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Effect of vascular endothelial growth factor and interleukin-1β on apoptosis in endometrial cell cultures from patients with endometriosis and controls

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ABSTRACT

The aim of this study was to evaluate the effect of vascular endothelial growth factor (VEGF) and interleukin-1β (IL-1β) on apoptosis induced by leuprolide acetate (LA) in endometrial epithelial cell cultures from patients with endometriosis. Primary endometrial epithelial cell cultures were obtained from uterine endometrial biopsies of patients with endometriosis and control women. Endometrial epithelial cells were incubated with LA; a combination of LA and VEGF; a combination of LA and IL-1β; or in basal conditions. LA was added 3 h prior to addition of VEGF and IL-1β. After stimulation, the percentage of apoptotic cells was evaluated by the acridine orange–ethidium bromide technique and Bax expression was assessed by western blot. Treatment with LA enhanced the percentage of apoptotic cells in endometrial epithelial cells from subjects with endometriosis and control subjects. Addition of either VEGF or IL-1β after exposure to LA restored the percentage of apoptotic cells to basal levels. Moreover, treatment with LA increased Bax expression in endometrial epithelial cells from patients with endometriosis. This effect was reverted by the addition of either VEGF or IL-1β. Our results show that VEGF and IL-1β reduce apoptosis and decrease Bax expression in endometrial epithelial cells from patients with endometriosis. This study suggests that VEGF and IL-1β may protect endometriotic cells from undergoing apoptosis in addition to exerting their pro-angiogenic role.

1. Introduction

Endometriosis is a chronic and recurrent disease characterized by the presence of functional endometrial tissue outside the uterine cavity (Crosignani et al., 2006). It is associated with pelvic pain and infertility and is estimated to occur in 6–10% of the general female population and in 35–50% of women presenting pelvic pain, infertility or both (Giudice and Kao, 2004). The most widely accepted theory for the development of endometriosis proposes the transplantation of exfoliated endometrium into the abdominal cavity (Sampson, 1927). According to this theory, the establishment of a new blood supply is essential for the survival and development of endometriotic lesions; therefore, angiogenesis is a mandatory process in the pathogenesis of endometriosis (Groothuis et al., 2005).

Vascular endothelial growth factor (VEGF) is a heparin-binding glycoprotein with potent angiogenic, endothelial cell-specific mitogenic and vascular permeability activities (Donnez et al., 1998). Studies have demonstrated that VEGF is involved in both the etiology and maintenance of peritoneal endometriosis (Donnez et al., 1998). Moreover, it

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has been shown that VEGF levels are increased in the peritoneal fluid from patients with endometriosis compared with control women (McLaren et al., 1996).

Besides being a potent pro-angiogenic factor, VEGF may also have a direct proliferative or anti-apoptotic effect on different cell types (Mercurio et al., 2005). In addition, it has been shown that in normal endometrium the expression of VEGF is potentiated by a variety of cytokines, especially interleukin-1β (IL-1β) (Lebovic et al., 2000).

IL-1β is a pleiotropic cytokine that participates in several immunological and reproductive processes that occur in normal endometrium during the menstrual cycle (Simon et al., 1995). It has been demonstrated that IL-1β expression is increased in the eutopic endometrium and in the peritoneal fluid from patients with endometriosis compared to control women (Kyama et al., 2008). In addition to its mitogenic effects, IL-1β induces the protection of different cells from apoptotic death (Markstrom et al., 2002; Simonart and Van Vooren, 2002).

Accumulating evidence indicates that apoptosis plays a critical role in the pathogenesis of endometriosis (Harada et al., 2007). We have demonstrated that spontaneous apoptosis is decreased in ectopic and eutopic endometrium from patients with endometriosis compared to control women (Meresman et al., 2000). This feature is related to its growth and survival in an ectopic site (Dmowski et al., 2001).

