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Endometriosis: hormone regulation and clinical consequences of chemotaxis and apoptosis

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BACKGROUND: The recruitment of immune cells by chemokines and the regulation of endometrial cell apoptosis are critical aspects of endometriosis biology. Here, we review the local (paracrine) and systemic hormone (endocrine) modulation of these two specific, but highly related phenomena.

METHODS: We searched Pubmed for items published in English between September 1991 and September 2011 and selected the studies evaluating the effects of hormones on chemokines or apoptosis in normal human endometrium and endometriosis.

RESULTS: Estradiol has proinflammatory and antiapoptotic effects in endometrial cells, and these effects appear to be exacerbated in women with endometriosis. In these women, physiological estradiol concentrations are able to induce an enhanced inflammatory response mediated by local chemokine production and to reinforce mechanisms of cell survival mediated by extracellular signal-regulated kinases and Bcl-2. The main effect of progestogens is to inhibit interleukin-8 and other chemokines in stromal cells from both eutopic and ectopic endometrium. Progesterone is also effective in inducing apoptosis in endometrial and endometriotic cells through the inhibition of Bcl-2 and nuclear factor-κB.

CONCLUSIONS: Estrogens and progestogens modulate chemotaxis and apoptosis in human endometrium and endometriotic cells and tissues. These endocrine and paracrine pathways are perturbed in women with endometriosis, contributing to inflammatory responses, abnormal tissue remodeling, therapeutic refractoriness and disease persistence. Ultimately, they promote adhesion formation and the clinical symptoms of pelvic pain and infertility. A more detailed understanding of the molecular mechanisms involved will offer new opportunities for novel pharmacological strategies to diagnose and treat endometriosis.

Key words: endometriosis / endometrium / chemokines / apoptosis / hormones

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Introduction

Endometriosis is a common gynecologic disorder characterized by the presence of hormonally responsive, ectopic implants of endometrial epithelium and stroma dispersed in extrauterine locations. It is estimated that >175 million women worldwide suffer from this disease and the symptoms of pelvic pain and infertility that commonly accompany its surgical findings (Adamson *et al.*, 2010). The precise etiology and pathogenesis of endometriosis remain controversial, however, many hypotheses have been put forward and several are considered in detail in this review. The primary objective of our report is to summarize two critical and related processes in the progression of endometriosis: chemotaxis, the recruitment of immune cells to anatomic sites of ectopic lesion invasion; and apoptosis, the regulation of programmed cell death leading to the remodeling of these dynamic tissues. Both phenomena have been uncovered in recent years and gaining an understanding of them promises to expand our comprehension of the pathogenesis of endometriosis.

Endometriosis as an inflammatory syndrome

In his definitive historical review, Batt (2011) reports that John Sampson himself attributed von Rokitansky (1861) as the first to comprehensively describe the appearance of an ovarian endometrioma. However, it was Meigs (1922) who originally appreciated that neoangiogenesis, fibrosis and hemosiderin accumulation were intrinsic microscopic characteristics of the lesion. His description of the infiltration of 'endothelial leukocytes' ushered in a modern understanding of the inflammatory response evoked by endometriosis. These findings set the stage for our consideration of the mechanisms and consequences of immune cell recruitment and activation within ectopic foci of endometrial tissue.

As argued from the turn of the 20th century, by Meyer (1924), Halban (1925) and Sampson (1927), respectively, it remains controversial whether the derivation of endometriotic lesions is metaplastic, lymphovascular or via retrograde menstruation. It is possible that a combination of mechanisms contributes to their genesis (Taylor, 2010). Nevertheless, even from early records in our medical literature, the histology and pathogenesis of endometriosis have been linked to its presumptive source and 'mother' of all the implants, the eutopic endometrium. When one considers the dramatic physiological remodeling that is required for normal cyclical endometrial function and gestational support in Old World primates, it is not surprising that this plastic tissue has evolved a remarkable capacity for ectopic translocation. In this review, we consider two specific, but highly related, aspects of endometriosis biology: the recruitment of immune cells and the regulation of mucosal apoptosis, including local (paracrine) and systemic hormone (endocrine) modulation of these phenomena. We contend that a fundamental understanding of the molecular pathways that mediate immune cell chemotaxis and programmed cell death in endometriosis lesions is required before devising rational new therapeutic strategies to reduce lesion burden and mitigate the symptoms associated with endometriosis.

Overview of immune response

As noted above, inflammation in the setting of endometriosis has been recognized for decades (reviewed in Batt, 2011), but the critical role of leukocytes, particularly macrophages and their products, did not receive attention until the 1980s.

Two arms of the immune system are currently recognized. The adaptive immune arm, composed of specialized T and B lymphocytes and antibody-producing plasma cells, recognizes specific antigens. Its characteristic anamnestic response allows the host to mount more avid attacks when the same pathogen is encountered subsequently.

In contrast, the innate immune arm is evolutionarily more ancient, lacking antigen specificity and memory, but providing a generic line of defense. Granulocytes, eosinophils, mast cells, natural killer (NK) cells and macrophages are recruited to sites of infection and injury, where they produce biochemical mediators, including histamine, leukotrienes and prostaglandins. These cells also secrete cytokines and complement, but we will focus on their production of chemokines, the primary stimuli for leukocyte attraction (Hornung *et al.*, 1997).

Peritoneal inflammation and endometriosis: a historical perspective

Although an association of inflammation with endometriotic histopathology was recognized and reported by Meigs (1922) and Sampson (1927) in their pioneering treatises, the intimate relationship between immune cell activation and endometriosis was not generally accepted until 30 years ago. In their seminal publication, Weed and Arquembourg (1980) extended Meigs' observation of perivascular leukocytes and proposed that endometriosis was an autoimmune phenomenon. Their microscopic observations of lymphocyte infiltration of endometriomas, and evidence of complement C3 deposition in ectopic and eutopic biopsies from affected women, transformed the thinking about the pathogenesis of endometriosis. Moreover, their hypothesis that endometriosis-associated infertility might be due to 'the rejection of early implantation of embryos' was highly insightful. This proposal now is supported by a decade of recent transcriptomic and proteomic data focused on the secretory differentiation of the eutopic endometrium (Kao *et al.*, 2003; Klemmt *et al.*, 2006).

Immune cell infiltration of normal endometrium and endometriotic lesions

Insight into the immune cell biology of normal human endometrium also was beginning to emerge by the late 1980s, particularly with respect to pregnancy (Bulmer *et al.*, 1988). Cluster determination (CD)14+ macrophages were noted to be prominent in the decidua and localized in close association with invading extravillous trophoblasts, where they were thought to play an immunoprotective role. Endometrial leukocytes were observed by confocal scanning laser microscopy to be concentrated in lymphoid aggregates consisting of a core of B cells surrounded by T cells and a circumferential halo of macrophages. The aggregates were small or absent during the early proliferative stage of the menstrual cycle, increasing in size during the secretory phase, and absent in post-menopausal women, suggesting that their development was hormonally influenced (Yeaman *et al.*, 1997). Leukocyte populations in women with endometriosis were evaluated with respect to steroid hormone receptors, proliferative activity and apoptosis markers in eutopic (intrauterine) and ectopic tissues. These studies revealed high estrogen receptor (ER) and Bcl-2 (an antiapoptotic gene product discussed in more detail

below) expression and increased density of leukocytes in ectopic lesions (Jones *et al.*, 1998).

