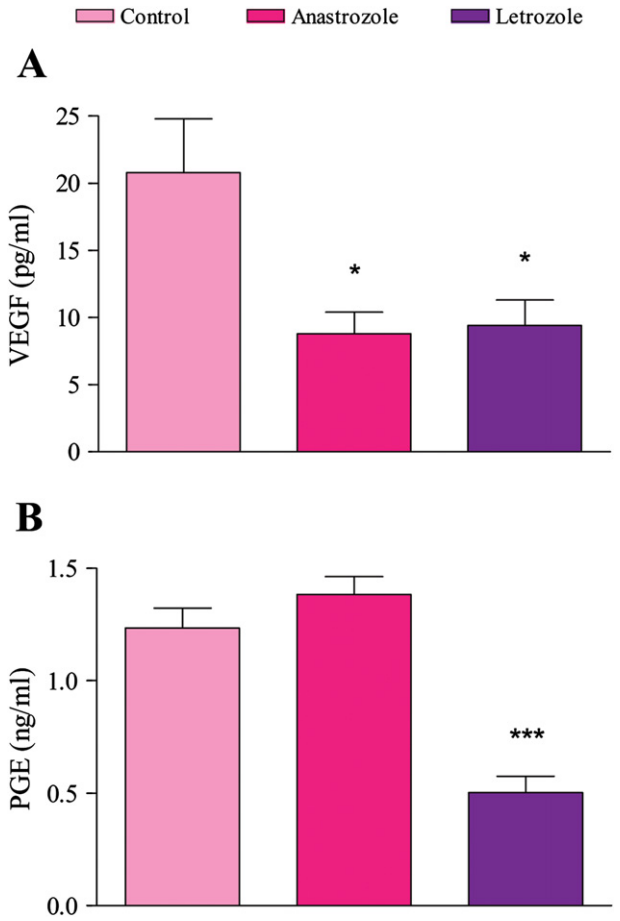
DISCUSSION

In the present study we evaluated the effect of anastrozole and letrozole on the establisment of endometriotic lesions and on established lesions in an endometriosis mouse model. We have observed that both anastrozole and letrozole did not prevent the establishment of endometriotic lesions but caused a significant decrease in their size. These results are in agreement with the ones observed by Fang et al. (30), who demonstrated that letrozole reduces endometriotic lesion size in a mouse model of endometriosis. Likewise, aromatase inhibitors relieved pain symptoms and decreased endometriotic lesion size in patients with endometriosis (22–28).

In the present work we observed that endometriosis lesions at different stages of their development (during and after establishment) responded in a similar way to treatment with aromatase inhibitors. In this study we have demonstrated that anastrozole and letrozole decreased cell proliferation in the epithelial fraction

FIGURE 4

Effect of aromatase inhibitors on vascular endothelial growth factor (VEGF) (**A**) and prostaglandin E (PGE) (**B**) levels in peritoneal fluid (PF). Mice with surgically induced endometriosis were treated either with anastrozole, letrozole, or saline (control) starting on postoperative day 28. After 4 weeks of treatment mice were sacrificed and the PF was collected. The levels of VEGF (**A**) were assessed by ELISA and the levels of PGE (**B**) were determined by RIA. *$P<.05$ vs. control; ***$P<.001$ vs. control.



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of endometriotic lesions in both stages of development. Also, we observed that anastrozole increases apoptosis levels in these cells in both experimental designs. Although letrozole increased apoptosis in the epithelial fraction of endometriotic lesions in both experimental designs, this effect was only statistically significant when treatment was initiated on postoperative day 1. There are reports of studies of the antiproliferative and proapoptotic effect of aromatase inhibitors in vitro and in vivo. It has been demonstrated that the treatment with letrozole diminishes cell proliferation in endometriotic lesions in a murine model of endometriosis (30). Furthermore, it has been observed that letrozole decreases cell proliferation and increases apoptosis in models of breast cancer (35, 36). Dowsett et al. (37) proved that the treatment with anastrozole decreases cell proliferation in breast cancer tumors, but that it has no effect on apoptosis. In addition, in previous work from our laboratory, we observed that treatment with anastrozole and letrozole diminishes cell proliferation and increases apoptosis levels in epithelial endometrial cells from patients with endometriosis in vitro (29). Other investigators have observed similar effects in uterine leiomyoma cells and in epithelial cells from breast cancer tumors in vitro (36, 38).

Our results demonstrate that the treatment with anastrozole and letrozole caused a decrease in VEGF levels in the PF of mice with surgically induced endometriosis. However, although letrozole was able to diminish the levels of PGE in the PF, the treatment with anastrozole had no significant effect. In agreement with our results, Bottini et al. (39) observed that letrozole decreases the expression of VEGF in patients with breast cancer. In addition, Weems et al. (40) demonstrated that treatment with an aromatase inhibitor decreases the plasma levels of PGE_2 in ovariectomized pregnant ewes.

As mentioned, we have observed that letrozole diminishes the levels of PGE in the PF, whereas anastrozole has no significant effect. Different effects of letrozole and anastrozole have been observed by other investigators. Letrozole appears to be more potent than anastrozole, both in vivo and in vitro (41). Also, it has been demonstrated that letrozole is more effective than anastrozole in inducing tumor regression, as well as in suppressing serum E levels and in inhibiting aromatization (31, 41). These differences between anastrozole and letrozole may be related to differences in pharmacokinetics as well as in effects on lipid levels and adrenosteroidogenesis (42).

In the present work we used daily doses of letrozole and anastrozole that were higher than the ones routinely used for endometriosis treatment (24, 26), but were similar to the doses used by other investigators in endometriosis mouse models and in breast cancer models in nude mice (30, 31, 43). Also it must be taken into account that in this study anastrozole and letrozole were administered alone. Administration of aromatase inhibitors to treat endometriosis in premenopausal women is always combined with other agents to suppress pituitary function (25–28).

In the present work we demonstrated that aromatase is expressed in endometriosis-like lesions in this mouse model of endometriosis. Immunoreactivity for aromatase was localized exclusively in the glands but not present in the stroma. This pattern of aromatase expression was observed in human ectopic endometrium by other investigators (3, 7). However, Matsuzaki et al. (5) have seen aromatase expression in the glandular and stromal fraction of human endometriotic lesions.

We have not analyzed the role of local aromatase expression in this model because we did not use ovariectomized mice to mimic endometriosis in premenopausal women. Although we cannot discard a local effect of aromatase inhibitors in endometriotic lesion, more studies are needed to demonstrate it.

In summary, our work suggests that aromatase inhibitors have a beneficial effect on endometriotic lesion growth. The treatment with these compounds decreases the size of endometriotic lesions by diminishing cell proliferation and increasing apoptosis levels. In addition, treatment with aromatase inhibitors normalizes the levels of proangiogenic and proinflammatory factors in the peritoneal environment. These data support the further investigation of aromatase inhibition as a treatment option for endometriosis.

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