

Effect of GnRH analogues on apoptosis and expression of Bcl-2, Bax, Fas and FasL proteins in endometrial epithelial cell cultures from patients with endometriosis and controls

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BACKGROUND: Our purpose was to evaluate the effect of the GnRH agonist (GnRHa), leuprolide acetate (LA), and the GnRH antagonist (GnRHant), Antide, on apoptosis and expression of apoptosis-related proteins in endometrial epithelial cell (EEC) cultures from patients with endometriosis and controls (infertile women without endometriosis). **METHODS:** Biopsy specimens of eutopic endometrium were obtained from 22 patients with endometriosis and from 14 women that served as controls. Apoptosis was examined in EEC after incubation with LA and Antide. Bax, Bcl-2, Fas and FasL expression was evaluated after exposure to LA, Antide or a combination of both. The percentage of apoptotic cells (%ApC) was assessed by the acridine orange–ethidium bromide technique, and protein expression was evaluated by western blot and immunocytochemistry. **RESULTS:** LA 100 and 1000 ng/ml increased the %ApC in EEC from patients with endometriosis (both $P < 0.05$) and controls ($p < 0.05$ and $P < 0.01$, respectively). Antide 10^{-5} M increased the %ApC in EEC from patients with endometriosis and controls ($P < 0.01$). In EEC from women with endometriosis, Bax expression increased after treatment with LA, Antide and LA + Antide ($P < 0.05$, $P < 0.001$ and $P < 0.001$), whereas Bcl-2 expression decreased after exposure to LA and Antide ($P < 0.001$ and $P < 0.01$). FasL expression increased after LA, Antide and LA + Antide treatments ($P < 0.01$, $P < 0.001$ and $P < 0.01$). No significant changes were observed on Fas expression. **CONCLUSIONS:** GnRH analogues enhanced apoptosis in EEC, and this was accompanied by an increase in expression of the pro-apoptotic proteins Bax and FasL and a decrease in expression of the anti-apoptotic protein Bcl-2.

Key words: apoptosis/endometriosis/GnRH analogues

Introduction

Endometriosis, defined as the presence of endometrial glands and stroma outside the uterine cavity, is an estrogen-dependent disease that affects 5–10% of women of reproductive age (Kitawaki *et al.*, 2002). The rationale behind the use of the GnRH analogues in the treatment of endometriosis is to achieve a regression of the endometriotic implants through the induction of hypoestrogenism by pituitary down-regulation (Vignali, 1998); however, a direct modulatory effect of GnRH analogues on endometriotic growth has been observed (Borroni *et al.*, 2000; Imai *et al.*, 2000; Sica *et al.*, 2001; Meresman *et al.*, 2003).

Apoptosis or programmed cell death is a physiological process that kills unwanted cells without inducing an immune response or inflammatory reaction (Garcia-Velasco and Arici, 2003) and is implicated in both normal development and disease (Assuncao and Linden, 2004). Accumulating evidence suggests that apoptosis helps to maintain cellular homeostasis

during the menstrual cycle by eliminating senescent cells from the functional layer of the uterine endometrium during the late secretory and menstrual phase of the cycle (Harada *et al.*, 2004).

Others and we have previously demonstrated that GnRH agonist (GnRHa) induces apoptosis in both eutopic and ectopic endometrial cells from patients with endometriosis (Imai *et al.*, 2000; Meresman *et al.*, 2003). Likewise, it has been shown that GnRH antagonist (GnRHant) induces apoptosis and reduces cell proliferation in ovarian, endometrial and breast cancer cell lines (Grundker *et al.*, 2002, 2004; Tang *et al.*, 2002) as well as in rat granulosa cells (Yano *et al.*, 1997). Also GnRHant enhance apoptosis by inducing the messenger RNA (mRNA) and protein expression of Fas, FasL and Bax as well as by reducing the mRNA and protein expression of Bcl-2 in leiomyoma and myometrial cells (Kwon *et al.*, 2005).

The first objective of the present work was to evaluate the effect of GnRHant on apoptosis in endometrial epithelial cells

from patients with endometriosis and controls. The second objective of this study was to evaluate the effect of GnRHa and GnRHant on the expression of apoptosis-related proteins Bcl-2, Bax, Fas and FasL.

Two major pathways towards apoptosis have been defined: the death receptor pathway and the mitochondrial pathway. The Bcl-2 family of proteins constitutes a critical intracellular checkpoint in the intrinsic pathway of apoptosis. The ratio of anti- to pro-apoptotic molecules, such as Bcl-2/Bax, constitutes a rheostat that sets the threshold of susceptibility to apoptosis for the intrinsic pathway, which utilizes organelles such as mitochondria to amplify death signal (Danial and Korsmeyer, 2004). Through this interaction, it has been proposed that Bax antagonizes Bcl-2 function abrogating the ability of Bcl-2 to prolong cell survival.

The Fas–Fas ligand (FasL) system is the most studied mechanism in the death receptor pathway, because it is the primary mechanism for induction of apoptosis in cells and tissues (Curtin and Cotter, 2003). Fas (also known as CD95 or Apo-1 and now as TNFRSF6) is a prototypical member of the tumour necrosis factor (TNF) receptor family (Locksley *et al.*, 2001) and induces apoptosis via cross-linking with FasL in various types of cells. When FasL binds to Fas, the target cell undergoes apoptotic cell death with characteristic nuclear condensation and DNA fragmentation (Nagata and Golstein, 1995).

In the human endometrium, several reports have clearly demonstrated that Bcl-2, Bax, Fas and FasL are expressed throughout the menstrual cycle (Tao *et al.*, 1997; Watanabe *et al.*, 1997; Yamashita *et al.*, 1999; Otsuki, 2001). Our previous work has shown the involvement of Bax and Bcl-2 in regulating the decreasing eutopic endometrial apoptosis in patients with endometriosis (Meresman *et al.*, 2000) and indicates that Fas and its ligand are diminished in eutopic endometrium from endometriosis patients when compared with control women (Meresman *et al.*, 2004). These results and several recent reports confirm the involvement of apoptosis as a mechanism in the pathogenesis of endometriosis (Dufournet *et al.*, 2006; Szymanowski, *in press*). In addition, it has been proposed that the anti-proliferative action of GnRH analogues would be mediated by the activation of the Fas–FasL system by increasing apoptotic cell death (Imai and Tamaya, 2000).

On the basis of the data reviewed, the aim of our study was to test the hypothesis that GnRH analogues induce apoptosis by both the mitochondrial and the death receptor pathways.