As the innate cell-mediated immune response became better understood, it was postulated that deficits in this function might compromise the ability to eliminate misplaced autologous cells in endometriosis. Further, it was suggested that impaired macrophage and NK cell function might facilitate ectopic implantation and the growth of endometriotic lesions (Dmowski *et al.*, 1994). Retrograde refluxed cells were thought to irritate the peritoneum, and elaborate chemotactic, angiogenic and immunosuppressive cytokines (discussed below), creating a vicious cycle (Vinatier *et al.*, 1996; Lebovic *et al.*, 2001b).

As described above, several laboratories contributed fundamental insights into the role of macrophage accumulation within the pelvic fluid of women with endometriosis. In particular, the studies of Haney *et al.* (1981) and Halme *et al.* (1983) indicated that macrophage concentration and degree of biochemical activation were higher in endometriosis cases compared with fertile controls. By the early 1990s investigators began to query how these peritoneal macrophages were recruited into the pelvic fluid.

Our laboratory in San Francisco took a candidate molecule approach, based on the discovery that members of the CC chemokine family attract monocytes to sites of chronic inflammation (Schall *et al.*, 1988). Using a newly developed, enzyme-linked immunosorbent assay, the first chemokine we characterized in the peritoneal fluid of subjects with endometriosis was RANTES (regulated on activation, normal T cell expressed and secreted, CCL5) and we observed that its concentration was correlated directly with the extent of active disease as assessed by laparoscopic staging (Khorram *et al.*, 1993). A few years later, another CC chemokine, MCP-1 (monocyte chemoattractant protein-1, CCL2), was identified in the pelvic fluid of endometriosis patients (Akoum *et al.*, 1996; Arici *et al.*, 1997). The CC chemokines comprise the largest family of these bioactive peptides and will be described in greater detail below. We went on to demonstrate that RANTES protein (Hornung *et al.*, 1997) and mRNA (Hornung *et al.*, 2001b) were synthesized *de novo* within the stromal cells of endometriotic lesions and that the protein was biologically active as a monocyte chemokine (Hornung *et al.*, 2001a).

In the same year as our first report, Lyttle and his collaborators at the University of Pennsylvania used a bioassay strategy and demonstrated that a histiocyte chemotactic protein was present at increased concentrations in peritoneal fluid specimens from women with endometriosis compared with controls (Leiva *et al.*, 1993). Subsequent work from that group suggested that a 20 kDa immunophilin-like protein might contribute to the chemotactic activity (Weil *et al.*, 1997).

Chemokines and their receptors in endometriosis

Chemokines (a term derived from the contraction of 'chemoattractant cytokines') are low molecular mass (8–10 kDa) peptides that recruit leukocytes from the circulation to sites of inflammation. These molecules operate by binding to transmembrane receptors expressed on the surface of inflammatory cells (Fig. 1). Ligation by its cognate chemokine typically activates one of several heptahelical G-protein-coupled receptors (GPCRs), resulting in inositol 1,4,5-trisphosphate (IP_3) generation and calcium release from intracellular compartments, facilitating cell motility. Leukocyte stimulation by chemokines also results in phosphatidic acid accumulation (Sozzani *et al.*, 1994), which stimulates a transient peak of diacylglycerol kinase activity. Chemokine receptor activation results in a respiratory burst, relaxation of leukocyte adhesion, cellular shape remodeling and migration of the immune cell toward a gradient of the chemotactic substance (Baggiolini *et al.*, 1989). While these steps are becoming appreciated, a sophisticated understanding of the biochemical structure–activity relationships of specific chemokines with their GPCRs remains obscure.

Approximately 50 human chemokines have been identified and these can be categorized into four distinct families based on their structure. Members of three of the four families have been reported in studies of endometriosis (Fig. 1). The largest family consists of the CC chemokines, so named for the adjacent location of the first two of four canonical cysteine residues near the carboxy terminus. Representatives of this family, which have been detected in cases of endometriosis, include MCP-1 (CCL2) (Akoum *et al.*, 1995), MIP-1α (monocyte inflammatory protein-1α, CCL3) (Na *et al.*, 2011), RANTES (CCL5) (Khorram *et al.*, 1993) and eotaxin (CCL11) (Hornung *et al.*, 2000). The primary targets of this class of chemokines are monocytes, T cells and eosinophils.

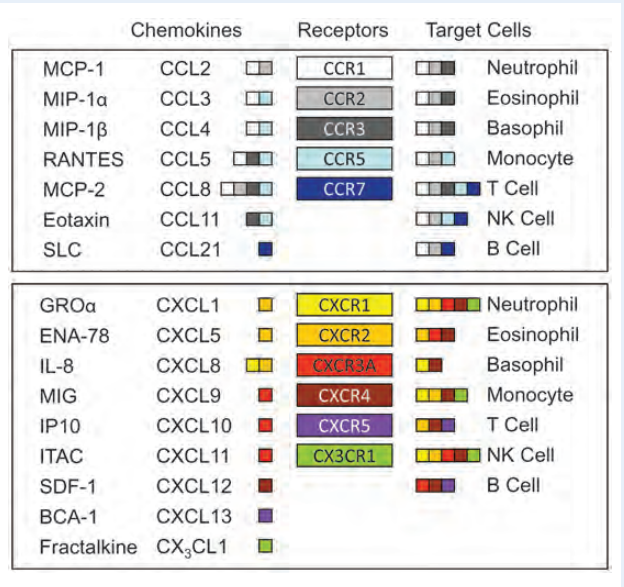

Figure 1 Chemokines identified in endometriosis. The upper box shows members of the CC family and the lower box shows members of the CXC family and one member of the CX3C family. Each receptor is represented by a different color, which is reproduced in its corresponding, high-affinity ligands and target cells. The chemokines are identified by their old names or abbreviations (first column) and by the current nomenclature (second column). MCP, monocyte chemoattractant protein; MIP, monocyte inflammatory protein; RANTES, regulated on activation, normal T cell expressed and secreted; SLC, secondary lymphoid tissue chemokine; GRO, growth-related oncogene; ENA, epithelial neutrophil-activating peptide; IL, interleukin; MIG, monokine induced by gamma interferon; IP, interferon-inducible protein; ITAC, Interferon-inducible T-cell alpha chemoattractant; SDF, stromal cell-derived factor; BCA, B-cell-attracting chemokine.

The regulation of CC chemokines in endometriosis is well exemplified by two representative molecules, which appear to be consistently altered in the disease: MCP-1 and RANTES. Endometrial glands from women with endometriosis express high mRNA and protein levels of MCP-1 (Jolicoeur *et al.*, 1998). These cells, when isolated and incubated *in vitro* with proinflammatory cytokines, also release MCP-1 to a much greater extent than endometrial epithelial cells obtained from normal individuals (Akoum *et al.*, 1995).

