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Gonadotropin-releasing hormone agonist induces apoptosis and reduces cell proliferation in eutopic endometrial cultures from women with endometriosis

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Objective: There is growing evidence that suggests a direct action of gonadotropin-releasing hormone agonist (GnRH-a) on endometrial growth. Consequently, our purpose was to evaluate the effect of GnRH-a on in vitro eutopic endometrial cell growth and apoptosis.

Design: Prospective study.

Setting: Research institute and clinical fertility center.

Patient(s): Sixteen women with untreated endometriosis and 14 controls.

Intervention(s): Biopsy specimens of eutopic endometrium were obtained from all subjects. Apoptosis and cell proliferation were examined in epithelial endometrial cell cultures after incubation with leuprolide acetate (LA), antide, and a combination of both.

Main Outcome Measure(s): The percentage of apoptotic cells was evaluated by the acridine orange–ethidium bromide technique; cell proliferation was assessed by ^{3}H-thymidine incorporation.

Result(s): Leuprolide acetate (LA) (100 ng/mL) enhanced apoptosis in endometrial cultures from patients with endometriosis and controls, and this effect was reversed by antide 10^{-7}M. Cell proliferation was down-regulated by LA at 1, 10, and 100 ng/mL in cultures from women without and with endometriosis. The addition of antide 10^{-7}M reversed this inhibition.

Conclusion(s): GnRH-a appears to have a direct effect by enhancing the apoptotic index and decreasing the cell proliferation in endometrial cells. (Fertil Steril® 2003;80(Suppl 2):702–7. ©2003 by American Society for Reproductive Medicine.)

Key Words: GnRH agonist, endometriosis, endometrial epithelial cells, apoptosis, cell proliferation

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Endometriosis, defined as the presence of endometrial tissue outside the uterine cavity, is one of the most common benign disorders of the female pelvis during the reproductive years. The mechanisms leading to the occurrence and progression of endometriosis are far from being completely understood, although there is growing evidence that the endometrium of women with endometriosis is not normal and behaves differently from the endometrium of women without endometriosis. Recently, a number of studies reported on the eutopic endometrium from patients with endometriosis and suggested an etiology of the possible source of the tissue ultimately responsible for the development of endometriotic implants (1).

Recent work from our laboratory showed an increased survival capability in the eutopic endometrium from patients with endometriosis, both at the epithelial and stromal levels, possibly regulated by an increased expression of Bc1-2 (2). Additionally, we found that combination oral contraceptives significantly diminished cell proliferation and induced apoptosis of eutopic endometrial tissue from patients with endometriosis (3).

Endometriotic implants are known to regress after the induction of hypoestrogenism.

To date, the rationale of most of the therapeutic agents for endometriosis, including GnRH agonists (GnRH-a) and antagonists, is to achieve a regression of the endometriotic implants through the induction of hypoestrogenism. Prolonged administration of these compounds result in down-regulation and desensitization of pituitary GnRH receptors and, therefore, in a complete suppression of gonadal function (4, 5).

There are reports indicating that GnRH-like peptides are involved in follicular apoptosis (6). Likewise, GnRH-a therapy suppresses cell proliferation in uterine leiomyomas (7). Additionally, experimental data have emerged during the past few years indicating that GnRH-a directly inhibits proliferation on various types of steroid-dependent cancers through a direct action on GnRH receptors expressed in these tumors (8, 9).

There is growing evidence supporting a direct action of GnRH-a on endometrial growth since both GnRH and GnRH receptors have been isolated from eutopic and ectopic endometrium (10). It was suggested that GnRH may act as a direct regulator of this growth (5). Based on these data, the objective of this research project is to study and compare the direct effect of GnRH-a on the growth and programmed cell death (apoptosis) of endometrial culture cells obtained from eutopic endometrial tissue from patients with or without endometriosis.

MATERIALS AND METHODS

Patients

A total of 30 patients who underwent laparoscopy participated in this study: 16 with untreated endometriosis (stage 1 and 2) and 14 controls. Determination of the stage of the disease was performed according to the revised American Fertility Society Classification (11). Control subjects were infertile women without endometriosis with tubal factor infertility or unexplained infertility who were undergoing diagnostic laparoscopy. All the patients were infertile, had regular menstrual cycles, and had not received any hormonal medical treatments during the past 6 months. Biopsy specimens of eutopic endometrium were obtained from all subjects in the proliferative phase as previously described (2).