The Bcl-2 family of proteins constitutes a critical intracellular checkpoint in the intrinsic pathway of apoptosis and their members can be subdivided into anti-apoptotic factors (such as Bcl-2) and pro-apoptotic factors (such as Bax) (Youle and Strasser, 2008). In the previous work, we have demonstrated that leuprolide acetate (LA), a GnRH agonist, diminishes the levels of VEGF and IL-1β in the supernatant of endometrial epithelial cell cultures from patients with endometriosis (Meresman et al., 2003). Also it has been reported that VEGF suppresses apoptosis in vascular endothelial cells because it regulates Bcl-2 family proteins (Nor et al., 1999).

Taking into account the reviewed data, the objective of the present work was to evaluate the effect of VEGF and IL-1β on apoptosis induced by LA in endometrial epithelial cell cultures from patients with endometriosis.

2. Materials and methods

2.1. Subjects

A total of 27 patients who underwent diagnostic laparoscopy for infertility participated in this study: 19 with untreated endometriosis (stages I and II) and 8 control patients. 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, had regular menstrual cycles and had not received any hormonal medical treatment for the last six 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 Instituto de Biología y Medicina Experimental, Buenos Aires, Argentina, and all subjects included in the study signed informed consents.

2.2. Isolation and culture of endometrial epithelial cells

Endometrial epithelial cells were obtained from uterine endometrial biopsies of patients with endometriosis and control women. The tissue was immediately placed into culture medium and processed within 60 min of collection. Epithelial cells were enzymatically separated, isolated by successive centrifugation and primary cultures were established for *in vitro* studies using the method previously described (Bilotas et al., 2007). Briefly, the explant was minced, washed and placed in basic medium (DMEM 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 2 h incubation at 37 °C in an atmosphere of 5% CO_2, the resulting suspension was centrifuged at 100 × *g* for 5 min. The pellet containing glands was re-suspended in nutrient medium and centrifuged again at 100 × *g* for 5 min. The final pellet mainly contained epithelial cells. After 1 h incubation, the medium containing enriched epithelial cells was removed and plated in fresh medium. Epithelial cell cultures were grown to sub-confluence (70–80%) at 37 °C for two days. The cells were cultured with 10% fetal bovine serum (FBS; Gibco) before the experiments.

The purity of epithelial cells in culture was evaluated by immunocytochemistry using a cocktail of two mouse monoclonal antibodies against human cytokeratin (clones AE1/AE3, Dako Ltd., Cambridge, UK). Clones AE1/AE3 react with cytokeratin polypeptide numbers 1–8, 10, 13–16 and 19. The broad spectrum cytokeratin antibody produced clear specific labelling in 90% of the cells in the endometrial epithelial cell cultures (data not shown). No staining of stromal cells was observed with the anti-cytokeratin antibody.

The *in vitro* model used in this work has been extensively employed by us and other authors as a model for endometriosis research (Bilotas et al., 2007; Borroni et al., 2000; Garcia-Velasco et al., 1999; Meresman et al., 2003; Olivares et al., 2008; Ryan et al., 1994)

2.3. Apoptosis assay

One hundred and twenty thousand endometrial epithelial cells were plated in Lab-Tek 8-well culture chambers (Nalge Nunc, Naperville, IL, USA). 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 (Lupron; Abbot, Cedex, France) (1000 ng/ml); a combination of LA (1000 ng/ml) and VEGF at different doses (0.1, 1 and 10 ng/ml); or a combination of LA and IL-1β at different doses (0.1, 1 and 10 ng/ml). LA was added 3 h prior to VEGF or IL-1β to induce apoptosis in endometrial epithelial cells as we have previously demonstrated (Bilotas et al., 2007). Basal conditions consisted of supplemented 2.5% FBS medium without any agent. The cells were incubated for a further 24 h and the percentage of apoptotic cells

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 by a fluorescence microscope and apoptotic cells were counted as percentage of the total by two observers.