Subjects and methods

Subjects

A total of 36 patients who underwent diagnostic laparoscopy for infertility participated in this study: 22 with untreated endometriosis (stages I and II) and 14 controls. Determination of the stage of the disease was performed according to the Revised American Society for Reproductive Medicine Classification (ASRM, 1997). Control subjects were infertile women without endometriosis, with tubal factor infertility or unexplained infertility, undergoing diagnostic laparoscopy. All patients were infertile, showed regular menstrual cycles and had not received any hormonal medical treatment for the last 6 months. Biopsy specimens of eutopic endometrium were obtained from all subjects in the proliferative phase, as previously described (Meresman *et al.*, 2000).

This study was approved by the Ethics and Research Committee of the Biology and Experimental Medicine Institute of Buenos Aires, Argentina, and all subjects included in the study signed informed consents.

Isolation and culture of endometrial epithelial cells

EEC were obtained from biopsies of endometriosis patients. The tissue was immediately placed into culture medium and processed within 60 min of collection. Epithelial cells were enzymatically separated and isolated by successive centrifugation, and primary cultures were established for *in vitro* studies using the method previously described (Meresman *et al.*, 2003). Briefly, the explant was minced, washed and placed in basic medium (Dulbecco's modified Eagle's medium F12, Gibco, Paisley, UK) 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 a 2-h incubation at 37°C in an atmosphere of 5% CO₂, the resulting suspension was centrifuged at 100 g for 5 min. The pellet containing glands was re-suspended in nutrient medium and spun again at 100 g for 5 min. The final pellet mainly contained epithelial cells. Any stromal cells remaining with the glands were further separated by selective adherence to plastic tissue culture dishes for 1 h. The enriched epithelial cells were cultured with 10% fetal bovine serum (FBS; Gibco) and were grown to sub-confluence (70–80%) at 37°C for 48-h before the experiments.

It has been previously shown that this method guarantees purity of epithelial cells in culture (Meresman *et al.*, 2003). Briefly, cultures of epithelial cells from endometrium were fixed in 100% methanol for 20 min and stained by indirect immunofluorescence using mouse monoclonal anti-cytokeratin 56 kDa (reacts with cytokeratin polypeptide nos. 1, 2, 5, 6, 7, 8, 11, 14, 16, 17, 18; Serotec Ltd, Oxford, UK) followed by anti-immunoglobulin-fluorescein isothiocyanate (Dako Ltd, Cambridge, UK) as second antibody. 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. Identical immunocytochemical evaluation was also done on endometrial stromal cells.

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

Apoptosis assay

A total of 120 000 EEC were plated in Lab-Tek 8-well culture chambers (Nalge Nunc, Naperville, IL, USA). After 48-h incubation with 10% FBS medium, the cells were washed and cultured with 2.5% FBS medium. Different agents were added to supplemented 2.5% FBS medium: Antide (Sigma Laboratories, St. Louis, MO, USA) (10^{-7} , 10^{-6} and 10^{-5} M) was used as GnRHant, and leuprolide acetate (LA, Lupron; Abbot, Cedex, France) (100 and 1000 ng/ml) was added as GnRHa. The concentrations in the study were based on our titrations assessing apoptosis with either LA or Antide for the endometrial epithelial cells and from several published references on other uterus-derived cells (Imai *et al.*, 1998b; Nagai *et al.*, 2002; Tang *et al.*, 2002; Chen *et al.*, 2005; Kwon *et al.*, 2005). Basal condition consisted of supplemented 2.5% FBS medium without any agent. The cells were incubated for 24 additional hours, and the percentage of apoptotic cells (%ApC) was assessed by the acridine orange–ethidium bromide technique. 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 (Abrams *et al.*, 1993; Ribble *et al.*, 2005). After addition of the acridine

orange (4 µg/ml)–ethidium bromide (4 µg/ml) mix, the cells were viewed using a fluorescence microscope, and apoptotic cells were counted as percentage of the total by two observers. There was no significant difference in the results obtained between the two observers ($P = 0.75$).

Immunocytochemistry

A total of 120 000 EEC were plated in Lab-Tek 8-well culture chambers (Nalge Nunc). After a 48-h incubation with 10% FBS medium, the cells were washed, and different agents were added to supplemented 2.5% FBS medium: LA (1000 ng/ml); Antide (10^{-5} M); a combination of Antide with LA, adding LA 3 h after the addition of Antide; or nothing (basal). The cells were incubated with the agents for 24 additional hours. Following treatments, chamber slides were fixed for 10 min in 4% paraformaldehyde/phosphate-buffered saline (PBS) and permeabilized with 0.2% Triton X-100/PBS. Endogenous peroxidase activity was quenched with 3% H_2O_2 for 20 min at room temperature, after which non-specific binding was blocked by incubation with 2% bovine serum albumin (BSA)/PBS. Cells were incubated overnight with rabbit polyclonal anti-human Bax antibody (1:400, N-20, Santa Cruz Biotechnology Inc., California, CA, USA) or goat polyclonal anti-human Bcl-2 antibody (1:500, N-19, Santa Cruz Biotechnology Inc.) at 4°C. After that, cells were treated for 60 min with the corresponding secondary antibody-peroxidase-conjugated (LSAB+ System, Dako Corporation, Carpinteria, CA, USA).

Binding was visualized by incubating slides with diaminobenzidine and lightly counterstained with haematoxylin. As a negative control, immunoglobulin of the same immunoglobulin class and concentration as the primary antibody was used. The negative control showed absence of specific staining. Bax- and Bcl-2-positive cells were identified by the presence of brown cellular reactivity.

Western blot

To analyse the apoptosis-related proteins by western blot after the treatment with GnRHa, a 1000 ng/ml dose of LA was used, because lower doses did not show clear differences in the expression of such proteins (data not shown).

