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The Role of GnRH Analogues in Endometriosis-Associated Apoptosis and Angiogenesis

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Key Words
Endometriosis · Apoptosis · Angiogenesis · GnRH analogues

Abstract
It has been postulated that gonadotropin-releasing hormone (GnRH) analogues may act directly on endometrial cells and inhibit their growth and proliferation by regulation of apoptotic and angiogenic mechanisms. Eutopic endometrial cells from patients with endometriosis show an increased proliferation rate and are less susceptible to cell death by apoptosis than those from subjects without the disease. Notably, the GnRH analogue, leuprorelin, inhibits cell proliferation and increases the apoptotic rate in eutopic endometrial cell cultures, an effect that appears to be mediated by an increase in the expression of the pro-apoptotic proteins Bax and FasL and a decrease in the expression of the anti-apoptotic protein Bcl-2. Angiogenesis is an important process in the development of endometrial tissue, and it is regulated by vascular endothelial growth factors (VEGFs) and angiopoietins. VEGF levels are elevated in peritoneal fluid and endometriotic tissue from patients with endometriosis. In addition, it has been demonstrated that the expression of VEGF is potentiated by a variety of cytokines, including IL-1β. Recent studies show that leuprorelin reduces the production of VEGF-A and IL-1β in eutopic endometrial cell cultures, suggesting a mechanism by which it could inhibit the development of endometriosis. Thus, GnRH analogues appear to be effective in reducing the growth of endometrial cells, not only due to their classical pituitary endocrine effects, but also via a direct effect on the endometrial cells themselves.

Introduction

It is generally accepted that the etiology of endometriosis (EDT) is associated with retrograde migration of eutopic endometrial tissue outside the uterine cavity. Mechanisms involved in the development of endometriotic lesions include immunological factors, apoptosis and cell growth, hormonal factors (e.g. estrogens), and angiogenesis.

Synthetic gonadotropin-releasing hormone (GnRH) analogues (both agonists and antagonists) are frequently used in the treatment of human reproductive pathologies, including EDT. The administration of long-lasting GnRH agonists reduces the endogenous secretion of gonadotropins by a mechanism of pituitary downregulation, and as a consequence they are frequently used to achieve regression of endometriotic implants due to the induction of hypoestrogenism. In addition to this central effect, it has also been demonstrated that GnRH and its agonists have a direct effect on extra-pituitary tissues,

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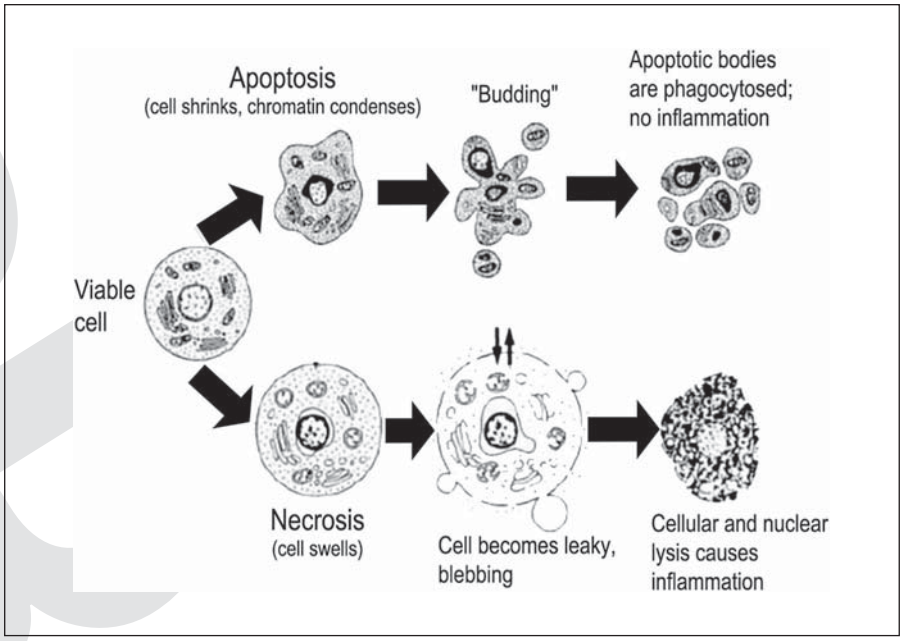

Fig. 1. Comparison of the cellular changes that occur during apoptosis and necrosis.

such as endometrium and ovary [1, 2]. There are reports demonstrating that GnRH agonists directly inhibit the proliferation of various types of steroid-dependent cancers, including those of the breast, ovary and endometrium [3–5]. In addition, a GnRH-like peptide and GnRH receptors have been isolated from both eutopic and ectopic endometrium [1].

This paper will review the underlying pathophysiological mechanisms implicated in the development of EDT and the effect of GnRH analogues in this setting.

Apoptosis

There are two different mechanisms of cell death: necrosis and apoptosis (fig. 1). During necrosis the viable cell becomes leaky, forms blebs and the resulting cellular and nuclear lysis causes a release of the cell's contents leading to inflammation. In contrast, apoptosis, or programmed cell death, also kills unwanted cells but without inducing an immune response or an inflammatory reaction. The viable cell shrinks, the chromatin condenses, budding occurs, and the apoptotic bodies are phagocytozed by white blood cells, so there is no resulting inflammation.