Endometrial RANTES protein and mRNA are mostly confined to the stromal compartment of endometrial and endometriotic tissues. *In vitro*, stromal cell cultures synthesize RANTES mRNA and secrete protein when stimulated by proinflammatory cytokines, whereas epithelial cells synthesize neither transcripts nor protein. In endometriosis, the pattern of RANTES protein distribution was similar to that found in normal endometrium, however, the production of this chemokine in endometriotic cultures was significantly greater than from cells derived from normal tissue (Hornung *et al.*, 1997; Wieser *et al.*, 2005). This phenomenon of enhanced chemokine production may provide a mechanism by which peritoneal implants increase pelvic fluid concentrations of RANTES in patients with moderate and severe endometriosis and manifest more monocyte chemotactic bioactivity. Expression of the RANTES gene is up-regulated in endometriotic stromal cells in response to tumor necrosis factor (TNF)-α, interferon-γ and interleukin (IL)-1β (Hornung *et al.*, 1997, 2001b; Lebovic *et al.*, 2001a; Fig. 2). The transcription factor nuclear factor (NF)-κB plays a critical role in the regulation of RANTES expression (Lebovic *et al.*, 2001a). These findings are consistent with a feed-forward inflammatory loop whereby cytokines secreted from activated macrophages can lead to RANTES production and stimulation of further monocyte chemotaxis into the peritoneal cavity. In addition to confirming many of the above data, Wang *et al.* (2010) demonstrated that recombinant RANTES can induce surface markers of macrophage tolerance *in vitro* and inhibits the apoptotic effects of macrophage-like U937 cells on endometrial stromal cells. The findings suggest that despite more immune cell recruitment, macrophages in the vicinity of endometriotic lesions may be less capable of phagocytosing and clearing the ectopic implants.

The next most numerous family of chemokines is the CXC family, in which a single, variable amino acid is interposed between the two conserved cysteines. Growth regulated oncogene (GRO)-α (CXCL1) (Oral *et al.*, 1996), epithelial cell-derived neutrophil-activating peptide (ENA)-78 (CXCL5) (Mueller *et al.*, 2003), IL-8 (CXCL8) (Ryan *et al.*, 1995; Arici *et al.*, 1996) and stromal cell-derived factor (SDF)-1 (CXCL12) (Ruiz *et al.*, 2010) are members of this family (Fig. 1). Primary targets for the CXC chemokines include monocytes and neutrophils. An important characteristic of this group of proteins is that they also stimulate angiogenesis (Strieter *et al.*, 2004), a process intimately linked with the pathogenesis of endometriosis (Taylor *et al.*, 2009). The recruitment of macrophages and neutrophils to sites of endometriosis may contribute indirectly to angiogenesis within the lesions and both these inflammatory cells are known to be potent sources of vascular endothelial growth factor production and release (McLaren *et al.*, 1996; Mueller *et al.*, 2000). The distribution of ENA-78 and IL-8 within endometriotic lesions tends to be more prominent in the epithelial compartment. The elevated concentrations of ENA-78 in the pelvic fluid of women with endometriosis (Mueller *et al.*, 2003) were confirmed by others (Suzumori *et al.*, 2004). As we observed for RANTES gene activation, the stimulation of endometrial cells with IL-1β and TNF-α also induces ENA-78 production (Bersinger *et al.*, 2011; Fig. 3).

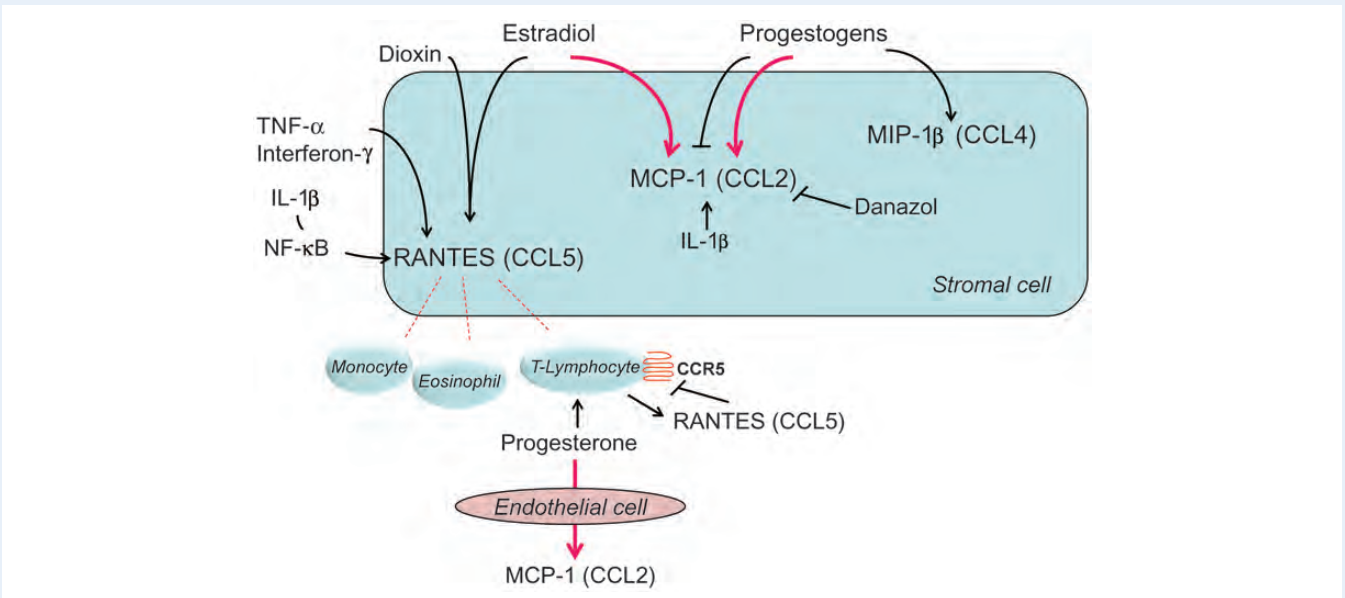

Figure 2 CC chemokines: endocrine and paracrine regulation in human endometrium and endometriosis. ↑ stimulation; ⊥ inhibition. The bold, pink signs indicate abnormal responses observed in endometriosis. Note that leukocytes are attracted by chemokines released by the endometrial or endometriotic cell in response to estradiol and/or proinflammatory cytokines, such as TNF-α. In endometriotic cells, sex steroids may abnormally stimulate, rather than inhibit MCP-1. NF, nuclear factor; CCR, CC chemokine receptor; MIP, monocyte inflammatory protein.