A total of 500 000 EEC were plated in 24-well plates. After incubation for 48 h with 10% FBS medium, the cells were washed, and different agents were added to supplemented 2.5% FBS medium: LA (1000 ng/ml); Antide (10^{-5} M); a combination of Antide with LA, adding LA 1000 ng/ml 3 h after the addition of Antide 10^{-5} M; or nothing (basal). Following treatment, cells were lysed by scraping in chilled lysis buffer [20 mM Tris-Cl (pH 8.0), 137 mM NaCl, 1% Nonidet P-40 and 10% glycerol] supplemented with protease inhibitors (0.5 mM phenylmethylsulphonyl fluoride, 0.025 mM N-CBZ-L-phenylalanine chloromethyl ketone, 0.025 mM N'-p-tosyl-lysine chloromethyl ketone, 0.025 mM L-1-tosylamide-2-phenyl-ethylchloromethyl ketone). After freeze thawing, the lysate was centrifuged at 15 000 g for 10 min at 4°C, and the pellet was discarded. Protein concentrations in the supernatant were measured by the Bradford assay (Bradford, 1976). Equal protein samples (20 µg) were solubilized using sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis sample buffer, boiled for 5 min and electrophoresed through a 12% SDS-polyacrylamide gel. The separated proteins were transferred to nitrocellulose membranes. Nitrocellulose membranes were blocked for 1 h in 5% low-fat powdered milk at room temperature and incubated with the primary antibody diluted in 1% low-fat powdered milk at 4°C. After overnight incubation, proteins were incubated with the appropriate peroxidase-conjugated secondary antibody diluted in 1% low-fat powdered milk at room temperature for 1 h. Protein bands were visualized by incubating the membranes with an enhanced chemiluminescence reagent (Perkin Elmer Life Science, Boston, MA, USA) followed by exposure to

Kodak X-Omat AR films. The levels of protein were compared and analysed by densitometric studies using Scion Image for Windows (Scion Corporation, Worman's Mill, CT, USA). Consistency of protein loading was evaluated by staining the membranes with Ponceau-S. As an internal control, the density of each protein was normalized to the density of a band that was observed in the protein transference pattern in all Ponceau-S stained membranes. This band was selected because it was unchanged under the different treatments (data not shown). Selected samples were validated with β -actin loaded as control, obtaining similar results (data not shown). Although Ponceau-S technique is actually not the method of choice for protein normalization, protein quantification was performed by Bradford's technique, identical protein quantity was loaded in each lane and Ponceau-S normalization did not show any significant difference from β -actin normalization (data not shown). Results are expressed as percentage of basal \pm SEM.

The following antibodies were used: rabbit polyclonal anti-human Bax (1:200, N-20; Santa Cruz Biotechnology Inc.), mouse monoclonal anti-human Bcl-2 (1:50, Clone 124, Dako Corporation), rabbit polyclonal anti-human Fas (1:3000, C-20, Santa Cruz Biotechnology Inc.) and rabbit polyclonal anti-human FasL (1:500, Q-20, Santa Cruz Biotechnology Inc.) as primary antibodies; goat peroxidase-conjugated anti-mouse immunoglobulin G (IgG) (1:4000, HAF007, R&D Systems Inc., Minneapolis, MN, USA) or goat peroxidase-conjugated anti-rabbit IgG (1:1000, A4914, Sigma Laboratories) as secondary antibodies.

Statistics

Statistical comparisons were performed by Kruskal–Wallis non-parametric analysis of variance test, followed by Dunn's multiple comparison test. Regardless of the statistical test, only a P -value ≤ 0.05 was considered significant.

Results

Effect of GnRHa on apoptosis in endometrial epithelial cells from patients with endometriosis and controls

Treatment with LA enhanced apoptosis in EEC from patients with endometriosis (Figures 1A and 2). The %ApC showed a significant increase from 23 ± 2 to $42 \pm 4\%$ ($P < 0.05$) after treatment with LA 100 ng/ml and to $46 \pm 8\%$ ($P < 0.05$) after exposure to LA 1000 ng/ml (Figure 1A). Also LA increased apoptosis in EEC from control women (Figure 1B): the %ApC increased from 21 ± 2 to $42 \pm 6\%$ ($P < 0.05$) after treatment with LA 100 ng/ml and to $48 \pm 3\%$ ($P < 0.01$) after exposure to LA 1000 ng/ml (Figure 1B). There was no significant difference in the %ApC between the EEC from endometriosis and control patients.

Effect of GnRHant on apoptosis in endometrial epithelial cells from patients with endometriosis and controls

Treatment with Antide 10^{-5} M enhanced apoptosis in EEC from patients with endometriosis and controls (Figure 3). The %ApC showed a significant increase from 22 ± 4 to $48 \pm 4\%$ ($P < 0.01$) in cultures from patients with endometriosis (Figures 3A and 2) and from 24 ± 1 to $46 \pm 2\%$ ($P < 0.01$) in cultures from control women (Figure 3B). After exposure to concentrations of 10^{-7} and 10^{-6} M of Antide, no significant changes were observed on the %ApC in EEC from patients with endometriosis and control women (Figure 3): in cultures

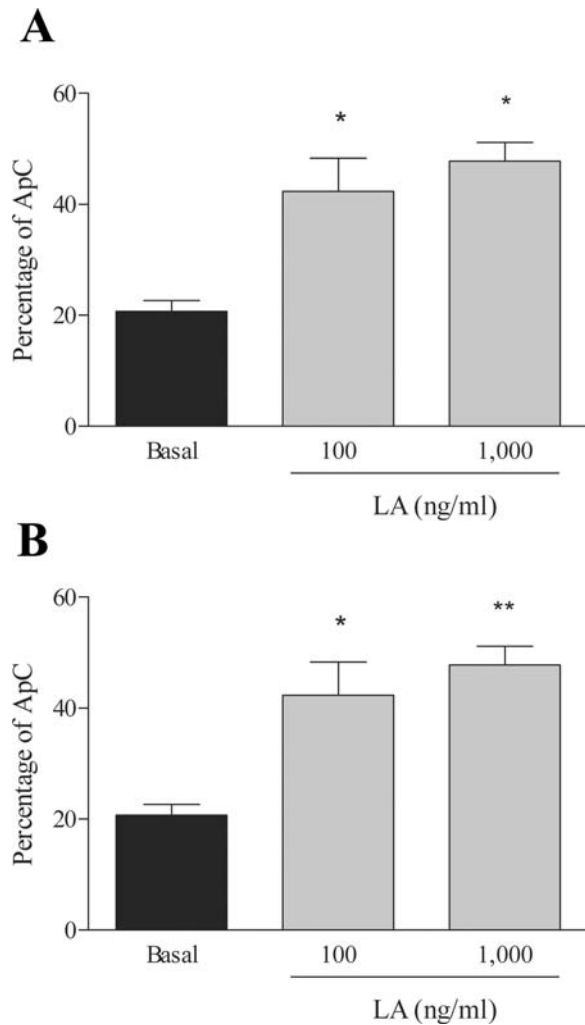


Figure 1. Effects of the GnRH agonist leuprolide acetate (LA) on apoptosis in endometrial cell cultures from subjects with endometriosis (A) and controls (B). Epithelial cell cultures from patients with endometriosis (A) and control women (B) were analysed for apoptosis by the acridine orange–ethidium bromide technique at basal conditions and after stimulation with LA 100 ng/ml and 1000 ng/ml. Values are expressed as percentage of apoptotic cells (%ApC) \pm SEM. The cells were viewed by a fluorescence microscope. ApC were counted as a percentage of the total. * $P < 0.05$ versus basal; ** $P < 0.01$ versus basal.

from control women, the %ApC was 26 ± 1 and $33 \pm 2\%$ after treatment with Antide 10^{-7} and 10^{-6} M, respectively (Figure 3B) and in cultures from patients with endometriosis, the %ApC was 28 ± 3 and $33 \pm 2\%$ after exposure to Antide 10^{-7} and 10^{-6} M, respectively (Figure 3A). In addition, we found no significant differences in the %ApC between EEC from endometriosis patients and EEC from controls. Taking all these results into account, we decided to utilize only EEC cultures from patients with endometriosis to evaluate the expression of apoptosis-related proteins.