2.4. Western blot

One million endometrial epithelial cells from patients with endometriosis were plated in 6-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; a combination of LA 1000 ng/ml and VEGF 1 ng/ml; a combination of LA 1000 ng/ml and IL-1β 1 ng/ml; or nothing (basal). LA 1000 ng/ml was added 3 h before the addition of VEGF and IL-1β. Following treatment, cells in plates 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 a protease inhibitor cocktail (P8340, Sigma, Saint Louis, MO, USA). After freeze-thaw 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 amounts of protein samples (20 μg) were solubilized with SDS polyacrylamide gel electrophoresis sample buffer, boiled for 5 min and electrophoresed through a 12% SDS 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 powered milk at room temperature for 1 h. Protein bands were visualized by incubating the membranes with an enhanced chemiluminescence reagent (PerkinElmer Life Science, Boston, MA, USA) followed by exposure to Kodak X-Omat AR films. The levels of protein were compared and analyzed by densitometric studies using Scion Image for Windows (Scion Corporation, Worman's Mill, CT). Consistency of protein loading was evaluated using β-actin as control loading. Results are expressed as percentage of basal ± SEM.

The following antibodies were used: rabbit polyclonal anti-human Bax (1:200, N-20; Santa Cruz Biotechnology Inc.) and mouse monoclonal anti-human β-actin (1:2000; Abcam Inc., Cambridge, MA, USA) as primary antibodies; and goat peroxidase conjugated anti-rabbit IgG (1:1000; A4914, Sigma) or goat peroxidase conjugated anti-mouse IgG (1:4000; HAF007, R&D Systems Inc., Minneapolis, USA) as secondary antibodies.

2.5. Statistics

Statistical comparisons were performed using two-way ANOVA followed by Tukey honest significant difference test. Western blot experiments were analysed by one-way ANOVA followed by Tukey's multiple comparison test. A p value ≤ 0.05 was considered significant.

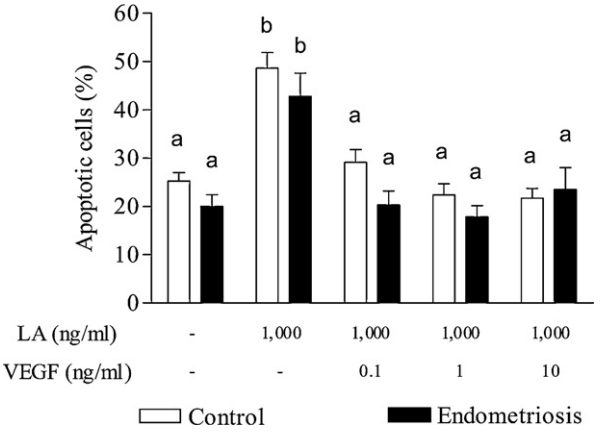

Fig. 1. Effects of vascular endothelial growth factor (VEGF) on apoptosis induced by leuprolide acetate (LA) in endometrial epithelial cell cultures from subjects with endometriosis and control subjects. Endometrial epithelial cell cultures from patients with endometriosis and control women were analyzed for apoptosis by the acridine orange–ethidium bromide technique at basal conditions and after stimulation with either LA (1000 ng/ml) or a combination of LA (1000 ng/ml) and different doses of VEGF. Values are expressed as percentage of apoptotic cells ± SEM. The cells were viewed by a fluorescence microscope. Apoptotic cells were counted as a percentage of the total. a vs. b $p < 0.001$

3. Results

3.1. Effect of LA on apoptosis in endometrial epithelial cells from patients with endometriosis and controls

Treatment with LA 1000 ng/ml caused a significant increase in the percentage of apoptotic cells in endometrial epithelial cell cultures from both subjects with endometriosis and controls ($p < 0.001$ vs. basal) (Figs. 1 and 2).