This study was approved by the Ethics and Research Committee of the Biology and Experimental Medicine Institute, and all subjects signed informed consent forms.

Endometrial Cell Culture

The tissue was immediately placed into culture medium and processed within 60 minutes of collection. Epithelial cells cycle were enzymatically separated and isolated by successive centrifugation, and primary cultures were established for in vitro studies on implantation using a modification of a method described by Bongso et al. (12).

Briefly, the explant was minced, washed, and placed in basic medium (MEM D-Val, GIBCO, Paisley, United Kingdom) containing 100 IU/mL penicillin, 100 μg/mL streptomycin, and 25 μg/mL amphotericin B (GIBCO) with 1 mg/mL collagenase (GIBCO, type I). After 2 hours of incubation at 37°C in an atmosphere of 5% CO_2, the resulting suspension was centrifuged at 100 $\times$ g for 5 minutes. The pellet containing glands was resuspended in nutrient medium and spun again at 100 $\times$ g for 5 minutes. The final pellet mainly contained epithelial cells. After a 1-hour incubation, the medium containing enriched epithelial cells was removed and plated in fresh medium. The cells were cultured in quadruplicate for each patient with 10% calf serum (CS; GIBCO) before the experiments.

Epithelial cells cultures were left undisturbed at 37°C for 2 days. Subsequently, the cells were washed and incubated with 2.5% CS medium for 48 additional hours.

Coverslip cultures of epithelial cells from endometrium were fixed in 100% methanol for 20 minutes and stained by indirect immunofluorescence using mouse monoclonal anti-cytokeratin 56 kD (reacts with cytokeratin polypeptides nos. 1, 2, 5, 6, 7, 8, 11, 14, 16, 17, 18; Serotec Ltd., Oxford, United Kingdom), followed by anti-immunoglobulin-fluorescein isothiocyanate conjugate (FITC) (Dako Ltd., Cambridge, United Kingdom) as the second antibody. The immunocytochemical study was based on that described by Matthews et al. (13). Cultures incubated without the primary antibody were included as controls in all experiments. Slides were viewed on a microscope equipped with fluorescence and differential interference contrast optics.

Alternatively, endometrial stromal cell cultures were performed as previously described (13) on coverslips. An identical immunocytochemical evaluation was done on these cells.

Cell Proliferation Assay

Fifty thousand epithelial endometrial cells were plated in 96 microwell plates and incubated with 10% CS medium. After 48 hours of incubation, the cells were washed and different agents were added to supplemented 2.5% CS medium: transforming growth factor β (TGFβ; GIBCO) in concentrations of 0.01, 0.1, 1.0, and 10 ng/mL; leuprolide acetate (LA; Lupron; Abbot, Cedex, France) as GnRH-a in concentrations of 1, 10, and 100 ng/mL; antide (ANT; Sigma, St. Louis, MO) in a concentration of 10^{-7} M, as GnRH antagonist; or a combination of ANT with LA, adding LA 100 ng/mL 3 hours after the addition of ANT 10^{-7}M. The cells were incubated with the agents for 48 additional hours.

Twenty-four hours before harvesting, 1μCi ^{3}H-thymidine (Nen, Dupont, Boston, MA) was added to each microwell, and DNA synthesis was assessed by ^{3}H-thymidine incorporation using a liquid scintillation counter (14).

Apoptosis Assay

The percentage of apoptotic cells was assessed by the acridine orange–ethidium bromide technique in endometrial cultures at basal conditions and after exposure to LA 100 ng/mL or ANT at a concentration of 10^{-7} M**,** or a combination of ANT with LA, adding LA 100 ng/mL 3 hours after the addition of ANT 10^{-7}M.

Acridine orange is a vital dye that is excluded from viable cells. It is specific for apoptotic forms of cell death and does not significantly label cells undergoing necrotic death provoked by injury (15). After addition of the acridine orange (1 mg/L)–ethidium bromide (250 mg/L) mix, the cells were viewed by a fluorescence microscope and the apoptotic cells were counted as a percentage of the total.