Effects of GnRH analogues on Bax and Bcl-2 protein expression in endometrial epithelial cells from patients with endometriosis

The expression of Bcl-2 and Bax was estimated using western blot analysis (Figure 4). LA 1000 ng/ml caused a $42 \pm 12\%$

increase in Bax expression when compared with basal levels ($P < 0.05$) and treatment with Antide 10^{-5} M, and the combination of both showed a 107 ± 24 and a $135 \pm 49\%$ increase in Bax expression, respectively ($P < 0.001$) (Figure 4A). EEC from patients with endometriosis treated with LA 1000 ng/ml showed a $31 \pm 4\%$ decrease in Bcl-2 expression when compared with basal levels ($P < 0.001$), whereas those treated with Antide 10^{-5} M caused a $24 \pm 8\%$ decrease in Bcl-2 expression ($P < 0.01$) (Figure 4B). Despite these findings, no significant differences were observed in Bcl-2 expression after treatment with the combination of LA and Antide (Figure 4B).

Bax and Bcl-2 protein expression patterns obtained by the immunocytochemical technique were generally similar to those from western blot analysis. Treatment with LA, Antide and the combination of both increased Bax immunostaining in endometrial epithelial cells from patients with endometriosis when compared with basal levels (Figure 5). Immunostaining for Bcl-2 decreased in cells treated with LA, Antide and the combination of both (unlike western blot, for LA + Antide) when compared with basal levels (Figure 6).

Effect of GnRH analogues on Fas and FasL proteins in endometrial epithelial cells from patients with endometriosis

The expression of Fas and FasL was analysed by western blot (Figure 7). After exposure to LA 1000 ng/ml, FasL expression increased by $61 \pm 13\%$ when compared with basal levels ($P < 0.01$) (Figure 7A). Treatment with Antide 10^{-5} M caused a $121 \pm 52\%$ increment in FasL expression ($P < 0.001$), whereas treatment with the combination of LA and Antide showed a $100 \pm 33\%$ increase ($P < 0.01$) (Figure 7A). No statistically significant effect was observed on Fas expression in EEC from patients with endometriosis after treatment with LA, Antide or the combination of both (Figure 7B).

Discussion

The GnRHa are used in the treatment of different estrogen-dependent disorders like endometriosis. These compounds act by producing a profound suppression in the gonadotropin output by the pituitary gland that causes a hypoestrogenic status with a subsequent reduction on the size of the endometriotic lesions. All of the current indications for GnRHa pituitary down-regulation may prove to be indications for GnRHant. Likewise, the use of GnRHant for the treatment of endometriosis has already been reported (Kupker *et al.*, 2002). GnRHant seem to offer important advantages due to their specific pharmacological mode of action. They do not cause the initial stimulation of gonadotropin (flare) and ovarian hormone release that is associated with GnRHa administration; therefore, they have the theoretical advantage of working faster and more effectively than GnRHa with an earlier improvement of symptoms (Ferrero *et al.*, 2005).

Apoptosis indices in the eutopic endometrium of patients with endometriosis were lower compared with women without endometriosis (Meresman *et al.*, 2000; Szymanowski, in press). The increased cell viability in eutopic endometrium from these patients as a consequence of a reduction in cell