Apoptosis in human endometrium and endometriosis

Programmed cell death, or apoptosis, is a physiological process required for the balanced growth, differentiation and renewal of many tissues. It is mediated by selective internucleosomal cleavage of chromatin, leading to cell blebbing, shrinkage and nuclear pyknosis (Kerr *et al.*, 1972). In human endometrium, apoptosis is particularly important due to the dynamic cycles of proliferation and shedding, where the characteristic nuclear DNA fragmentation has been clearly documented (Tabibzadeh, 1996; Nasu *et al.*, 2011). The presumed role of apoptosis in the normal endometrium is to eliminate senescent or dysfunctional cells and pave the way for tissue repair at each menstrual cycle. Apoptotic cells predominate in the glandular epithelium but also can be found to a lesser extent in the stroma. The number of apoptotic cells in the functional layer of the normal endometrium increases in the late secretory phase and peaks at menstruation. This is followed by proliferation of new cells from the basal layer during the proliferative phase of the subsequent cycle (Tabibzadeh, 1996; Vaskivuo *et al.*, 2000; Nasu *et al.*, 2011).

One of the most intriguing aspects of endometriosis is the perturbation of the apoptotic mechanism (Gebel *et al.*, 1998; Izawa *et al.*, 2006). In women with endometriosis, endometrial cells regurgitated into the peritoneal cavity lack the appropriate mechanisms of programmed cell death and therefore escape clearance and survive to invade the peritoneum, induce neovascularization and establish an endometriotic implant. This abnormal survivability has been associated with concomitant overexpression of antiapoptotic factors and underexpression of proapoptotic factors (Nasu *et al.*, 2011). Microarray studies revealed down-regulation of several apoptosis-related genes in endometriotic tissues. However, the changes observed in apoptosis gene expression vary markedly according to the localization or type of endometriosis (Harada *et al.*, 2004). For instance, the Bcl-2 gene family is variably altered in peritoneal versus ovarian endometriosis (Nezhat and Kalir, 2002; Dufournet *et al.*, 2006; Table I).

According to the implantation theory, intrinsic characteristics of the eutopic endometrium in women with endometriosis will be carried into the peritoneal endometriotic implants and contribute to abnormal cell survival in ectopic sites. The physiological increase in the apoptotic rate in the late secretory phase is missing in the eutopic endometrium of women with endometriosis (Szymanowski, 2007). This abnormal characteristic of the intrauterine endometrium is probably retained by the ectopic tissue, which partly explains the excess proliferation and insufficient apoptosis of endometriotic cells.

Methods

We searched Pubmed for items published in the English language between September 1991 and September 2011, including clinical and experimental, *in vivo* and *in vitro* studies but restricted to the human species, using the following search terms: 'Chemokines'[Mesh] AND (endometrium OR endometriosis) AND (hormone OR steroid OR estradiol OR estrogen OR progesterone OR progestogen). This search returned 94 articles. Reference lists of the preselected articles and from other reviews were also searched. After detailed screening of titles, abstracts and full texts, we selected the studies evaluating the effects of hormones on chemokines in endometrial or endometriotic cells or tissues, and excluded the studies performed only in pregnancy, resulting in 38 articles being reviewed.

A second search was performed using the same criteria but substituting 'Apoptosis'[Mesh] for; Chemokines [Mesh], which returned 143 items. We then selected the studies evaluating the effects of hormones on apoptosis in endometrial or endometrium-like cells or tissues, and excluded studies performed only in pregnancy or only in endometrial cancer, which resulted in 44 articles meeting the inclusion criteria. The data

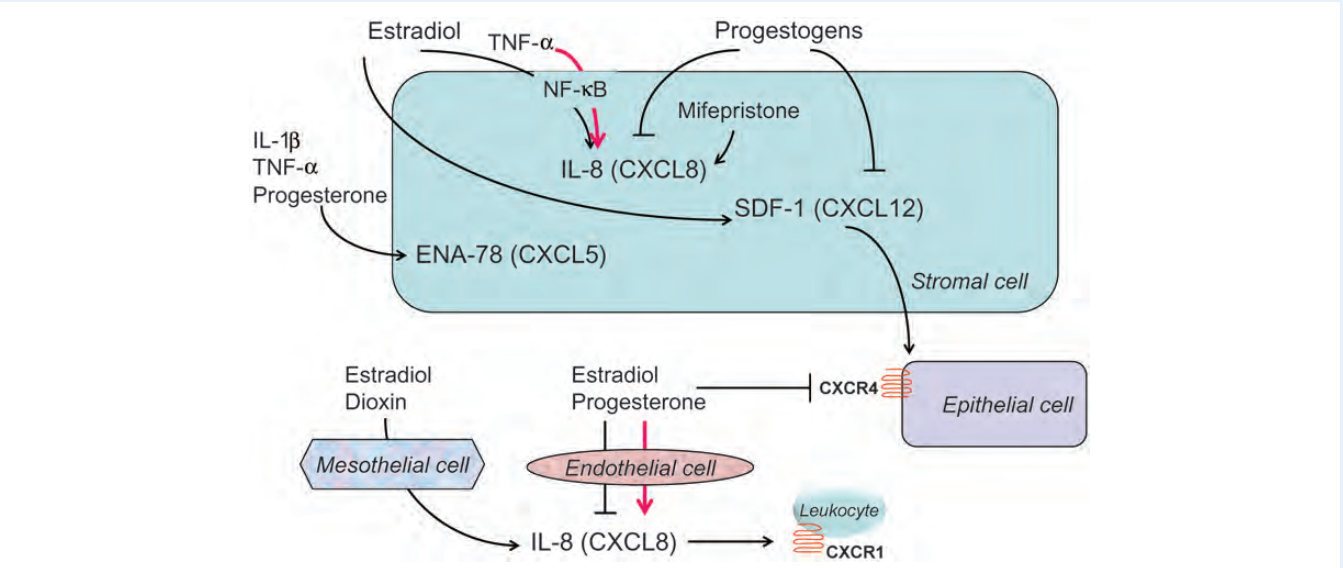

Figure 3 CXC chemokines: endocrine and paracrine regulation in endometrial cells (stromal, epithelial and endothelial) and mesothelial cells. ↑ stimulation; ⊥ inhibition. The bold, pink signs indicate abnormal responses observed in endometriosis. Observe that both stromal cells and peritoneal (mesothelial) cells release IL-8 after estradiol stimulation, which recruits leukocytes and enhances the local inflammatory response in endometriosis. TNF, tumor necrosis factor; CXCR, CXC chemokine receptor.

Table I Abnormal expression of Bcl-2 family proteins in women with endometriosis.

Protein	Lesion Localization	Compartment	Phase	Expression	References
Bcl-2	Eutopic, peritoneal, ovarian, deep	Glands (+++) and stroma (+)	Proliferative and secretory	Increased in eutopic endometrium and further increased in endometriotic lesions, except cysts	Braun *et al.* (2007), Dufournet *et al.* (2006), Goumenou *et al.* (2004), Hassa *et al.* (2009), Nezhat and Kalir (2002)
Bcl-X_L	Eutopic, peritoneal, ovarian	Undefined	Proliferative and secretory	Increased in eutopic endometrium and in endometriotic lesions	Braun *et al.* (2007), Nishida *et al.* (2005)
Bax	Eutopic, peritoneal, ovarian, deep	Glands (+++) and stroma (+)	Proliferative and secretory	Stronger in ovarian cysts versus other endometriotic lesions	Goumenou *et al.* (2004), Zubor *et al.* (2009)
Bcl-X_S	Eutopic	Undefined	Proliferative	Increased in women with endometriosis	Zubor *et al.* (2009)

Bcl-2 and Bcl-X_L prevent apoptosis and increase cell survival, whereas Bax and Bcl-X_S induce apoptosis.
+++, high levels of protein.
+, low levels of protein.

were then extracted, interpreted and summarized by all authors. No quantitative or statistical analysis was performed.