Apoptosis can be triggered in a cell through either the extrinsic pathway or the intrinsic pathway (fig. 2) [6]. Whichever pathway is triggered, the cell changes which result are mediated by a family of cysteine aspartic acid-specific proteases (caspases), which occur as inactive precursors (procaspases) that are proteolytically converted to an active state following an apoptotic stimulus. The extrinsic pathway is initiated through binding of the transmembrane death receptors, such as the Fas, TNF, and TRAIL receptors, located on the cell membrane with their respective ligands. This binding triggers aggregation of the receptors and recruitment of an adaptor protein known as Fas-associated death domain protein (FADD) within the cell cytoplasm. FADD, in turn, recruits caspase-8 to form the death-inducing signal complex (DISC) which initiates the caspase activation cascade.

In contrast, the intrinsic pathway requires disruption of the mitochondrial membrane and the release of mitochondrial proteins, such as cytochrome C. Cytochrome C is released into the cytoplasm, along with the other two cytosolic protein factors, apoptotic protease activating factor-1 (Apaf-1) and procaspase-9, promoting the assembly of a caspase-activating complex termed the apoptosome, which in turn initiates the apoptotic caspase cascade. Known regulators of cytochrome C release are the Bcl-2 family of proteins, which can be either pro-apoptotic or anti-apoptotic. In addition, cross-communication occurs between the extrinsic and intrinsic pathways, and factors produced in one pathway can stimulate the other (fig. 2) [6].

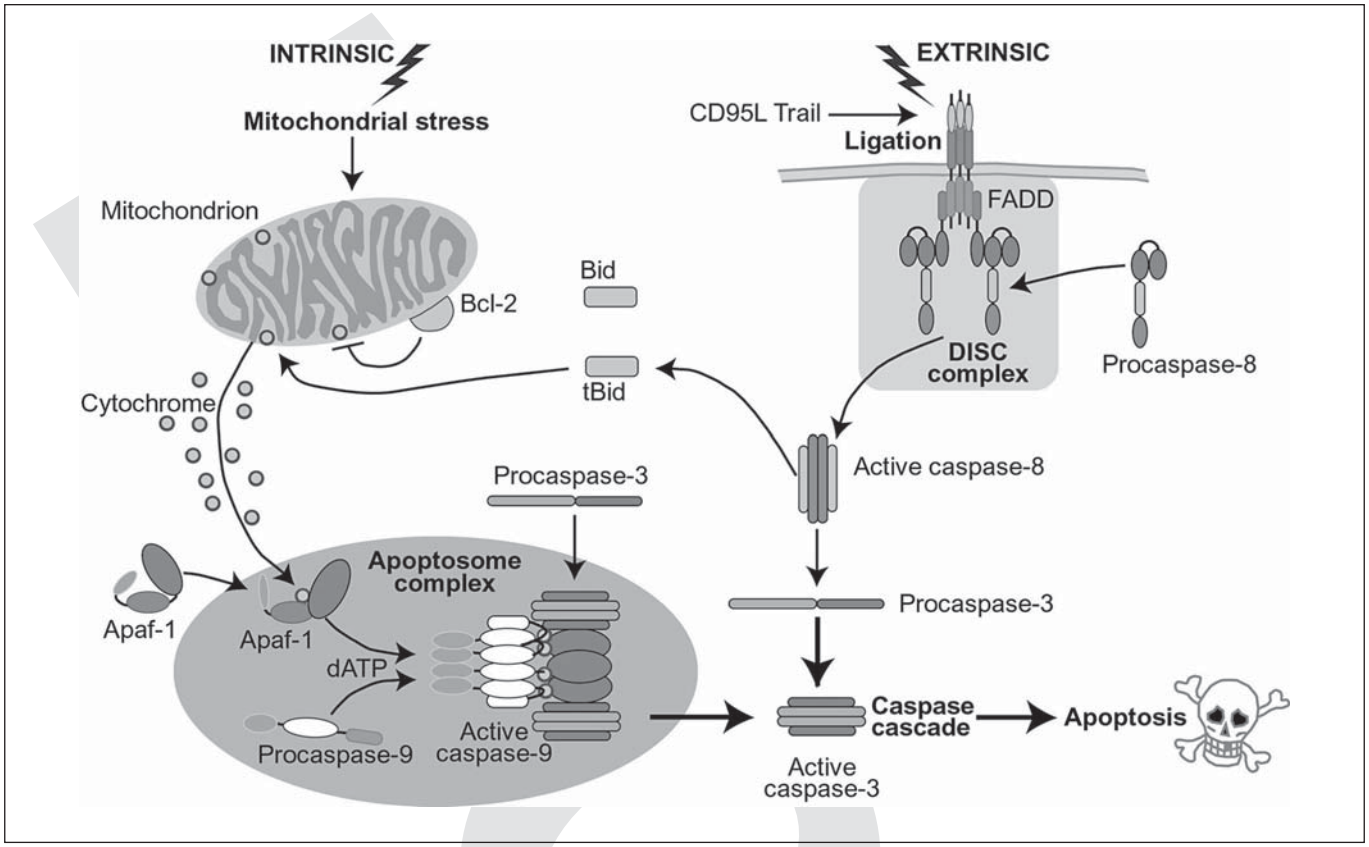

Fig. 2. The intrinsic and extrinsic apoptosis pathways. Reproduced with permission from MacFarlane and Williams [6].