3.2. Effect of VEGF on apoptosis induced by LA in endometrial epithelial cells from patients with endometriosis and controls

Treatment with VEGF 0.1 ng/ml, 1 ng/ml and 10 ng/ml after exposure to LA 1000 ng/ml restored the percentage of apoptotic cells to basal levels in endometrial epithelial cell cultures from both endometriosis patients and control women ($p < 0.001$ vs. LA 1000 ng/ml; $p > 0.05$ vs. basal) (Fig. 1). In addition, there was no significant difference in the percentage of apoptotic cells between the endometrial cell cultures from endometriosis and control patients ($p > 0.05$).

3.3. Effect of IL-1β on apoptosis induced by LA in endometrial epithelial cells from patients with endometriosis and controls

Addition of IL-1β 0.1 ng/ml, 1 ng/ml and 10 ng/ml after treatment with LA 1000 ng/ml decreased the percentage of

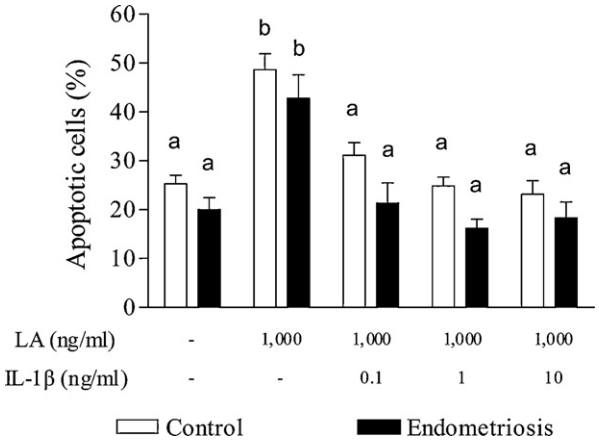

Fig. 2. Effects of interleukin-1β (IL-1β) on apoptosis induced by leuprolide acetate (LA) in endometrial epithelial cell cultures from subjects with endometriosis and controls. Endometrial epithelial cell cultures from patients with endometriosis and control women were analyzed for apoptosis by the acridine orange–ethidium bromide technique at basal conditions and after stimulation with either LA (1000 ng/ml) or a combination of LA (1000 ng/ml) and different doses of IL-1β. Values are expressed as percentage of apoptotic cells ± SEM. The cells were viewed by a fluorescence microscope. Apoptotic cells were counted as a percentage of the total. a vs. b $p < 0.001$

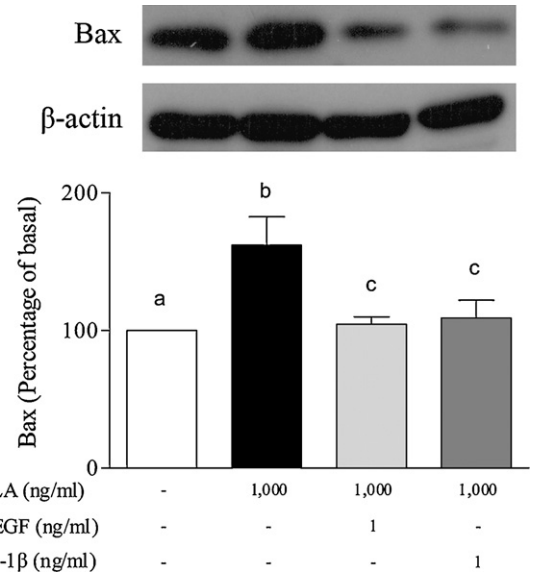

Fig. 3. Effects of vascular endothelial growth factor (VEGF) and interleukin-1β (IL-1β) on Bax expression in endometrial cell cultures from patients with endometriosis. Western blot analysis was performed on cell homogenates from endometrial epithelial cell cultures from patients with endometriosis at basal conditions and after treatment with LA (1000 ng/ml), a combination of LA (1000 ng/ml) and VEGF (1 ng/ml) or a combination of LA (1000 ng/ml) and IL-1β (1 ng/ml). Representative blots for Bax and β-actin are presented in upper panels. The lower panels show quantification results for Bax normalized against β-actin expressed as a percentage of basal values ± SEM. a vs. b $p < 0.01$; b vs. c $p < 0.05$; a vs. c $p > 0.05$.