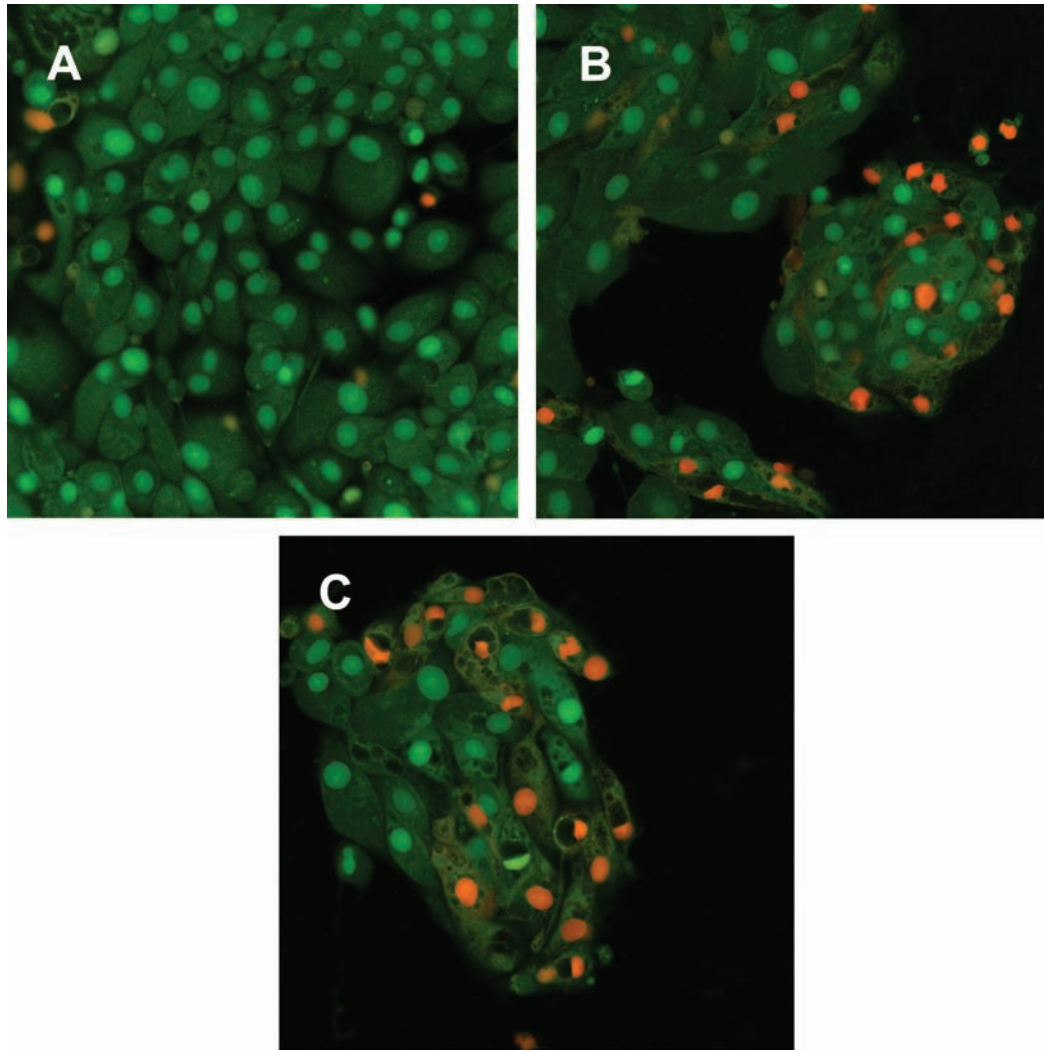


Figure 2. Effects of leuprolide acetate (LA) and Antide on apoptosis in endometrial cell cultures from subjects with endometriosis. Endometrial epithelial cells from patients with endometriosis were plated in cell chamber slides under basal conditions (A) or after treatment with LA 1000 ng/ml (B) or Antide 10^{-5} M (C). Apoptosis was assessed by acridine orange and ethidium bromide staining and evaluated by fluorescent microscopy (magnification: $\times 400$).

death by apoptosis, as well as the increase in cell proliferation, indicates that this condition may facilitate the invasive feature of the endometrium (Johnson *et al.*, 2005).

In this study, EEC cultures of human endometrium from control women and from patients with endometriosis were used as a model to evaluate cell proliferation and apoptosis in response to the addition of GnRH analogues. Although the cells obtained from patients with endometriosis were not derived from endometriotic implants and their *in vitro* response may not be identical, the relevance for the use of endometrial cells in short-term culture as a model for endometriotic implants has been previously described (Surrey and Halme, 1990; Meresman *et al.*, 2003).

In the present study, we confirmed that the GnRH_a, LA (100 and 1000 ng/ml), increases the %ApC in EEC from patients with endometriosis and control women. In agreement with us, other authors have demonstrated that GnRH_a increase the apoptotic rate in different cellular types. GnRH_a trigger apoptosis in a single suspension of endometrial stromal and glandular

cells from patients with endometriosis (Imai *et al.*, 2000) and also stimulate apoptosis in rat granulosa cells (Andreu *et al.*, 1998) and in cultured uterine leiomyoma cells (Wang *et al.*, 2002). Lately, many publications have suggested that the GnRH_a have a direct effect on the ectopic endometrial growth (Vignali, 1998; Borroni *et al.*, 2000; Sica *et al.*, 2001). Also, previous results from our laboratory suggest that treatment with LA diminishes the cell proliferation and enhances apoptosis in endometrial cell cultures (Meresman *et al.*, 2003).

The results obtained suggest that the GnRH_{ant}, Antide (10^{-5} M) increases the %ApC in EEC from control women and patients with endometriosis. These results are in agreement with other publications that demonstrated the effects of GnRH_{ant} on different cell types, by enhancing apoptosis. Cetrorelix increases the %ApC in cell lines from ovarian cancer (Tang *et al.*, 2002). Also, it has been observed that Cetrorelix induces apoptosis in granulosa cell cultures obtained from rat pre-ovulatory follicles (Yano *et al.*, 1997) and in leiomyoma cells in culture (Chen *et al.*, 2005; Kwon *et al.*, 2005). The fact that

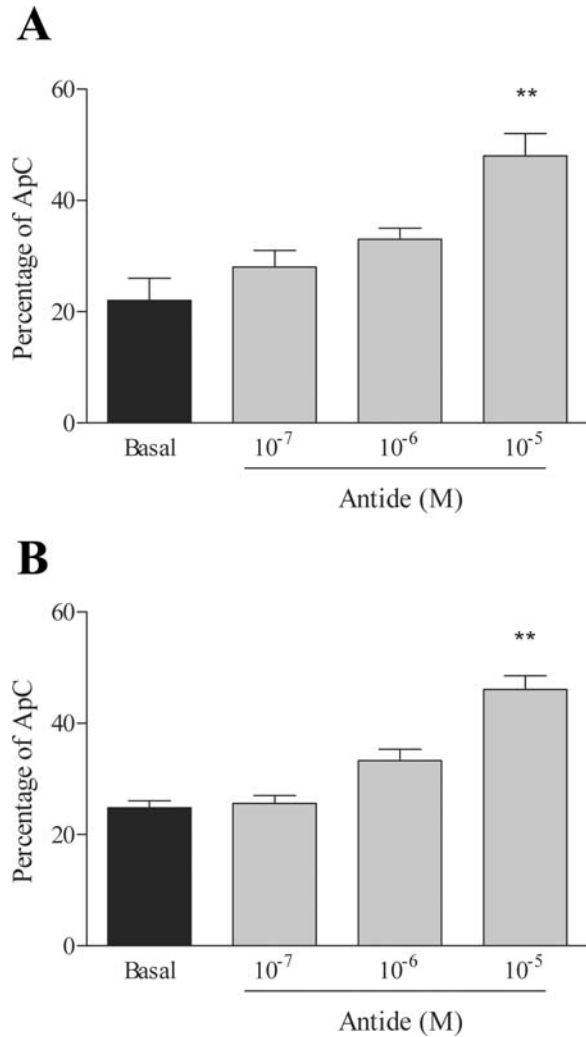


Figure 3. Effects of the GnRH antagonist Antide on apoptosis in endometrial cell cultures from subjects with endometriosis (A) and controls (B). Epithelial cell cultures from patients with endometriosis (A) and control women (B) were analysed for apoptosis by the acridine orange–ethidium bromide technique under basal conditions and after stimulation with Antide 10^{-7} , 10^{-6} and 10^{-5} M. Values are expressed as a percentage of apoptotic cells (%ApC) \pm SEM. The cells were viewed by a fluorescence microscope. ApC were counted as a percentage of the total (** $P < 0.01$ versus basal).

serum levels of GnRH analogues in patients differ from the doses used in our experiments (Erb *et al.*, 2001; Periti *et al.*, 2002) is not surprising due to the different environmental conditions usually observed between *in vivo* and *in vitro* studies. Nevertheless, this discrepancy does not affect the relevance of our *in vitro* experiments, which is related to the direct action of the analogues on endometrial cell growth independently of a possible effect through pituitary interactions.