Angiogenesis

There are two main processes of vascular development: vasculogenesis and angiogenesis. Vasculogenesis involves the differentiation of endothelial cells from embryologic mesoderm and the assembly of these differentiated cells into discrete blood vessels whereas angiogenesis involves the proliferation and migration of endothelial cells from pre-existing vessels and leads to the sprouting or remodeling of new vessels in a given area.

The highly regulated process of angiogenesis that occurs within the female reproductive tract is critical for normal reproduction, including follicular maturation, selection and normal function of the corpus luteum, and endometrial growth and remodeling. The pathogenesis of EDT involves the implantation of exfoliated endometrium which is dependent for its survival on the development of an extensive blood supply – by the process of angiogenesis – both within and surrounding the ectopic tissue.

There are two main regulators of angiogenesis – vascular endothelial growth factors (VEGFs) and angiopoietins. VEGF-A is a potent mitogen and migratory stimulus for endothelial cells and has been found to be involved in both physiological and pathological conditions [7]. Studies have shown that it is produced by endometrial cells and is a critical factor involved in the pathogenesis of the disease [8, 9]. VEGF levels are elevated in peritoneal fluid from patients with EDT and in the endometriotic tissue itself [8, 9]. In addition the expression of VEGF in endometrial cells has been found to be potentiated by a variety of cytokines, including IL-1 [10, 11]. Visualization of ectopic endometrial implants by laparoscopy shows them to be highly vascularized.

In view of the above evidence it has therefore been postulated that GnRH analogues interfere with endometrial cell growth by regulation of apoptotic and angiogenic mechanisms.

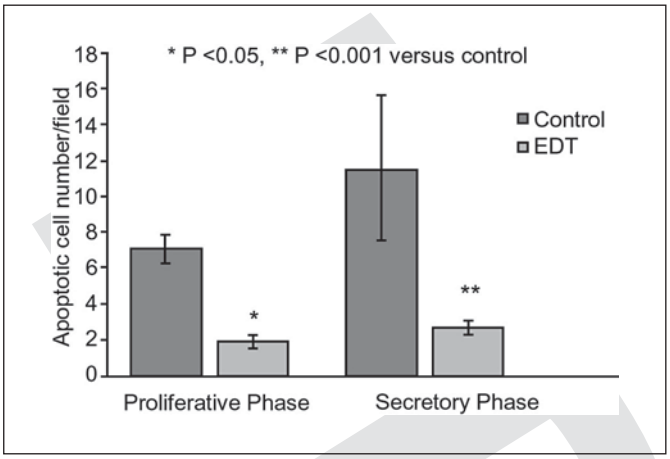

Fig. 3. Apoptosis during the proliferative and secretory phases in biopsy specimens of eutopic endometrium from control subjects and women with endometriosis (EDT) [12]. Reproduced with permission from Meresman et al. [12].

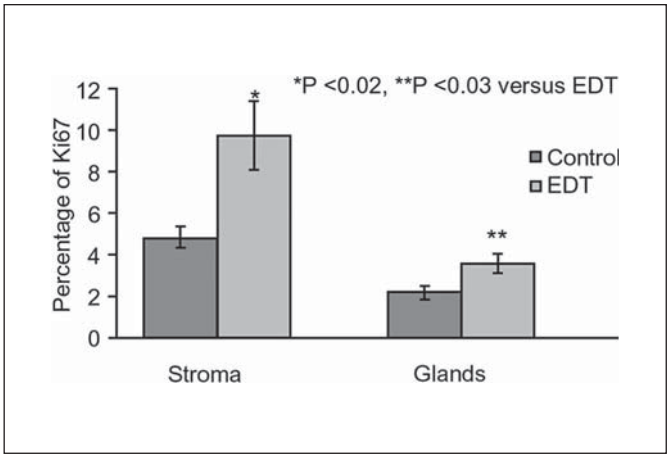

Fig. 4. Cell proliferation in stromal and glandular cells in biopsy specimens of eutopic endometrium from control subjects and women with EDT [13].

Studies of GnRH Analogues and Apoptosis

Initial studies were undertaken to evaluate apoptosis and cell proliferation in eutopic endometrium from women with and without EDT [12]. Thirty-six patients undergoing diagnostic laparoscopy for infertility participated in the study. All had regular menstrual cycles and had not received any hormonal therapy in the previous six months. Twenty-two patients had untreated EDT (stages I and II) and 14 control subjects had tubal factor infertility or unexplained infertility. Biopsy specimens of eutopic endometrium were obtained from all subjects during the proliferative phase. Apoptosis and cell proliferation were examined in these tissue sections.

The proportion of apoptotic cells in situ was determined using the TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) method, in which terminal deoxynucleotidyl transferase (TdT) transfers biotin-dUTP to strand breaks of cleaved nuclear DNA that occur as a result of the apoptotic process. The biotin-labeled cleavage sites are then detected by reaction with horseradish peroxidase-conjugated streptavidin and visualized by diaminobenzidine showing brown color under the microscope. Figure 3 shows quantification of the degree of apoptosis in eutopic endometrial tissue during the proliferative and secretory phases of the menstrual cycle. In both phases, apoptosis in cells from women with EDT was significantly lower than in cells obtained from control subjects ($p < 0.05$ in the proliferative phase and $p < 0.001$ in the secretory phase vs. controls).