apoptotic cells to basal levels in endometrial epithelial cell cultures from both patients with endometriosis and controls ($p < 0.001$ vs. LA 1000 ng/ml; $p > 0.05$ vs. basal) (Fig. 2). In addition, there was no significant difference in the percentage of apoptotic cells between the endometrial cell cultures from endometriosis and control patients ($p > 0.05$).

3.4. Effect of IL-1β and VEGF on Bax expression in endometrial epithelial cells from patients with endometriosis

Taking the previous results into account we decided to evaluate the expression of Bax in cultures from patients with endometriosis. Treatment with LA 1000 ng/ml caused a significant increase in Bax expression in endometrial epithelial cell cultures from patients with endometriosis ($p < 0.01$ vs. basal) (Fig. 3). Addition of VEGF 1 ng/ml or IL-1β 1 ng/ml after LA exposure restored Bax expression to basal levels in the same cultures ($p < 0.05$ vs. LA 1000 ng/ml; $p > 0.05$ vs. basal) (Fig. 3).

4. Discussion

In any auto-transplanted tissue or organ the development of an adequate blood supply is critical for the survival of the tissue (Groothuis et al., 2005). In this regard it has been shown that endometriotic lesions are highly vascularized, and it is widely accepted that angiogenesis is essential for their establishment and growth in ectopic sites (Groothuis et al., 2005; Nisolle et al., 1993).

VEGF is probably the most studied angiogenic factor, and it has been demonstrated that it is involved in the pathophysiology of endometriosis. VEGF expression is high in endometriotic lesions, and its levels are increased both in eutopic endometrium and in the peritoneal fluid of patients with endometriosis compared to control women (Donnez et al., 1998; Pupo-Nogueira et al., 2007). Besides the well-known role of VEGF as a pro-angiogenic factor, growing evidence suggests its involvement as a pro-mitogenic and pro-survival factor (Mercurio et al., 2005). This led to the idea that the suppression of blood vessel formation by inhibition of VEGF may be a novel therapeutic opportunity in the treatment of endometriosis (Park et al., 2004)

In this study, epithelial cell cultures of human endometrium from control women and from patients with endometriosis were used as a model to evaluate apoptosis induced by LA in response to the addition of VEGF and IL-1β. 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).

We used the GnRH agonist LA to induce apoptosis in endometrial cells. We confirmed that LA at a concentration of 1000 ng/ml increases the percentage of apoptotic cells in endometrial epithelial cell cultures from women with endometriosis and controls (Bilotas et al., 2007). In agreement with our findings, other authors have shown that GnRH agonists increase the apoptotic index in different cellular types (Andreu et al., 1998; Castellon et al., 2006; Imai et al., 2000; Wang et al., 2002).

In the present work we demonstrated that VEGF reversed the apoptosis induced by LA in endometrial

epithelial cells from patients with endometriosis and control women. This anti-apoptotic effect of VEGF has been previously observed in several cell types. In agreement with our results, Berkkanoglu et al. (2004) have demonstrated that the addition of VEGF decreases apoptosis in human endometrial stromal cells *in vitro*. Also, it has been shown that VEGF suppresses apoptosis in bovine granulosa cells and prevents human umbilical vein endothelial cells (HUVEC) apoptosis from high glucose exposure (Kosaka et al., 2007; Yang et al., 2008). In addition, blockage of VEGF increases apoptosis in embryonic stem cells and in HepG2 human hepatoblastoma cells during hypoxia (Baek et al., 2000; Brusselmans et al., 2005).