Statistics

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

RESULTS

Immunocytochemical Characterization

The broad-spectrum cytokeratin antibody 56 kD produced clear labeling in the epithelial cells. No staining of stromal cells was observed with the anti-cytokeratin antibody. No fluorescent labeling was observed in control cultures incubated with the second antibody alone.

Effects of the Addition of TGFβ on Epithelial Cell Proliferation

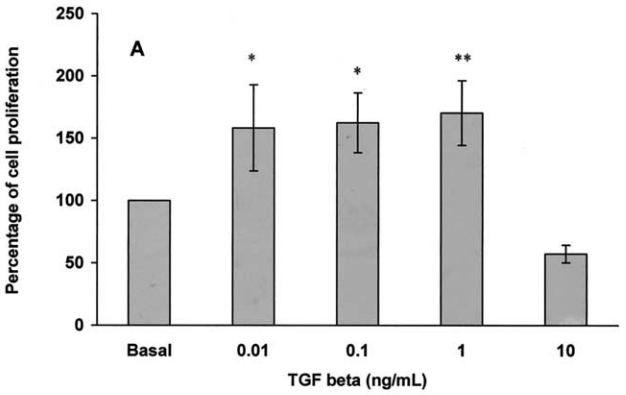
The effects of different concentrations of TGFβ on epithelial cell proliferation are displayed in Figures 1A and 1B. We found that in endometrial cultures from both patients and controls, TGFβ at low concentrations leads to a significant increase in cell proliferation. There was a concentration-dependent stimulation of cell proliferation with TGFβ concentrations at 0.01, 0.1, and 1.0 ng/mL: 58.3 ± 34; 62.5 ± 24, and 70.6 ± 26, respectively (expressed as a percentage of cell proliferation), in endometriosis cultures; and 53.3% ± 26.2%; 77.0% ± 3%, and 74.5% ± 3.5%, respectively, in cultures from control women (not significant [NS]).

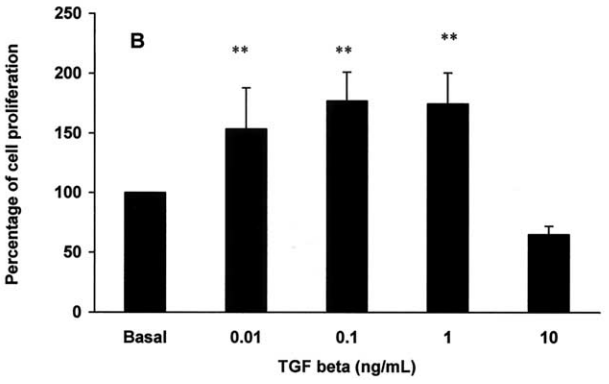
In contrast, at higher concentrations (10 ng/mL), TGFβ inhibited the ^{3}H-thymidine incorporation: −42.7% (expressed as a percentage of inhibition), in endometriosis cultures ($P<.01$ vs. TGFβ 0.01 and 0.1 ng/mL, and $P<.001$ vs. TGFβ 1 ng/mL, respectively), and −35% in cultures from control subjects ($P<.001$ vs. TGFβ 0.01, 0.1, and 1ng/mL).

This is consistent with the general observation of a bimodal effect of TGFβ on cell proliferation for several cell types (16–18).

FIGURE 1

Effects of TGFβ on cell proliferation in endometrial cell cultures from subjects with (**A**) and without (**B**) endometriosis. Epithelial cell cultures from patients with endometriosis and control subjects were analyzed for cell proliferation by ^{3}H-thymidine incorporation after exposure to increasing concentrations of TGFβ (see text). Values are expressed as a percentage of basal cell proliferation set as 100%. *$P<.01$ vs. TGFβ 10 ng/mL. **$P<.001$ vs. TGFβ 10 ng/mL.





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Effects of the Addition of GnRH-a on Epithelial Cell Proliferation

The GnRH-a LA alone had no effect on basal DNA synthesis. Therefore, for cell proliferation assays, TGFβ (0.1 ng/mL) stimulated cell cultures (SCCs) were considered as basal.