Cell proliferation was determined by evaluation of the nuclear protein Ki-67, which is strongly associated with cell proliferation. During interphase, it can be exclusively detected within the nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. The fact that the Ki-67 protein is present during all active phases of the cell cycle, but is absent from resting cells, makes it an excellent marker for determining growth of a cell population. Ki-67 staining was observed in eutopic endometrial cells from women with EDT, suggesting that cell proliferation was occurring. This was confirmed by quantification of Ki-67, as shown in figure 4 [13]. The degree of cell proliferation (the proportion of Ki-67-positive cells) was significantly higher in both stromal and glandular cells of endometriotic tissue obtained from women with EDT than in tissue obtained from control subjects ($p < 0.02$ in stromal cells and $p < 0.03$ in glandular cells vs. controls). It can be concluded from these studies that eutopic endometrial cells from patients with EDT are less susceptible to apoptotic cell death and show an increased proliferation rate compared with cells from subjects without the disease.

As a result of these interesting findings further studies were undertaken to assess the in vitro effects of GnRH analogues on the production of factors associated with apoptosis and angiogenesis in epithelial endometrial cell cultures [14].

Biopsies from patients with EDT or control subjects were obtained as described in the earlier study. Endometrial cells were enzymatically separated and epithelial

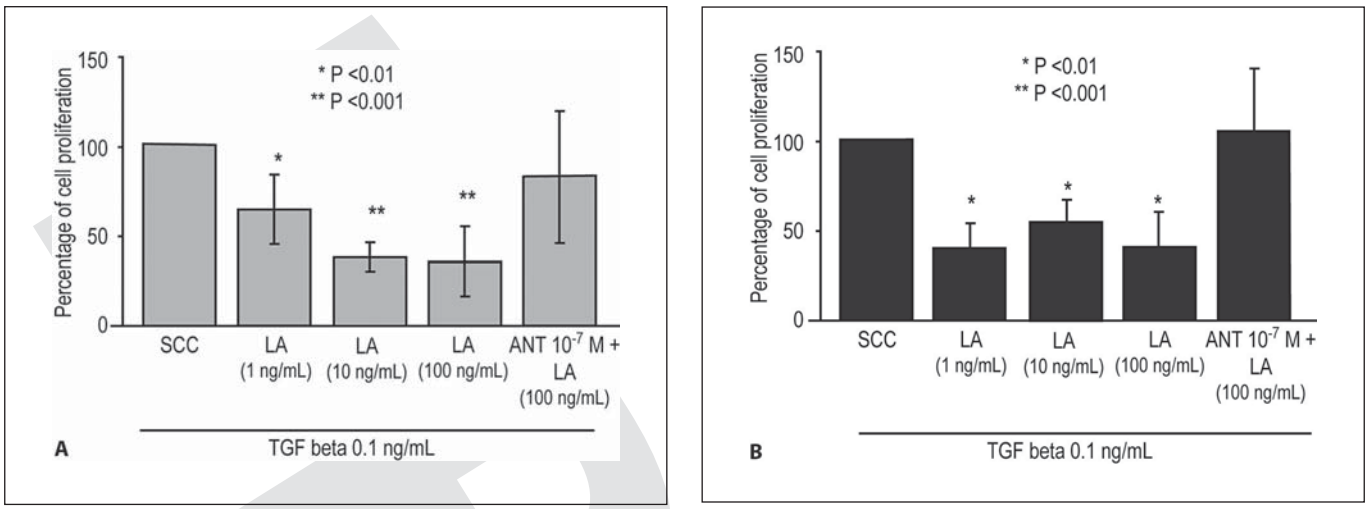

Fig. 5. Effect of leuprorelin acetate (LA) and antide (ANT) on cell proliferation in epithelial endometrial cell cultures obtained from EDT patients (**A**) or control subjects (**B**). Values are expressed as percentage of TGFβ 0.1 ng/ml stimulated cell cultures (SCC) proliferation set as 100% [15]. Reproduced with permission from Meresman et al. [15].

cells were isolated by successive centrifugation. Primary cultures were established as previously described [15]. Cells were cultured in 10% fetal bovine serum (FBS) culture medium (DMEM-F12) for 48 h and GnRH agents (a GnRH agonist: leuprorelin acetate [LA] and a GnRH antagonist: antide [ANT]) were added for 24 h to supplemented 2.5% FBS medium. Cell epithelial purity was assessed by cytokeratin immunocytochemistry (indirect immunofluorescence using mouse anti-cytokeratin 56 kDa).

Cell proliferation was measured by a thymidine incorporation method. As shown in figure 5A and B, cell proliferation in epithelial endometrial cell (EEC) cultures from both control women and EDT patients was inhibited at a range of concentrations of LA (1, 10 and 100 ng/ml), and this inhibition was reversed by the addition of ANT.