Results

Endocrine and paracrine regulation of chemokines in endometriosis

CC Chemokines

The endocrine and paracrine modifiers of RANTES in endometriosis have been evaluated by several investigative groups (Fig. 2). Despite higher concentrations of immunodetectable RANTES in secretory phase biopsies *in situ*, our group reported that endometrial and endometriotic stromal cells *in vitro* failed to respond directly to acute stimulation with estradiol, with or without progestogens (Hornung *et al.*, 1997). Similar negative findings were noted with estradiol for RANTES secretion from isolated endometrial epithelial cells (Caballero-Campo *et al.*, 2002). However, in subsequent reports, Akoum *et al.* (2002) and Yu *et al.* (2008) showed that combinations of estradiol plus IL-1β and estradiol plus the endocrine disruptor dioxin, respectively, indeed increased stromal cell RANTES synthesis. Interestingly, when stromal cells were exposed to chronic progestogen treatment, RANTES mRNA and protein levels were decreased; this effect appeared to be mediated via the NF-κB pathway (Zhao *et al.*, 2002). Conversely, in cultured T lymphocytes isolated from the endometrium, progesterone stimulated RANTES expression, while recombinant RANTES down-regulated its own receptor CCR5 (Ramhorst *et al.*, 2006).

In cultured endometrial stromal cells, both estradiol and dioxin induced MIP-1α (CCL3) release. This chemokine appeared to be involved in promoting endometrial cell invasiveness (Yu *et al.*, 2008), which is an important mechanism in the onset of peritoneal endometriotic lesions. In addition, progesterone stimulated the release of MIP-1β (CCL4), a ligand of the CCR5 receptor (Kitaya *et al.*, 2003).

Estradiol, progesterone and medroxyprogesterone acetate did not modify the MCP-1 (CCL2) output from normal endometrial stromal cells (DeLoia *et al.*, 2000; Matta *et al.*, 2007). However, in cells from women with endometriosis, estradiol (Akoum *et al.*, 2000) and progesterone increased IL-1β-induced MCP-1 synthesis and release (Boucher *et al.*, 2000b). Luk *et al.* (2010) reported that ovarian steroids directly up-regulated MCP-1 mRNA and protein in cultured human endometrial endothelial cells. Interestingly, this effect was only present in cells obtained from women with endometriosis (Luk *et al.*, 2010). Danazol, a synthetic steroid with anti-estrogenic and androgenic activity, inhibited MCP-1 release by endometrial and endometriotic cells *in vitro* (Boucher *et al.*, 2000a; Jolicoeur *et al.*, 2001), indicating a direct anti-inflammatory action (Fig. 2).

It emerges from these studies that women with endometriosis have an aberrant CC chemokine response to sex steroids in their endometrial stroma and microvascular endothelium, which might explain the therapeutic failure and inadequate pain relief in some women with endometriosis who are treated with progestogens or hormonal contraceptives.

CXC Chemokines

The endocrine and paracrine control of the CC chemokine family in human endometrium has been addressed in several *in vitro* models. SDF-1 mRNA and protein have been detected in primary stromal cells, whereas its receptor CXCR4 was abundant in epithelial cells (Tsutsumi *et al.*, 2011). In cultured endometrial stromal cells, estradiol stimulated SDF-1 production dose-dependently. This chemokine promoted the proliferation of an epithelial cell line, suggesting that it may be one of the paracrine mediators of the proliferative action of estrogen in the endometrium (Tsutsumi *et al.*, 2011). According to this hypothesis, estrogen stimulation of stromal cells induces SDF-1 release, which acts in a paracrine fashion to increase epithelial/glandular cell proliferation (Fig. 3). Recent reports indicate that progesterone inhibits the expression of SDF-1 in an endometrial epithelial cell line (Ruiz *et al.*, 2010) and this hormone as well as synthetic progestogens reduced SDF-1 secretion from primary endometrial stromal cells (Okada *et al.*, 2011). In epithelial cells, CXCR4 was down-regulated by treatment with estradiol, progesterone or estradiol plus progesterone. Interestingly, *in vivo* assessment of CXCR4 showed that this chemokine receptor was more abundant in endometriotic lesions than in normal endometrium (Ruiz *et al.*, 2010). Altogether, this body of evidence suggests that the chemotactic signal produced by SDF-1 through the receptor CXCR4 is potentially sensitive to inhibition by

anti-estrogenic and/or progestogenic compounds used to treat endometriosis.

IL-8 concentrations *in situ* were observed to be highest in premenstrual endometrium (Dominguez *et al.*, 2003) and *in vivo* administration of the progesterone antagonist mifepristone induced its up-regulation (Critchley *et al.*, 2003). *In vitro* studies in endometriotic stromal cells showed that the combination of TNF-α and estradiol increased IL-8 mRNA and protein, and that this effect was mediated by NF-κB activation and could be reversed in the presence of natural progesterone, danazol and dienogest (Horie *et al.*, 2005). Moreover, IL-8 production by endometrial stromal cells markedly decreases during progesterone-induced decidualization (Lockwood *et al.*, 2004). A similar down-regulation of IL-8 release was induced by progesterone in endometrial tissue explants (Kelly *et al.*, 1994). As expected, progesterone withdrawal leads to IL-8 release by endometrial stromal cells *in vitro* (Kizilay *et al.*, 2008). In addition, estradiol inhibits aminopeptidase N activity in cultured endometrial stromal cells, an enzyme which limits the bioavailability of IL-8 in the endometrium (Seli *et al.*, 2001). Thus, inhibition of aminopeptidase N might be a mechanism by which estradiol could increase IL-8 activity independent from regulating its gene expression or translation.

In addition to hormone effects on epithelial and stromal cells, the endocrine regulation of IL-8 and its receptor also has been evaluated in mesothelial and endothelial cells. While not extensively characterized in this field, these two cell types represent critical barriers to the invasion and nourishment, respectively, of nascent peritoneal endometriotic implants. Estradiol plus dioxin stimulated IL-8 release by human pelvic mesothelial cells, while the IL-8 receptor CXCR1 was shown to be highly expressed in endometriotic tissue, suggesting that IL-8 secreted by peritoneal mesothelial cells in response to estrogen could act on CXCR1 receptors in the endometriotic implants to maintain the inflammatory status in the peritoneum of women with endometriosis (Shi *et al.*, 2006). The expression and release of IL-8 by normal human endometrial endothelial cells was inhibited by estradiol and progesterone, whereas both steroids paradoxically stimulated IL-8 release from the same cell type isolated from women with endometriosis (Luk *et al.*, 2005).