After exposure to different concentrations of LA, the epithelial endometrial cultures showed a significantly lower degree of cell proliferation.

In endometrial cultures from patients with endometriosis, ^{3}H-thymidine uptake was down-regulated by LA 1 ng/mL: −35.0 ± 20.0 ($P<.01$ vs. SCC); LA 10 ng/mL: −61.6 ± 8.4; and LA 100 ng/mL: −64.0 ± 20.0, expressed as a percentage of inhibition ($P<.001$ vs. SCC) (Fig. 2A). Likewise, this decrease in cell proliferation was observed in cell cultures

FIGURE 2

Effects of LA and ANT on cell proliferation in endometrial cell cultures from subjects with (**A**) and without (**B**) endometriosis. Epithelial cell cultures from patients with endometriosis and control subjects were analyzed for cell proliferation by ^{3}H-thymidine incorporation after exposure to increasing concentrations of LA and ANT 10^{-7}M (see text). Values are expressed as a percentage of TGFβ 0.1 ng/mL SCC proliferation set as 100%. *$P<.01$ vs. SCC. **$P<.001$ vs. SCC ANT 10^{-7}M + LA 100 ng/mL vs. SCC, $P>.05$, NS.

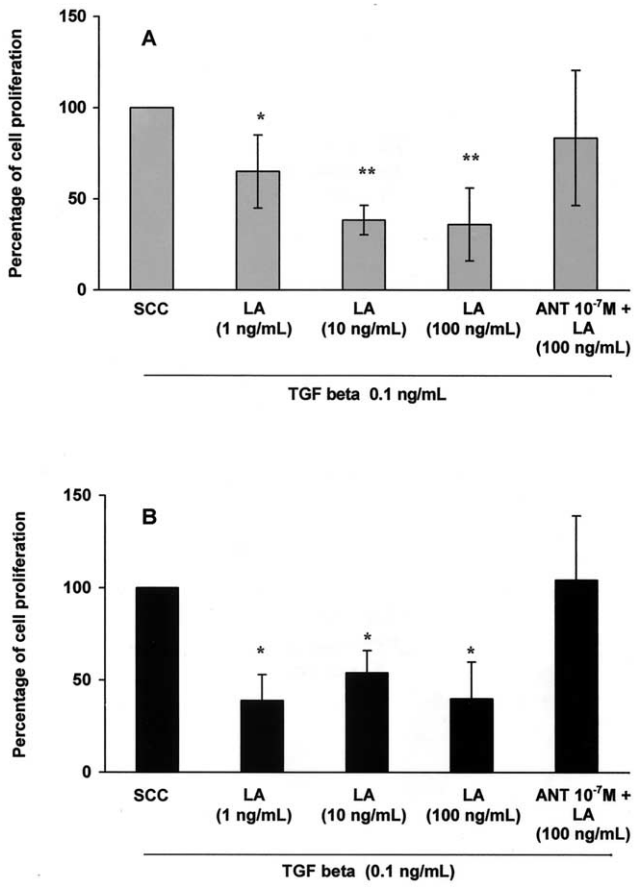

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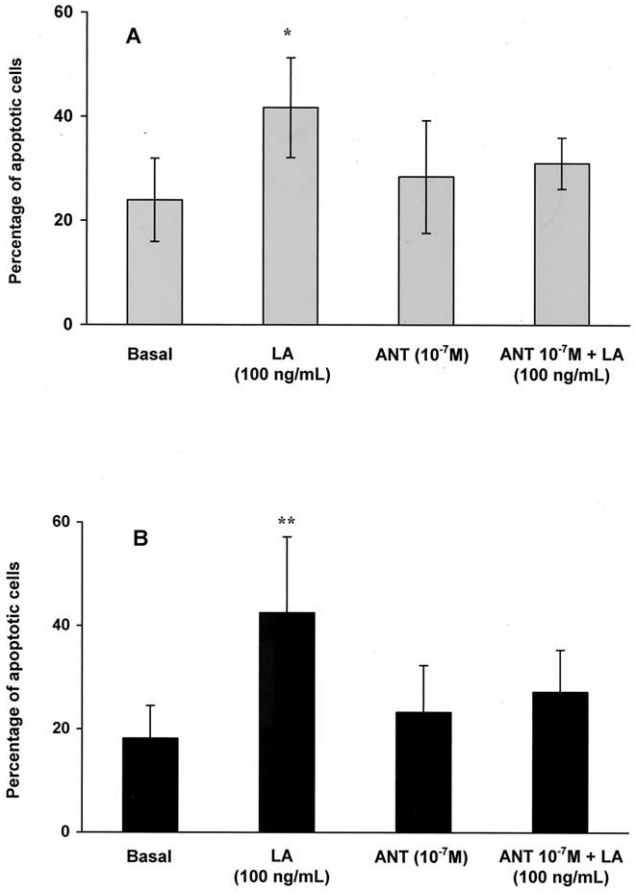
FIGURE 3

Effects of LA and ANT on apoptosis in endometrial cell cultures from subjects with (**A**) and without (**B**) endometriosis. Epithelial cell cultures from patients with endometriosis and control subjects were analyzed for apoptosis by the acridine orange–ethidium bromide technique at basal conditions and stimulated with LA 100 ng/mL or ANT in a concentration of 10^{-7} M or a combination of ANT with LA. Values are expressed as a percentage of apoptotic cells. The cells were viewed by a fluorescence microscope, and the apoptotic cells were counted as a percentage of the total (see text). *$P<.01$ vs. basal. **$P<.05$ vs. basal. ANT 10^{-7}M and ANT 10^{-7}M + LA 100 ng/mL vs. basal, $P>.05$, NS.



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from control women: LA 1 ng/mL: -61.0 ± 14.0; LA 10 ng/mL: -46.0 ± 12.0; and LA 100 ng/mL: -60.0 ± 20.0 ($P<.001$ vs. SCC) (Fig. 2B).