The percentage of apoptotic cells was assessed by the acridine orange–ethidium bromide technique and cells were viewed by a fluorescence microscope. When this effect was quantified (apoptotic cells counted as a percentage of the total) it was revealed that the proportion of apoptotic cells in EEC cultures from both control women and EDT patients was significantly increased in the presence of LA at concentrations of 100 ng/ml ($p < 0.05$ and $p < 0.01$, respectively) (fig. 6A, B). This apoptotic stimulation was reversed by the addition of ANT (10^{-7} M). The effect of ANT was found to be dose-dependent: at a concentration of 10^{-5} M it was found to increase the proportion of apoptotic cells in EEC from patients with EDT and controls ($p < 0.01$), but lower concentrations were not effective (fig. 7).

These results led the investigators to question whether the endometrial cell apoptosis induced by GnRH analogues related to changes in the expression of pro- or anti-apoptotic proteins.

As mentioned previously, two major pathways towards apoptosis have been defined: the extrinsic death receptor pathway and the intrinsic mitochondrial pathway. The Bcl-2 family of proteins constitutes a critical intracellular checkpoint in the intrinsic pathway of apoptosis; some of these proteins, such as Bax, Bcl-xS, Bak, and Bok, have pro-apoptotic effects and others, such as Bcl-2, Bcl-xL, and Mcl-1, have anti-apoptotic effects. The ratio of pro- to anti-apoptotic molecules, such as Bcl-2/Bax, constitutes a rheostat that sets the threshold of susceptibility to apoptosis for the intrinsic pathway. Through this interaction, it has been proposed that Bax antagonizes Bcl-2 function, diminishing the ability of Bcl-2 to prolong cell survival.

To investigate this, the expression of Bax and Bcl-2 protein in EEC cultures from EDT patients were evaluated by Western blot and immunocytochemistry. Western blot analysis was performed on cell homogenates from EEC cell cultures from patients with EDT under basal conditions and after treatment with LA (1,000 ng/ml), ANT (10^{-5} M) or a combination of ANT with LA. The results of this investigation are shown in figure 8. In EEC from women with EDT, Bax expression increased after

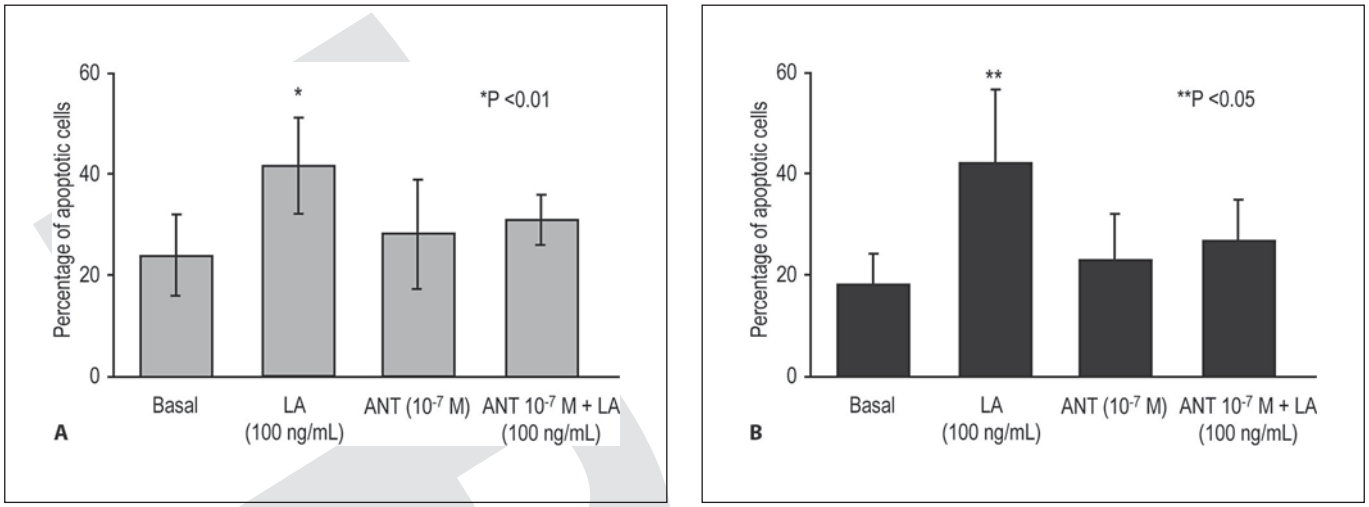

Fig. 6. Effect of leuprorelin acetate (LA) and antide (ANT) on the percentage of apoptotic cells in epithelial endometrial cell cultures obtained from EDT patients (**A**) or control subjects (**B**) [15]. Reproduced with permission from Meresman et al. [15].