While it is premature to extrapolate these *in vitro* data to a therapeutic context, they consistently point to an antagonistic relationship between estrogens and progestogens in the regulation of IL-8 in endometrial stromal cells, with stimulation by estrogens and inhibition by progestogens. This is not the case in endothelial cells, where the effect of natural progesterone appears to be synergistic with that of estradiol and to be reversed in women with endometriosis. Therefore, the effects of progestogenic compounds designed to treat or prevent endometriosis should be tested not only in endometrial cells but also in endothelial cells before any conclusion is reached about their potential clinical effectiveness in blocking IL-8 production at critical sites.

CX3C chemokines

Fractalkine (CX3CL1) is a member of a third family of chemokines, wherein three amino acids separate the two canonical cysteines. In a single report that described fractalkine in the peritoneal fluid of women with endometriosis, its concentrations were lower than in control samples (Shimoya *et al.*, 2005). This chemokine was noted to be present in endometrial epithelium and stroma, with the latter prominent in the secretory phase of the normal cycle and further stimulated after therapeutic treatment with progestogen contraceptives (Hannan *et al.*, 2004). To date, *in vitro* manipulation with ovarian steroids has not been reported, but several studies indicate that fractalkine can be suppressed in respiratory cells by glucocorticoids (Bhavsar *et al.*, 2008). IL-1β and interferon-γ both are potent activators of fractalkine in lung fibroblasts (Isozaki *et al.*, 2011).

In vivo *evidence for endocrine modulation of endometrial chemokines*

A study of endometrial fluid samples collected during controlled ovarian hyperstimulation for IVF showed the up-regulation of several endometrial cytokines and chemokines in stimulated cycles compared with previous natural cycles. However, it is not possible to determine in this model if the intrauterine milieu might have been altered by direct effects of GnRH analog or gonadotropins, or by excess estradiol or other ovarian products (Boomsma *et al.*, 2010). In a similar study of endometrial biopsies, B-cell-attracting chemokine (BCA)-1 (CXCL13) was up-regulated in stimulated versus natural cycles, but again the endocrine pathway involved could not be determined (Macklon *et al.*, 2008). In women receiving donor oocytes and treated with ovarian steroids to prepare the endometrium for embryo transfer, the immunoreactive levels of endometrial MCP-1 increased following the administration of progesterone (Caballero-Campo *et al.*, 2002).

Several chemokines were found to be overexpressed in the endometrium of women using contraceptive subdermal implants or intrauterine systems containing levonorgestrel, showing correlations with increased leukocyte recruitment and, hypothetically, endometrial atrophic changes and susceptibility to irregular bleeding (Hannan *et al.*, 2004; Jones *et al.*, 2005). In levonorgestrel implant users, uterine washings revealed a progressive increase in ENA-78 concentrations over time (Chegini *et al.*, 2007). This is consistent with *in vitro* findings that progesterone stimulates ENA-78 expression in endometrial stromal cells (Nasu *et al.*, 2001). Long-term users of the levonorgestrel-releasing intrauterine system also manifest high expression of chemokines with six cysteines (6Ckine, CCL21) and IL-8 in the endometrial glands and stroma (Peloggia *et al.*, 2006). In contrast, MCP-1 and MCP-2 (CCL8) appear to be down-regulated in levonorgestrel implant users (Hampton *et al.*, 2001). Although the lack of RCTs limits any conclusion about the impact of levonorgestrel itself on endometrial chemokine production, the persistence of these chemokines in levonorgestrel-releasing contraceptive users suggests that leukocyte recruitment leads to a low-grade chronic inflammatory response.

From the endometriosis perspective, these studies might suggest that long-term levonorgestrel-releasing systems induce increased chemokine production and leukocyte recruitment to the endometrium, which could counter the therapeutic goal of reducing the proinflammatory environment in endometriotic lesions. At this point we should remember that eutopic endometrium does not necessarily mirror the endometriotic implants. Thus, it remains to be investigated directly whether chronic treatment with progestogens modulate chemokine production and leukocyte infiltration within the endometriotic tissue. This hypothesis is difficult to test in humans because endometriotic lesions cannot be sampled repeatedly to compare chemokine expression before and after progestogen treatment, while parallel controls taking placebo or other treatments would be hard to match due to the large variability in endometriotic lesions. Thus, the *in vitro*

culture of endometriotic cells, despite their intrinsic limitations, may provide the most expeditious answers. In these cells, progesterone, danazol and dienogest all counteracted estradiol-induced IL-8 production (Horie *et al.*, 2005). In a similar *in vitro* model, estradiol enhanced but progesterone did not affect RANTES expression (Akoum *et al.*, 2002). In other studies, exposure of endometrial stromal cells to 100 nM medroxyprogesterone acetate for 8 days resulted in significant repression of RANTES protein secretion and gene expression, whereas no effect was seen in cells exposed to the same dose for only 2 days (Zhao *et al.*, 2002). It seems clear that more clinical research is needed to assess the chemokine profile of endometriotic lesions in women treated with progestogens and combined contraceptive pills, in order to elucidate the effects of such hormonal interventions on the local inflammatory response, and their relationship to the cardinal endometriotic symptoms, pain and infertility.

Endocrine and paracrine regulation of apoptosis in endometriosis

Sex steroid hormones and apoptosis

In most human tissues and under most physiological conditions, the activation of ER by estradiol or other ligands results in the inhibition of apoptosis (Amaral *et al.*, 2009). This is particularly true for the endometrium, as suggested by the following evidence. In natural cycles, unopposed estrogen stimulation in the proliferative phase is characterized by absence of apoptosis in endometrial glandular cells (Otsuki *et al.*, 1994). Furthermore, estrogen stimulation *in vitro* increases endometrial cell viability, and this effect is strongly reversed by estrogen withdrawal (Song *et al.*, 2002). The antiapoptotic effects of estrogens follow several pathways (Fig. 4) and appear to involve both nuclear and extranuclear ER signaling. For instance, through the classical nuclear mechanism, estrogen increases the transcription of the antiapoptotic protein Bcl-2, while the activation of extranuclear kinases by the estrogen-ER complex triggers a rapid non-genomic signaling cascade resulting in apoptosis inhibition (Amaral *et al.*, 2009).

The effects of progesterone, conversely, are more complex. In healthy volunteer women, the *in vivo* administration of progesterone decreases endometrial apoptosis observed in the late secretory phase (Lovely *et al.*, 2005). The data suggest that hormone withdrawal in the late luteal phase triggers apoptosis and that progesterone supplementation may prolong endometrial epithelial cell survival and prevent the premenstrual surge of apoptosis. However, the same strategy of progesterone supplementation, when used in endometrial explants, does not affect apoptosis, despite effectively maintaining the histological integrity of explanted tissue (Li *et al.*, 2005). In isolated endometrial cells in culture, progesterone actually induces apoptosis (Li *et al.*, 2001; Choksuchat *et al.*, 2009; Tang *et al.*, 2009), an effect that may be consistent with reports of antiapoptotic actions of mifepristone in endometrial biopsies from progestogen users (Jain *et al.*, 2006). Furthermore, progesterone physiologically down-regulates endometrial Bcl-2 expression, which decreases at the early secretory phase (von Rango *et al.*, 1998) and increases upon mifepristone administration (Critchley *et al.*, 1999; Fig. 5).