We observed a reversal in the inhibition of cell proliferation by LA 100 ng/mL after addition of the GnRH antagonist, ANT 10^{-7}M, since the values were not significantly different than in SCCs (-16.6 ± 34 in endometriosis cultures; and 4.2 ± 35 in cultures from control women, $P>.05$, NS, vs. SCC) (Figs. 2A and 2B). Antide (ANT) 10^{-7}M alone had no effect on basal or SCC proliferation (data not shown).

Effects of the Addition of GnRH-a on Epithelial Cell Apoptosis

Exposure to LA significantly increased the level of apoptosis in cultures from patients and controls. LA 100 ng/mL showed an effect on endometrial growth, enhancing apoptosis in endometrial cultures from patients with endometriosis from $23.9\% \pm 8.0\%$ to $41.7\% \pm 9.6\%$ (expressed as a percentage of apoptotic cells, $P<.01$) (Fig. 3A) and from $18.2\% \pm 6.3\%$ to $42.5\% \pm 14.7\%$ in cultures from control women ($P<.05$) (Fig. 3B). In both experiments, this effect was reversed by ANT 10^{-7}M: in endometriosis, $31.0\% \pm 4.9\%$; and in controls, $27.3\% \pm 8.1\%$ (vs. basal, $P>0.05$, NS) (Figs. 3A and 3B). ANT 10^{-7}M alone had no effect on basal apoptosis (data not shown).

DISCUSSION

It is now widely accepted that eutopic endometrium from women with endometriosis has some fundamental differences compared with eutopic endometrium from women without the disease. These differences give support to the concept that eutopic endometrium from women with endometriosis is inherently aberrant and plays an important role in the pathogenesis of the disease (2, 19). In a recent publication, we showed a significant decrease in apoptosis in the eutopic endometrium from patients with endometriosis (2). The low levels of apoptosis observed by our group in the epithelial and stromal levels were first described by Dmowski et al. (20) on the whole endometrium, who proposed that the survival of endometrial cells misplaced in ectopic locations may depend in part on the inherent ability of the cells to undergo apoptosis. Cell proliferation and apoptosis are two of the major biologic pathways that normally regulate cell growth and tissue homeostasis. Even though they are not biologically separate, each of these pathways can be defined by a unique set of molecular events and perturbations in one pathway cannot have consequences on another every time (21).