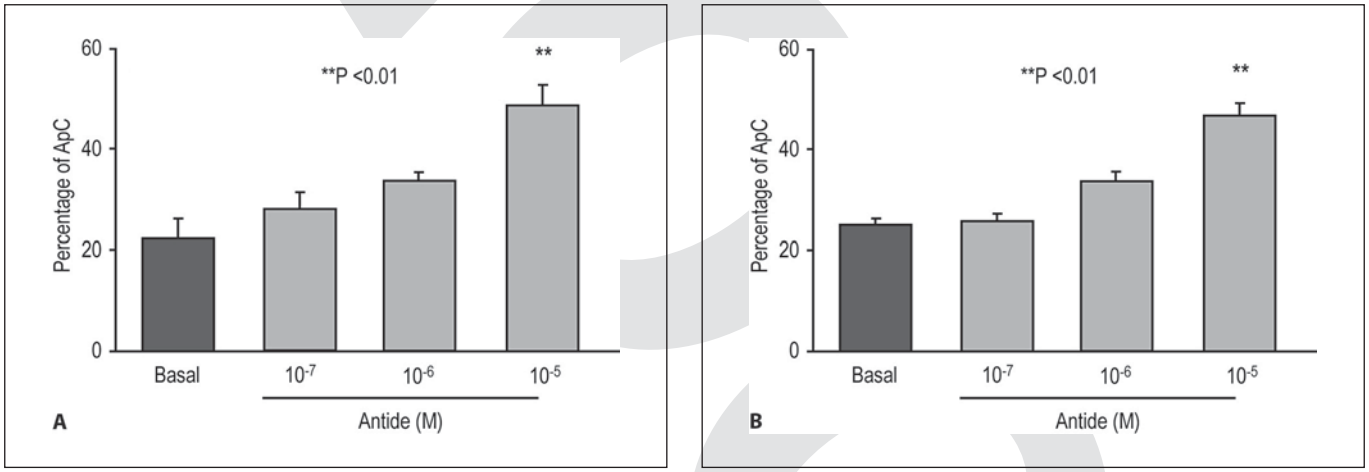

Fig. 7. Effects of antide on apoptosis in endometrial cell cultures from subjects with EDT (**A**) and controls (**B**). Values are expressed as percentage of apoptotic cells (ApC) ± SEM [14]. Reproduced with permission from Bilotas et al. [14].

treatment with LA, ANT and LA + ANT ($p < 0.05$, $p < 0.001$ and $p < 0.001$) (fig. 8A), whereas Bcl-2 expression decreased after exposure to LA and ANT ($p < 0.001$ and $p < 0.01$) (fig. 8B).

In relation to the extrinsic apoptosis pathway, the Fas–Fas ligand (FasL) system is the primary mechanism for induction of apoptosis in cells and tissues. Expression of Fas and FasL protein in EEC cultures from EDT patients were evaluated by Western blot and immunocytochemistry. FasL expression increased after LA, ANT and LA + ANT treatments ($p < 0.01$, $p < 0.001$ and $p < 0.01$) (fig. 9A), but no significant changes were observed in Fas expression (fig. 9B).

From these results it can be concluded that GnRH analogues enhance apoptosis in EEC, and that this is accompanied by an increase in the expression of the pro-apoptotic proteins Bax and FasL and a decrease in expression of the anti-apoptotic protein Bcl-2.

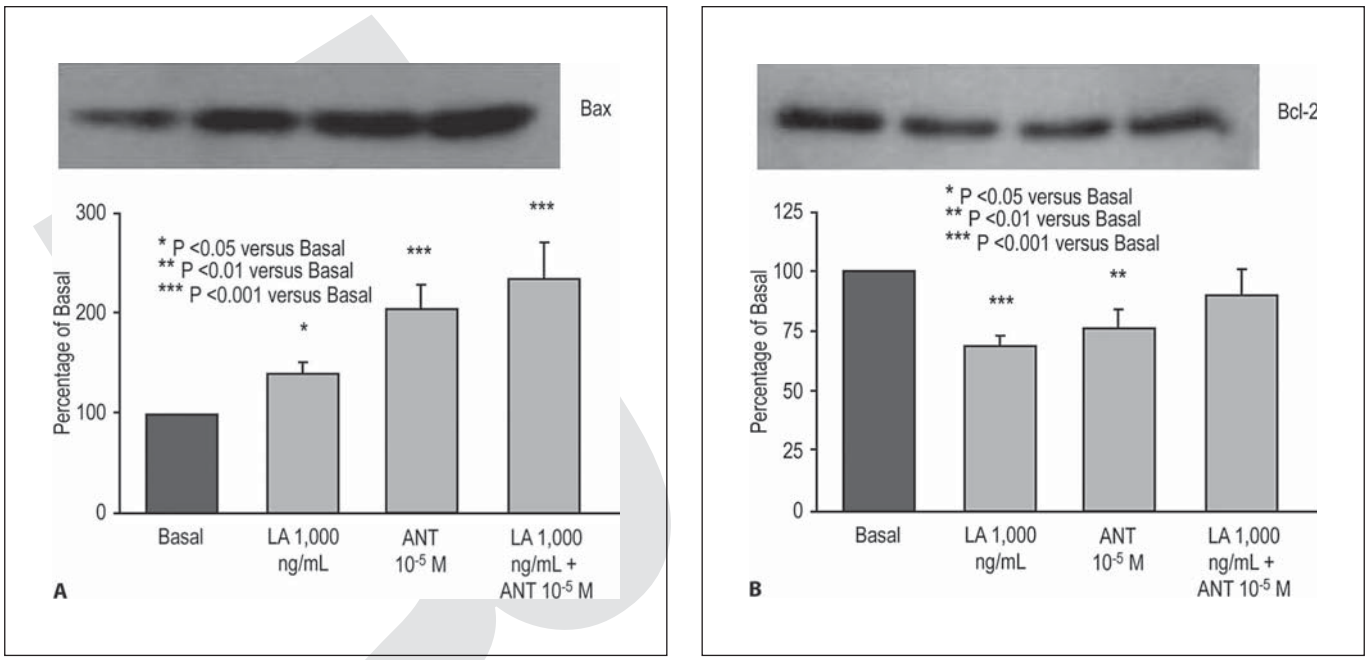

Fig. 8. Effects of leuprolide acetate (LA) and antide (ANT) on Bax (**A**) and Bcl-2 (**B**) protein expression in endometrial cell cultures obtained from EDT patients. Representative Western blots are presented in upper panels and quantification of results for protein expressed as a percentage of basal values ± SEM in the lower panels [14]. Reproduced with permission from Bilotas et al. [14].