To reconcile these findings, we suggest that part of the growth-limiting effect of progesterone on the human endometrium includes the modulation of apoptosis-related genes in favor of increased apoptosis (Tao *et al.*, 1998; von Rango *et al.*, 1998), but the process of glandular cell death is somehow precipitated by progesterone withdrawal at the late secretory phase (Lovely *et al.*, 2005).

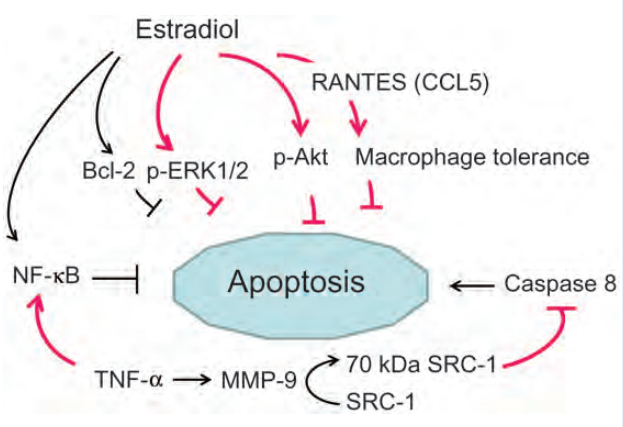

Figure 4 Apoptosis regulation by estradiol in human endometrium and endometriosis. ↑ stimulation; ⊥ inhibition. The bold, pink signs indicate abnormal responses observed in endometriosis. These include an enhanced activation (phosphorylation) of protein kinases (ERK1/2, Akt), increased levels of NF-κB and the cleavage of SRC-1, all converging to inhibit apoptosis. Bcl, B-cell leukemia/lymphoma; p-ERK, phosphorylated extracellular signal-regulated kinase; p-Akt, phosphorylated serine/threonine protein kinase B; MMP, matrix metalloproteinase.

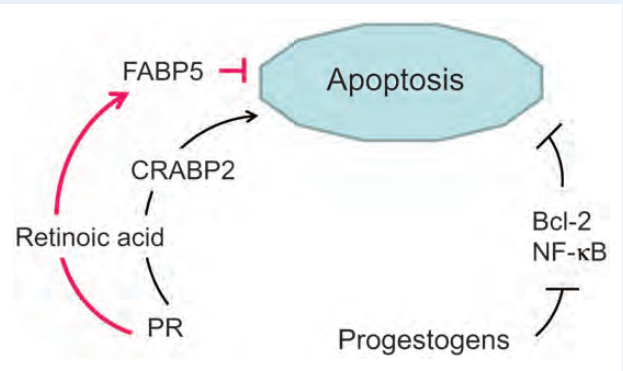

Figure 5 Apoptosis regulation by progestogens in human endometrium and endometriosis. ↑ stimulation; ⊥ inhibition. The bold, pink signs indicate abnormal responses observed in endometriosis. FABP, fatty acid-binding protein; CRABP, cellular retinoic acid-binding protein; PR, progesterone receptor.

Effects of sex steroid hormones on apoptosis in endometriosis

Considering that endometriotic cells are resistant to apoptosis, and that sex steroids modulate apoptosis in the endometrium, the next question is whether endometriotic cells respond abnormally to the apoptosis modulation by sex steroids and, if so, which mechanisms underlie this abnormal response. Indirect *in vitro* evidence suggests that endometriotic cells respond to estrogen-induced antiapoptotic

signaling more intensely than normal cells (Fig. 4). For example, the antiapoptotic protein extracellular signal-regulated kinase (ERK)1/2 is highly activated (i.e. phosphorylated) *in vivo* in eutopic and ectopic glandular endometrial cells from women with endometriosis throughout the menstrual cycle (Murk *et al.*, 2008). Moreover, estrogen stimulates ERK1/2 phosphorylation in eutopic endometrial stromal cells isolated from patients with endometriosis, but not in endometrial stromal cells from women without endometriosis. This abnormal response to estrogen probably explains the excess ERK1/2 activation and contributes to apoptosis resistance in endometriotic cells (Murk *et al.*, 2008; Fig. 4).

The serine/threonine protein kinase B, or Akt, is a pleiotropic regulator of cell proliferation and apoptosis. Estrogen induces a rapid phosphorylation of Akt in endometrial stromal cells, which is indicative of non-genomic action (Cinar *et al.*, 2009). Akt activity (phosphorylation) is increased in both eutopic and ectopic endometrial cells from women with endometriosis (Cinar *et al.*, 2009). Akt may play a central role in endometriosis by increasing cell survival through decreased apoptosis (Fig. 4). One of the putative factors responsible for higher Akt phosphorylation in endometriotic cells may be the continuous local stimulation of ERs due to intralesional production of estradiol, as suggested by studies showing local expression of aromatase (Bulun *et al.*, 2012).

The increased sensitivity of endometriotic cells to the survival message mediated by estradiol does not appear to be related to the abundance of ERα, which is expressed in endometriotic lesions at normal or reduced levels (Fujishita *et al.*, 1997; Brandenberger *et al.*, 1999; Morsch *et al.*, 2009). In contrast, ERβ is up-regulated in endometriosis and seems to contribute to implant survival (Cavallini *et al.*, 2011; Bulun *et al.*, 2012). A recent study tested the hypothesis that endometriotic cells overexpress estrogen-related receptors (ERRs), a subfamily of orphan receptors that shares sequence homology with ERs. However, the study reached the opposite conclusion, that ERRα and ERRγ mRNA and protein levels are significantly lower in endometriotic lesions compared with eutopic endometrium from women with endometriosis and normal endometrium (Cavallini *et al.*, 2011).

Recently, Han and coworkers (2012) discovered a novel potential mediator of resistance to apoptosis in endometriosis. They observed that endometriotic tissues contain a 70-kDa truncated isoform of steroid receptor coactivator (SRC)-1, which is generated by matrix metalloproteinase 9 cleavage of full-length SRC-1 (Fig. 4). The proteolytic process is induced by TNF-α released by macrophages and NK cells as part of the inflammatory response to endometriotic implants. The truncated SRC-1 isoform still acts as a transcriptional coactivator and boosts nuclear ER-β effects, but also has the unique ability of stabilizing procaspase-8 and thereby blocking apoptosis (Dyson and Bulun, 2012).