LA is a GnRH-a used to treat a wide range of estrogen-dependent disorders including endometriosis and uterine fibroids. It acts primarily on the anterior pituitary, initially inducing a transient rise in gonadotropin release. With continued administration, LA causes pituitary desensitization and/or down-regulation, leading to suppressed circulating levels of gonadotropins and estrogen (22). The antiproliferative effects of GnRH-a seem to be not only through the suppression of gonadal steroids, but also through a direct effect on cell growth. There is convincing in vitro evidence showing that GnRH-a can directly inhibit the proliferation of endometrial, breast, and ovarian cancer cells (23).

In this study, epithelial cell cultures of human endometrium were used in a model to evaluate cell proliferation and apoptosis in response to the addition of GnRH-a. Although the cells employed in this investigation were not derived from endometriotic implants and their in vitro response may not be identical, the rationale for the use of endometrial cells in short-term culture as a model for endometriotic implants has been previously described (24).

The results of our work indicate that GnRH-a alone had no effect on basal DNA synthesis. In agreement with our data, the results from experiments performed with the addition of LA to endometrial stromal cells showed a similar response (24).

However, when epithelial endometrial cells were cultured with TGFβ, the addition of GnRH-a produced a dose-dependent inhibition of thymidine incorporation. TGFβ (0.01–1.0 ng/mL) stimulated the growth of endometrial cell cultures from patients with endometriosis and from controls, whereas a high concentration of TGFβ (10 ng/mL) had an inhibitory effect on cell proliferation. These data are comparable to those reported by Arici and Sozen (17) and Kimura et al. (18), who investigated the effect of TGFβ on cell proliferation in leiomyomas and human bone marrow fibroblasts, respectively, and found a similar biphasic response after incubating the cells with TGFβ.

TGFβ and epidermal growth factor (EGF) have been used in our work as well, or in other reports on cell proliferation (19, 23, 25), since in vitro the cell proliferation of nonmalignant cells is very low and the incorporation of tritiated thymidine detected by the liquid scintillation counter is minimal. Because of this it is commonly accepted that a previous stimulation be done to trigger the cell proliferation and afterward establish the degree of inhibition.

Outside this strictly methodological aspect, some published reports on patients with leiomyomas treated with LA suggest that the GnRH-a modulates the growth of leiomyomas through down-regulation of the expression of the components in the TGFβ system (molecule and receptor) (26). Furthermore, Küpker et al. (27) showed that patients with endometriosis have a low concentration of TGFβ in the peritoneal fluid after GnRH-a treatment. These reports highlight the physiologic importance of TGFβ, and the GnRH-a may be working through these messengers to modulate endometrial growth.

In addition, we found that LA (100 ng/mL) increased the apoptotic rate in eutopic endometrial cells from controls and patients with endometriosis. This is in agreement with a report showing that incubation with GnRH-a stimulated apoptosis in rat granulosa cells (6) as well as with a recent study reporting that GnRH-a triggered apoptosis in a single suspension of stromal and glandular epithelial endometrial cells from patients with endometriosis (28).

In our study, the GnRH antagonist ANT was able to reverse the inhibitory action of GnRH-a on DNA synthesis and the stimulatory action of GnRH-a on programmed cell death. These findings combined strongly suggest that the effects of GnRH-a on DNA synthesis and apoptosis are mediated by homologous receptors.

We found no differences in the rate of apoptosis and thymidine incorporation between endometrial cell cultures from patients with endometriosis and controls; this is in contrast with the results in whole endometrial sections observed in our previous study (2). We could speculate that after 4 days of culture of the glandular fraction, the epithelial endometrial cells would alter their initial conditions and we would not be able to detect maintenance of their apoptotic characteristics in comparison with when we used the entire tissue.

Lastly, our findings seem to suggest that GnRH-a may be effective in reducing the growth of endometrial cells through a direct effect. This knowledge could contribute to a better understanding of the mechanisms implicated in the therapeu-

tic action of GnRH-a and of the medical treatment of endometriosis.

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