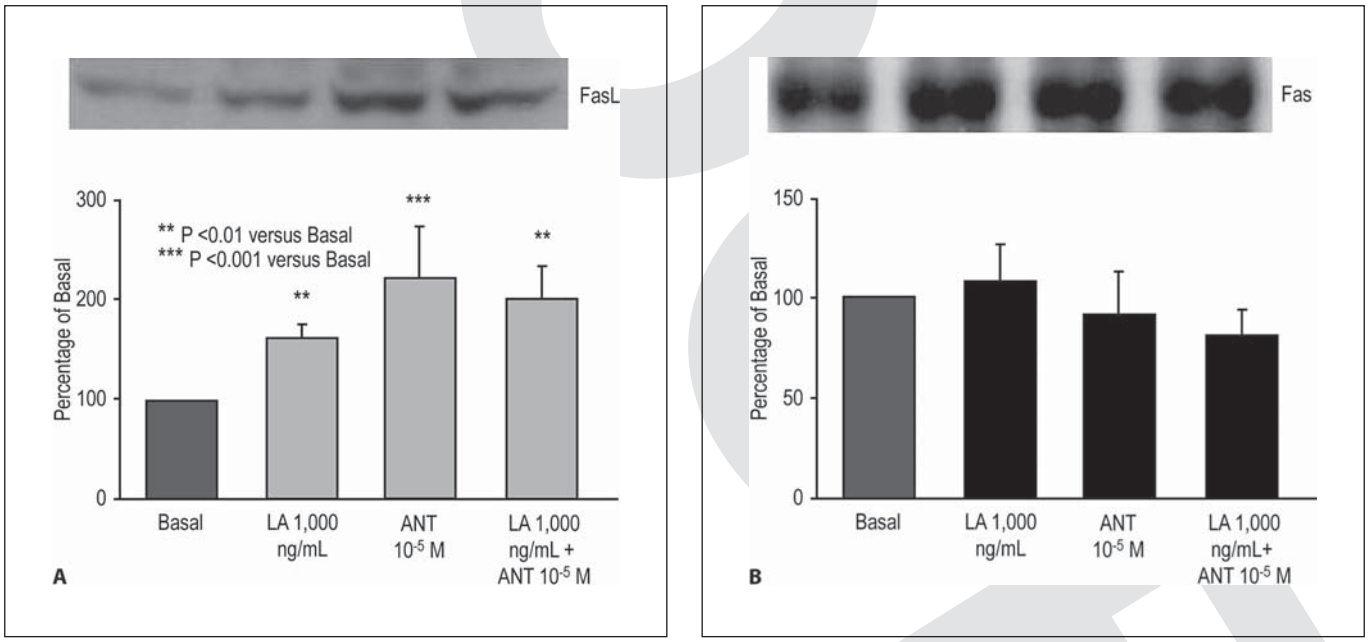

Fig. 9. Effects of leuprolide acetate (LA) and antide (ANT) on FasL (**A**) and Fas (**B**) protein expression in endometrial cell cultures obtained from EDT patients. Representative Western blots are presented in upper panels and quantification of results for protein expressed as a percentage of basal values ± SEM in the lower panels [14]. Reproduced with permission from Bilotas et al. [14].