IL-1β is an important pleiotropic factor that is thought to play a role in the establishment and progression of endometriosis (Lebovic et al., 2000). It has been shown that human endometrium and endometriotic tissue express significant levels of IL-1β (Bergqvist et al., 2001). Furthermore, IL-1β expression is elevated in peritoneal fluid from patients with endometriosis (Mori et al., 1992). In this work we have demonstrated that IL-1β inhibited apoptosis induced by LA in endometrial epithelial cell cultures from patients with endometriosis and control women. Conflicting data indicates that IL-1β elicits such opposite responses as induction of survival or cell death. Simonart and Van Vooren have shown that IL-1β supplementation reduces the apoptotic index in serum deprived Kaposi's Sarcoma cells (Simonart and Van Vooren, 2002). In addition, it has been demonstrated that IL-1β inhibits spontaneous apoptosis of neutrophils *in vitro* and that blocking of IL-1β accelerates the rate of spontaneous apoptosis in the promyelocytic HL60 cells (Marshall et al., 2008; McNamee et al., 2005). However, other studies report that IL-1β induces apoptosis in a glioblastoma-derived human cell line and in primary cultured thyroid cells (Castigli et al., 2000; El et al., 2006).

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 speculate that the cell culture conditions may have affected the apoptotic characteristics of endometrial epithelial cells compared to whole endometrial sections.

Alterations in the regulation of apoptosis and in the expression of apoptosis related proteins have been widely associated with endometriosis (Harada et al., 2007; Meresman et al., 2000). The B-cell lymphoma-2 (Bcl-2) family of proteins regulates the mitochondrial pathway of apoptosis that converge in the activation of caspases ending into cell death (Youle and Strasser, 2008). We have previously shown that Bax, a pro-apoptotic member of this family, is altered in the endometrium of women with endometriosis (Meresman et al., 2000). In addition, it has been suggested that VEGF inhibits apoptosis by regulating the Bcl-2 family of proteins. In this study we confirmed that LA at 1000 ng/ml increases Bax expression in endometrial epithelial cells from patients with endometriosis as we have previously shown (Bilotas et al., 2007). Also we observed that the addition of either VEGF or IL-1β diminished the expression of Bax in endometrial epithelial cells from women with endometriosis. These results are in agreement with the reduction in apoptosis observed after the addition of VEGF and IL-1β. Other authors have reported that these growth factors regulate the expression of Bcl-2 family of proteins in different cell types. In agreement with our results, Simonart and Van Vooren (2002) have demonstrated that IL-1β decreases Bax expression in Kaposi's Sarcoma cells. Also, it has been observed that the addition of VEGF augments the Bcl-2/Bax ratio by reducing Bax and increasing Bcl-2 expression in HepG2 human hepatoblastoma cells and in human umbilical vein endothelial cells (HUVEC) (Baek et al., 2000; Yang et al., 2008). However, other authors have not seen changes in Bax expression after VEGF addition (Kosaka et al., 2007).

Our results demonstrate that VEGF and IL-1β have similar effects on epithelial cell apoptosis. Previous studies suggest that IL-1β induces VEGF expression in human endometriotic stromal cells (Lebovic et al., 2000). We could then speculate that the effects caused by IL-1β in endometrial epithelial cells are either direct or mediated by the expression of VEGF. However future studies are needed to more clearly determine the mechanism of action of IL-1β at this level.

In summary, we have demonstrated that VEGF and IL-1β reduce apoptosis and decrease Bax expression in endometrial epithelial cells from patients with endometriosis. Our results suggest that VEGF and IL-1β may protect endometriotic cells from undergoing apoptosis in addition to exerting their pro-angiogenic role. We can therefore speculate that the high levels of VEGF and IL-1β in the peritoneal fluid of patients with endometriosis would favor the establishment and progression of endometriotic lesions by promoting the formation of new blood vessels and by protecting endometriotic cells from undergoing cell death.

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