We found no significant differences in the rate of apoptosis between endometrial cell cultures from endometriosis patients and those from controls. This is in contrast with the results in whole endometrial sections observed in our previous study (Meresman *et al.*, 2000). We could speculate that after 4 days of culture of the glandular fraction, the endometrial epithelial cells altered their initial conditions, and we could not detect

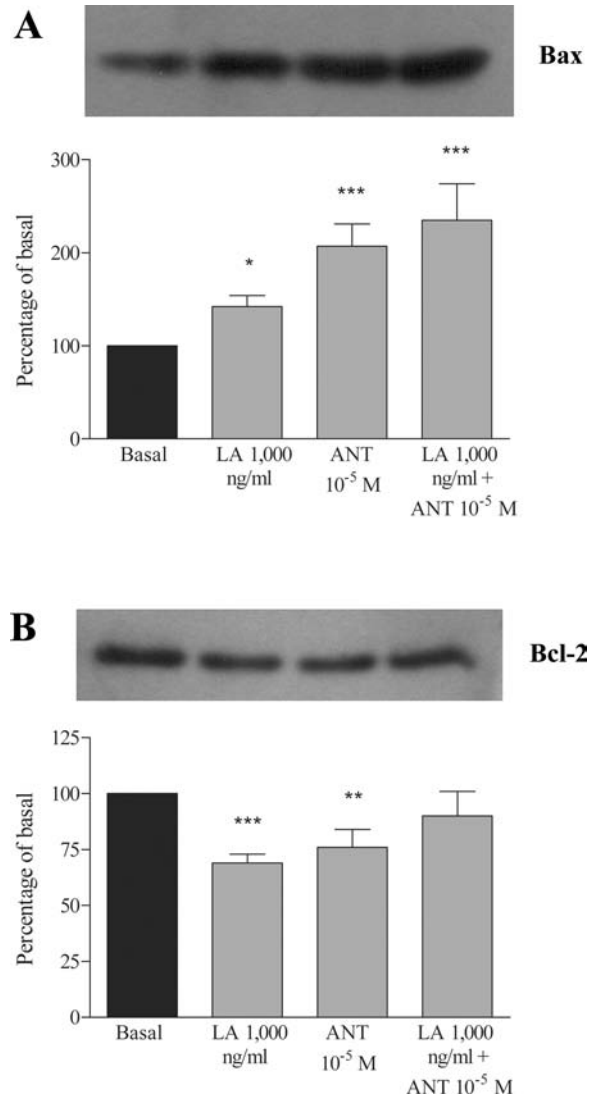


Figure 4. Effects of leuprolide acetate (LA) and Antide (ANT) on Bax (A) and Bcl-2 (B) protein expression in endometrial cell cultures from subjects with endometriosis. Western blot analysis was performed on cell homogenates from endometrial epithelial cell cultures from patients with endometriosis under basal conditions and after treatment with LA 1000 ng/ml, ANT 10^{-5} M or a combination of ANT with LA. Representative blots for Bax (A) and Bcl-2 (B) are presented in upper panels. The lower panels show quantification of results for Bax (A) and Bcl-2 (B) expressed as a percentage of basal values \pm SEM. Consistency of protein loading was evaluated by staining the membranes with Ponceau-S (* $P < 0.05$ versus basal; ** $P < 0.01$ versus basal; *** $P < 0.001$ versus basal).

maintenance of their apoptotic characteristics in comparison with when we used the entire tissue.

Fas is the best-characterized member of the TNF super-family receptors. Its main and best-known function in signalling is the induction of apoptosis. Fas is expressed on the surface of cells as pre-associated homotrimers (Peter and Krammer, 2003). Fas is expressed in most tissues and also plays an important role in the function/regulation of those same tissues (Curtin and Cotter, 2003). The balance of expression between Fas and FasL determines the predisposition of a cell to die by apoptosis involving the death receptor pathway. In this study,