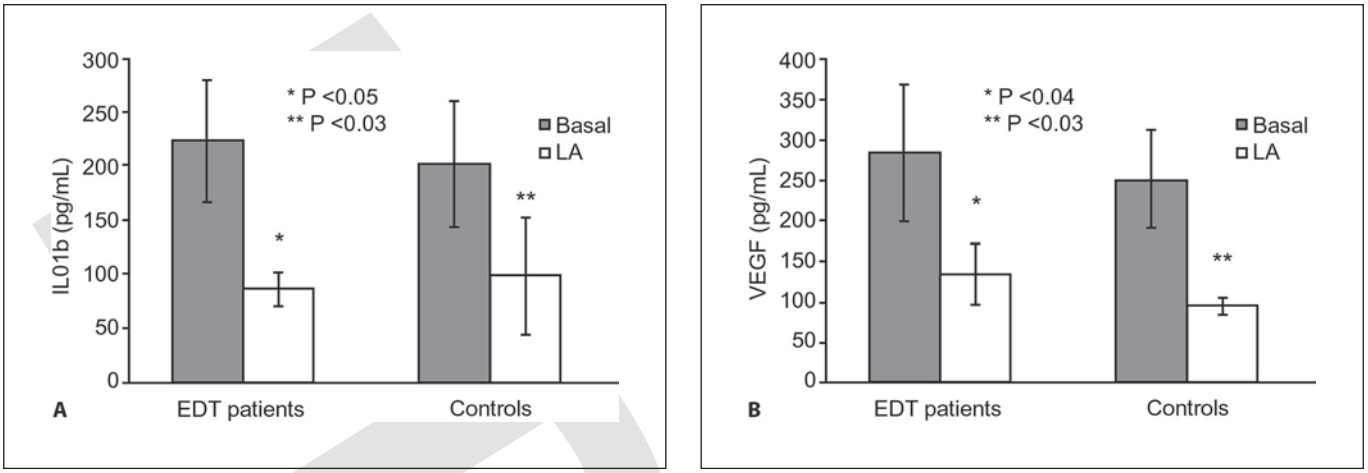

Fig. 10. Effects of leuprolide acetate (LA) on IL-1β (**A**) and VEGF (**B**) production by endometrial cell cultures obtained from EDT patients and control subjects [16]. Reproduced with permission from Meresman et al. [16].

Studies of GnRH Analogues and Angiogenesis

Studies have also been undertaken to evaluate the effect of GnRH analogues on the production of angiogenic factors in endometrial tissue [16]. It was questioned whether the production of VEGF and/or IL-1β is regulated by the presence of LA in the culture medium of endometrial cells.

Biopsy specimens of eutopic endometrium obtained from 16 women with untreated EDT and 14 controls were studied. IL-1β and VEGF release were evaluated in EEC cultures under basal conditions or with the addition of LA (10^{-7} M). IL-1β and VEGF concentrations were assessed by using commercial enzyme-linked immunosorbent assay (ELISA) kits. IL-1β and VEGF release were downregulated by LA in cultures from controls and EDT patients (fig. 10A, B).

The authors concluded that GnRH agonists appear to have a direct effect in endometrial cell cultures, in part by decreasing the release of pro-mitogenic cytokines such as IL-1β and VEGF.

Conclusions

From these preclinical studies of pathogenic mechanisms involved in EDT it can be concluded that LA inhibits cell proliferation and increases the apoptotic rate in EEC cultures. The GnRH antagonist, ANT, is able to reverse these effects, suggesting that the effect of GnRH analogues on DNA synthesis and apoptosis is mediated by homologous receptors. Higher ANT concentrations (10^{-5} M) enhance apoptosis in EEC cultures, most likely via another receptor distinct from the classic GnRH receptor. GnRH analogues also increase the expression of the pro-apoptotic proteins Bax and FasL and decrease the expression of the anti-apoptotic protein Bcl-2. LA inhibits the release of VEGF-A and IL-1β in eutopic endometrial cell cultures, factors known to be involved in angiogenesis. Thus, GnRH analogues are effective in reducing the growth of endometrial cells not only by the classical pituitary endocrine effect, but also via a direct effect on the endometrial cells themselves. Ultimately, this knowledge may contribute to the development of improved, targeted therapies for this debilitating disease.

Post-Presentation Discussion

Prof. Terakawa, Tottori University, Japan: Are there differences between GnRH agonists and antagonists in the induction of apoptosis?

Dr. Tesone: The effect is the same but in our experiments the GnRH antagonists, antide, only produced effects at higher concentrations (10^{-5} M). It is thought that the effect of antide is mediated through another receptor. When we add antide at lower concentrations, the effect of LA is blocked, suggesting that they are acting at the same receptor and LA is not able to bind in the presence of antide. At higher concentrations it appears that antide binds

to another receptor, the identity of which is currently unknown.

Dr. Kajitani, Keio University, Japan: You showed the direct effect of GnRH analogues mediated via the GnRH receptor on endometrial cells. As a consequence of binding to the GnRH receptor, the protein kinase-C pathway will be activated; however, you did not show any direct effect on products of the protein kinase-C pathway. Have you any information about the protein kinase-C pathway in this context?

Dr. Tesone: That is a very interesting question. We have not studied the protein kinase-C pathway in this experimental model, but have done so in ovarian cells. We found a direct effect of GnRH analogues on ovarian cells and an activation of the protein kinase-C pathway.

Dr. Harada, Tottori University, Japan: Did you investigate the effects of other GnRH agonists, such as buserelin or nafarelin?

Dr. Tesone: We have only undertaken studies with LA as it is the agonist most frequently used in the clinical setting in Argentina for the treatment of different pathologies.

Dr. Harada, Tottori University, Japan: The reason I ask is that we undertook studies with buserelin and found no effect on endometrial cell proliferation, suggesting this effect could be specific to LA.

Dr. Kahn, Nagasaki University, Japan: You investigated the pro-apoptotic marker, Bax, and the anti-apoptotic marker, Bcl-2, in EEC cultures from both control women and EDT patients. Which biological effect is stronger? Did you measure the ratio of Bax/Bcl-2? Unless you know this ratio, it is difficult to understand the overall effect of the GnRH agonist.

Dr. Tesone: We did measure the ratio and confirmed that with LA there is an increase in pro-apoptotic proteins and a decrease in anti-apoptotic proteins.

Dr. Kitawaki, Kyoto University of Medicine, Japan: The molecular structure of antide is similar to LA. In view of this, you might anticipate that antide would have some effect of its own on apoptosis, in addition to antagonizing the effect of LA. It may be valuable to evaluate the effect of antide at lower doses.

Dr. Tesone: We have investigated lower doses of antide and it has no effect other than inhibiting the action of LA.

Disclosure Statement

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