Progesterone resistance is one of the pathogenic mechanisms potentially involved in the survival of endometriotic implants. Progesterone receptor (PR) mRNA levels, particularly those of transcripts encoding the PR-B isoform, are reduced in extraovarian ectopic lesions relative to matched eutopic endometrium from the same subjects. These effects also are manifested at the level of PR-B protein as detected by western blots (Attia *et al.*, 2000). Immunohistochemical studies also suggested that PR levels tended to be low and without menstrual cycle variation in peritoneal ectopic lesions (Beliard *et al.*, 2004). In addition, the establishment of endometriotic lesions in the pelvis is able to induce long-lasting progesterone resistance in the eutopic endometrium (Fazleabas, 2010; Al-Sabbagh *et al.*, 2012).

The apoptotic index in mid-luteal phase endometrium is reduced in women with endometriosis (Szymanowski, 2007), suggesting a subnormal response to progesterone. Conversely, the number of apoptotic cells in endometrial biopsies from women with endometriosis increased following treatment with combined oral contraceptives (Meresman *et al.*, 2002) or the levonorgestrel-releasing intrauterine system (Gomes *et al.*, 2009). However, from a therapeutic perspective, it is relevant to know if endometriotic lesions, rather than the eutopic endometrium, are refractory to progestogen-induced apoptosis. As noted above, ethical constraints prevent the investigation of this response *in vivo*, which would require performing two laparoscopic biopsies of the same lesion, before and after a progestogen treatment course. Hence, we are left to extrapolate experimental findings obtained with stromal cells derived from endometriotic cysts.

In normal cells, PR stimulates the apoptotic pathway triggered by retinoic acid nuclear signaling, through the up-regulation of the retinoic acid shuttling protein CRABP2 (Fig. 5). Endometriotic cells have deficient retinoic acid production due to insufficient retinol uptake. In addition, they have an aberrant profile of retinoic acid shuttling proteins, leading to a paradoxical retinoic acid action mediated by fatty acid-binding protein 5 (FABP5), a prosurvival nuclear receptor (Pavone *et al.*, 2010). Wieser *et al.* (2005) demonstrated the constitutive activation of NF-κB, a master transcriptional regulator of inflammatory responses, in endometriotic cells. It has been suggested that NF-κB may promote the growth and survival of endometriotic lesions, since NF-κB inhibitors block the proliferation of endometriotic stromal cells and induce apoptosis (Gonzalez-Ramos *et al.*, 2008). NF-κB activation in endometriotic stromal cells is induced by TNF-α and estradiol (Fig. 4) and inhibited by progestogens (Horie *et al.*, 2005; Fig. 5), which is an additional downstream mechanism involved in steroid hormone control of apoptosis in endometriosis.

Genomic variants may also be implicated in the progesterone resistance of women with endometriosis. The PR gene polymorphism PROGINS codifies a variant PR that is less responsive to progestogens, when compared with wild-type PR, resulting in reduced biological activity. As predicted, *in vitro* experiments have demonstrated that progesterone fails to induce apoptosis in endometrial cells harboring the PROGINS variant allele (D'Amora *et al.*, 2009). This finding is relevant because the PROGINS variant has been reported to be more common in women with surgically confirmed endometriosis in some studies, compared with general populations (Wieser *et al.*, 2002; Lattuada *et al.*, 2004; De Carvalho *et al.*, 2007). However, these findings may not be consistent, as another larger collaborative survey by Near *et al.* (2011) failed to find an association, but it should be noted that in the latter study, the endometriosis diagnosis was based on self-report alone.

We conclude that normal apoptosis mechanisms are suppressed in endometrial cells from women with endometriosis and within endometriotic lesions. Increased estrogen availability due to local estradiol synthesis and increased estrogen sensitivity due to ERβ and SRC-1 lead to exaggerated protein kinase activation and apoptosis inhibition. Progesterone resistance further contributes to endometriotic cell survival, and may reduce the therapeutic effects of progestogens.

Effects of peptide hormones on apoptosis in endometriosis

GnRH receptors are not exclusive to the pituitary gland, but also are found in peripheral reproductive tissues, including the human endometrium (Wu *et al.*, 2009). Biopsies of rectovaginal endometriotic lesions obtained before and during treatment with the GnRH agonist leuprolide acetate showed increased apotosis during treatment (Mizutani *et al.*, 1999). However, this approach does not allow a distinction between systemic (ovarian suppression) and local action of the GnRH analog. To address this question, leuprolide acetate was incubated with human endometrial cells *in vitro* and directly stimulated apoptosis (Imai *et al.*, 2000; Meresman *et al.*, 2003). This effect was observed in cells from women with endometriosis as well as from normal controls, and was reversed by a GnRH antagonist (Meresman *et al.*, 2003). If confirmed, these experiments suggest that GnRH agonists may have a unique peripheral mechanism of inhibiting endometriosis by directly inducing apoptosis, but they question the role of GnRH antagonists in this setting.

Summary and conclusions

The mechanisms underlying the onset, progression and maintenance of endometriosis include an abnormal inflammatory response, which is established in the early stages of the disease and sustained by persistent immune cell recruitment to the endometriotic foci. Production of proteases, mitogens, angiogenic factors and activating cytokines and chemokines by infiltrating leukocytes appears to promote lesion invasiveness. A complementary and possibly synergistic mechanism of endometriosis formation derives from an imbalanced regulation of cell fate, with reduced susceptibility to apoptosis. Some chemokines, such as RANTES and IL-8, may be involved not only in the amplification of the local inflammatory response but also in the survival of endometriotic cells through increased proliferation and/or attenuation of apoptosis (Selam *et al.*, 2006; Wang *et al.*, 2010). Despite the unequivocal recognition that sex steroids play a central role in endometriosis biology and remain the first-line targets for medical therapies, the effects of estrogens and progestogens on chemotaxis and apoptosis in endometriotic cells are complex and only partially known.

As reviewed here, considerable evidence indicates that estrogen is not only proliferative but also proinflammatory and antiapoptotic in endometrial epithelial cells and stromal cells. These effects appear to be exacerbated in women with endometriosis, in whom local estradiol reinforces both inflammation and cell survival, mediated by chemokines, kinases and Bcl-2. Although endothelial cells from the endometrium of women with endometriosis may release IL-8 in response to progestogens, it is reassuring that, in stromal cells from both eutopic and ectopic endometrium, the predominant effect of progestogens is to inhibit IL-8 and other chemokines. Progestogens also are effective in inducing apoptosis in endometrial epithelial cells through the inhibition of Bcl-2 and the stimulation of Bax and ERK pathways. Finally, the role of peptide hormones, in particular GnRH, in regulating endometrial apoptosis deserves further investigation in order to clarify whether their direct proapoptotic effects on endometrial cells, indicated by some experiments *in vitro*, might be clinically relevant *in vivo*. Further elucidation of the fundamental mechanisms of leukocyte chemotaxis and endometrial and immune cell apoptosis will undoubtedly lead to the future development of adjuvant medical therapies for the clinical management of endometriosis.

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Authors' roles

All authors (F.M.R., F.P. and R.N.T.) participated in the design, bibliographic search and selection, data analyses, manuscript writing and final manuscript approval.

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Conflict of interest

None declared.

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