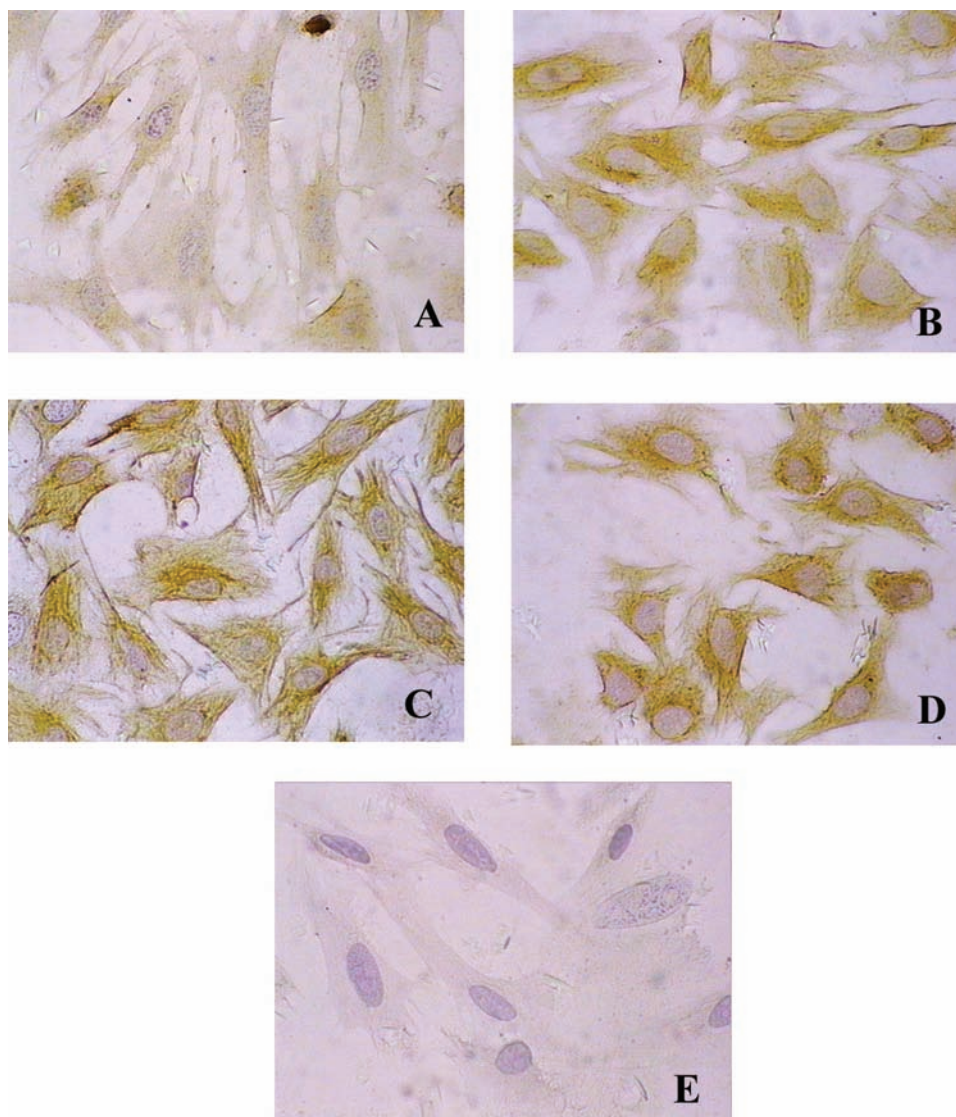


Figure 5. Immunocytochemistry of Bax expression in endometrial cell cultures from subjects with endometriosis after treatment with leuprolide acetate (LA) and Antide. Endometrial epithelial cells from patients with endometriosis were plated in cell chamber slides under basal conditions (A) or after treatment with LA 1000 ng/ml (B), Antide 10^{-5} M (C) or a combination of Antide with LA (D). In negative control (E), normal rabbit immunoglobulin G (IgG) was used instead of primary antibody (magnification: $\times 600$).

we noticed that LA (1000 ng/ml), Antide (10^{-5} M) and the combination of both increased the expression of FasL in the EEC from patients with endometriosis. In agreement with these results, Imai *et al.* (1998a) have demonstrated that the GnRH analogue busserelin acetate induces the expression of FasL mRNA and FasL protein in isolated cells from ovarian cancer and endometrial cancer, as well as in cell lines from those carcinomas. Also, it has been demonstrated that GnRH analogues increase Fas expression and induce the expression of FasL in cells from leiomyomas in culture (Wang *et al.*, 2002). Cetrorelix enhances the mRNA and protein expression of Fas and FasL in leiomyoma and myometrial cells (Kwon *et al.*, 2005).

Our hypothesis was that GnRH analogues increase apoptosis by the Fas/FasL pathway. Although in this study we did not observe significant changes in the expression of Fas by any of the treatments tested, we cannot discard the induction of apoptosis through the receptor pathway because the increased

expression of FasL would demonstrate that this apoptotic pathway remains active (Nagata and Golstein, 1995). Recent data have demonstrated a similar profile to the one shown in our study in the expression of Fas/FasL in the endometrium from Rhesus monkeys (Wei *et al.*, 2005) and humans (Watanabe *et al.*, 1997; Yamashita *et al.*, 1999; Otsuki, 2001). During the menstrual cycle, Fas and FasL are co-expressed in the endometrial epithelium. The expression of Fas does not change in the different phases of the cycle, whereas the expression of FasL is increased during the secretory and menstrual phases, when apoptosis takes place in the endometrial tissue (Watanabe *et al.*, 1997; Yamashita *et al.*, 1999; Otsuki, 2001). More studies are needed to further investigate the involvement of Fas/FasL pathway in apoptosis mediated by GnRH analogues in EEC.

As reviewed recently, the Bcl-2 family of intracellular proteins is the central regulator of caspase activation, and its

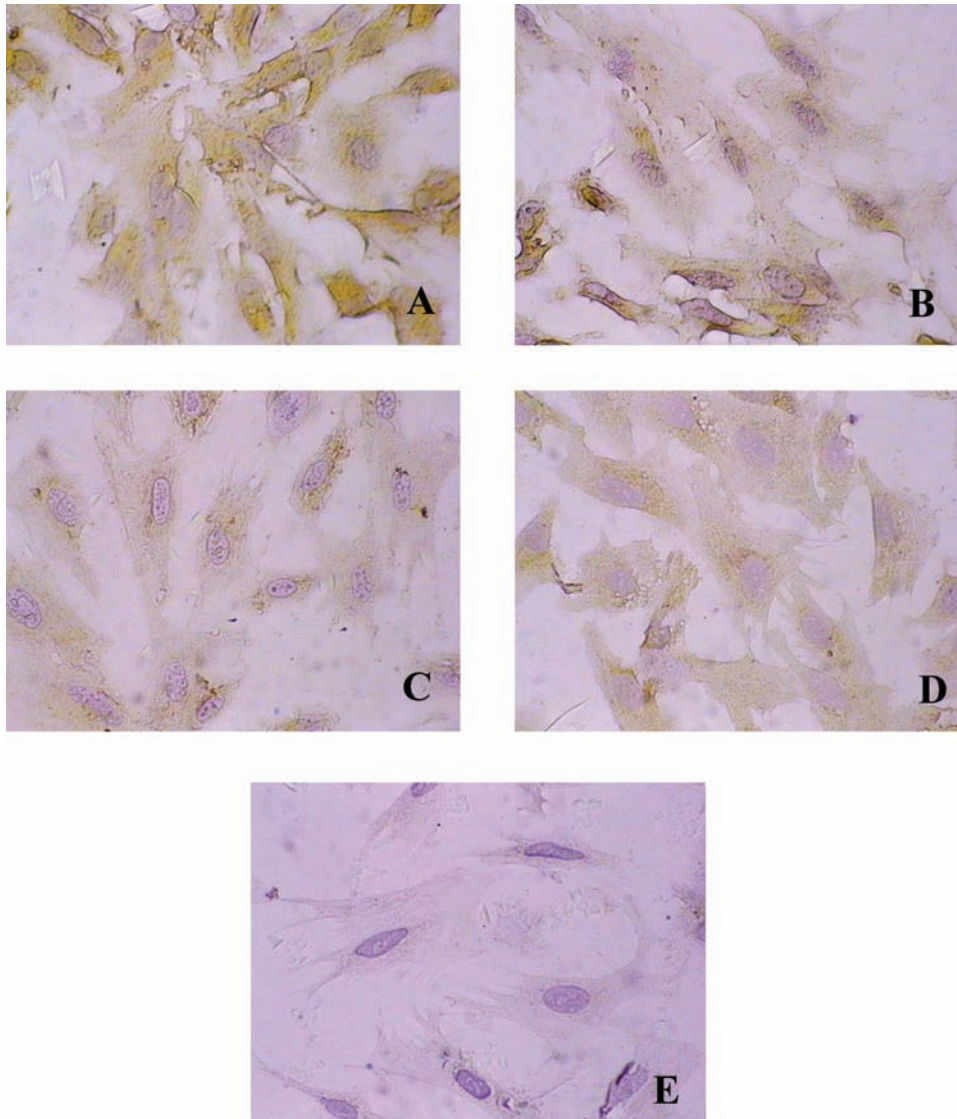


Figure 6. Immunocytochemistry of Bcl-2 expression in endometrial cell cultures from subjects with endometriosis after treatment with leuprolide acetate (LA) and Antide. Endometrial epithelial cells from patients with endometriosis were plated in cell chamber slides under basal conditions (A) or after treatment with LA 1000 ng/ml (B), Antide 10^{-5} M (C) or a combination of Antide with LA (D). In negative control (E), normal goat immunoglobulin G (IgG) was used instead of primary antibody (magnification: $\times 600$).

opposing factions of anti- and pro-apoptotic members arbitrate the life or death decision (Cory and Adams, 2002). Another feature of the Bcl-2 family members is their capacity to make homo- and heterodimers. Bax can interact with Bcl-2 by antagonizing its function and preventing cell survival (Burlacu, 2003). In this study, we demonstrate that LA (1000 ng/ml) and Antide (10^{-5} M) increase Bax protein expression and decrease Bcl-2 protein expression in the EEC from patients with endometriosis. The treatment with a combination of LA and Antide increased Bax expression but did not cause significant changes in Bcl-2 expression. Papadopoulos *et al.* (1999) found that GnRHa induce apoptosis in the *corpus luteum* in pregnant rats with an increased Bax expression but without changes in Bcl-2 expression. Other authors have observed no differences in Bcl-2 expression in leiomyomas between LA-treated and non-treated patients (Huang *et al.*, 2002). In addition, Kwon has shown increased Bax mRNA

and protein levels after treatment with Cetrorelix in leiomyoma and myometrial cells (Kwon *et al.*, 2005). On the basis of our results, it appears to be that the apoptosis induced by GnRH analogues is mediated by the mitochondrial pathway as we hypothesized.

In this study, we observed that the GnRHant Antide causes similar effects to LA on endometrial apoptosis. In addition, we observed similar expression profiles of Bax, Bcl-2, Fas and FasL after treatment with LA, Antide or both. There are a few reports in the literature about different types of cells, where GnRHa and GnRHant appear to have the same effects (Segal-Abramson *et al.*, 1992; Yano *et al.*, 1997; Tang *et al.*, 2002; Grundker *et al.*, 2004). The absence of opposing actions by the agonists and antagonists could be because of the presence of other binding sites, different from the classic GnRH receptor Type I, over which LA is an agonist. Antide at high concentrations might potentially bind to this different binding site acting

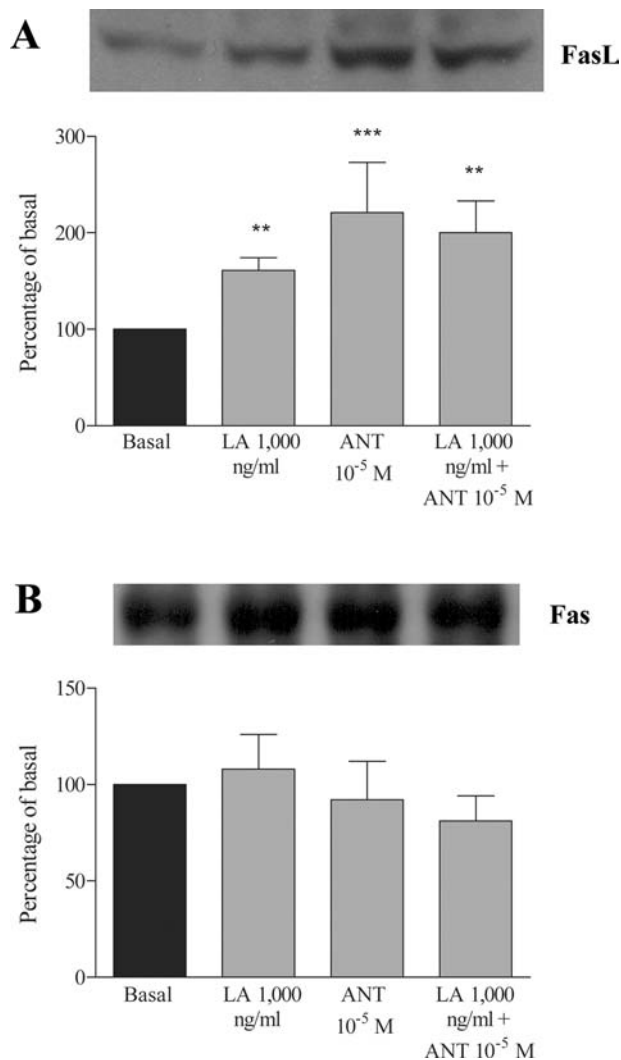


Figure 7. Effects of leuprolide acetate (LA) and Antide (ANT) on FasL (A) and Fas (B) protein expression in endometrial cell cultures from subjects with endometriosis. Western blot analysis was performed on cell homogenates from endometrial epithelial cell cultures from patients with endometriosis under basal conditions and after treatment with LA 1000 ng/ml, ANT 10⁻⁵ M or a combination of ANT with LA. Representative blots for FasL (A) and Fas (B) are presented in upper panels. The lower panels show quantification results for FasL (A) and Fas (B) expressed as a percentage of basal values \pm SEM. Consistency of protein loading was evaluated by staining the membranes with Ponceau-S (** $P < 0.01$ versus basal; *** $P < 0.001$ versus basal).

as an agonist and mediating the anti-proliferative and pro-apoptotic effects, as has been reported in other cell types (Segal-Abramson *et al.*, 1992; Grundker *et al.*, 2004). Alternatively, it has been proposed that the intracellular environment could modulate the ligand-binding characteristics of the GnRH receptor Type I and the nature of the intracellular signalling elicited by analogues (Millar and Pawson, 2004). In this manner, Antide would selectively bind to a specific active conformation of the GnRH receptor stabilized by specific intracellular protein complexes, which would result in a specific signalling. Future studies are needed to more clearly determine the mechanism of action of the analogues at this level.

In summary, we have demonstrated that both Antide and LA enhance apoptosis in EEC and that Antide and LA increase the expression of pro-apoptotic proteins Bax and FasL and diminish the expression of the anti-apoptotic protein Bcl-2. We suggest that the apoptosis induced by GnRH_a and GnRH_{ant} in EEC is mediated by the Bax/Bcl-2 pathway. On the basis of the data obtained in this study, we cannot discard the involvement of the Fas/FasL pathways, but more studies are needed to further clarify this issue.

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