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REVIEW

Regulation of apoptotic pathways during endometriosis: from the molecular basis to the future perspectives

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Abstract
Purpose Endometriosis is defined as the presence of endometrial-like endometrial cells, glands and stroma outside the uterus, causing a strong inflammatory-like microenvironment in the affected tissue. This may provoke a breakdown in the peritoneal cavity homeostasis, with the consequent processes of immune alteration, documented by peripheral mononuclear cells recruitment and secretion of inflammatory cytokines in early phases and of angiogenic and fibrogenic cytokines in the late stages of the disease. Considering the pivotal role of interaction between immune and endometriotic cells, in this paper, we aim to shed light about the role of apoptosis pathways in modulating the fine-regulated peritoneal microenvironment during endometriosis.
Methods Narrative overview, synthesizing the findings of literature retrieved from searches of computerized databases.
Results In normal conditions, endometriotic cells, refluxed through the fallopian tubes into the peritoneal cavity, should be attacked and removed by phagocytes and NK cells. During endometriosis, the breakdown of peritoneal homeostasis causes the failure of scavenging mechanisms, allowing the survival of endometriotic cells. The consequent so-called "immunoescaping" of endometriotic cells could be due, at least in part, to the reduction of apoptotic-mediated pathways previously described.
Conclusion Considering the large amount of evidence retrieved from in vitro as well as in vivo models, the reduced apoptosis of endometriotic cells together with the increased apoptosis of peritoneal fluid mononuclear cells may address the peritoneal homeostasis to a permissive environment for the progression of the disease.

Keywords Endometriosis · Apoptosis · Immunity · Peritoneal fluid

Introduction

Endometriosis is defined as the presence of endometrial-like endometrial cells, glands and stroma outside the uterus, causing a strong inflammatory-like microenvironment in the affected tissue [1, 2]. The exact prevalence of endometriosis is unknown, but the estimates range from 2–10 % of women of reproductive age, to 50 % of infertile women [3]. The risk of endometriosis appears to increase for reproductive health factors that may relate to increased exposure to menstruation (i.e., shorter cycle length, longer duration of flow, or reduced parity). The risk appears to decrease for personal habits that may relate to decreased estrogen levels (i.e., smoking, exercise) [4, 5]. Endometriosis can cause several symptoms and signs, including acute and chronic pelvic pain, dysmenorrhea, dyspareunia, abnormal vaginal bleeding, infertility/sterility and, in the severe stage, gastrointestinal and urological symptoms [6–8]. The etiopathogenesis of endometriosis still remains controversial: immune, hormonal, genetic, and

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epigenetic factors may be all involved, and several theories have been proposed to explain it.

According to Sampson's implantation theory [9], for example, during retrograde menstruation, eutopic endometrial cells reflux throughout the tubes to the peritoneal cavity, adhere to the peritoneal wall, proliferate and form endometriotic lesions, thereby triggering and advancing the disease. This theory was confirmed since the retrograde transport of endometrial cells was actually shown, and the sites of greater frequency of the disease are fallopian tubes, ovaries, and pouch of the Douglas, those most easily reached by the refluxed cells. Moreover, there is evidence that nulliparous women and women with heavy and short menses are at higher risk of developing endometriosis [10]. However, this phenomenon could be observed in 90 % of endometriosis-free women in reproductive age with patient's fallopian tubes and contrast with the relative low incidence of the disease [11]. In this view, other (multiple) factors seem to be mandatory to allow the development and progression of the disease. To date, accumulating evidence suggests that during postpubertal age, under the influence of different stimuli, misplaced and quiescent ectopic endometrial cells could acquire new phenotype, biological functions, and immunogenicity. These kinds of cells may differentiate, specializing in epithelium, glands, and stroma to form a functional ectopic endometrial tissue [12, 13]. This may provoke a breakdown in the peritoneal cavity homeostasis, with the consequent processes of immune alteration, documented by peripheral mononuclear cells recruitment and secretion of inflammatory cytokines in early phases and of angiogenic and fibrogenic cytokines in the late stages of the disease [14–16]. Once the endometriotic foci are established, in fact, the strict interaction between endometriotic and immune cells addresses toward a prevalence of Th1 profile cytokines in the peritoneal fluid (PF) at minimal and mild stages, whereas Th2 profile cytokines prevailed in severe stages [17–19]. Moreover, in the progression of the disease, a key role could be played by impaired ratio of Th17 [20, 21] and Tregs populations [22, 23]. In particular, the high level of estradiol, typical of endometriosis, can also play an important role in the expansion and activation of Tregs and cause a local decrease of immunosurveillance [24–26]. In this review, considering the pivotal role of interaction between immune and endometriotic cells, we aim to shed light about the role of apoptosis pathways in modulating the fine-regulated peritoneal microenvironment during endometriosis.

Molecular basis of apoptosis

Apoptosis is commonly described as a morphological phenomenon which includes chromatin condensation and nuclear fragmentation (pyknosis), plasma membrane blebbing, and cell shrinkage. Although the single definition, this cellular mechanism could occur through several pathways, which may underlie the formation of small membrane-surrounded fragments (apoptotic bodies). These particular fragments are typically removed by phagocytosis, without addressing a pro-inflammatory response [27]. Apoptosis occurs during normal physiological condition, as, for example, during embryonic development, as well as serves to clear cells which underwent damage and/or escaped from cell cycle regulation [28]. As widely evidenced, this machinery if triggered by caspases, a class of enzymes expressed as inactive zymogens (procaspases), cleaves C-terminal to aspartic acids residues upon activation. Caspases' activation strictly depends on the proteolytic cleavage of the zymogens, causing the elimination of N-terminal domain and the formation of two subunits. Later, those two subunits form a heterotetramer that acts as protease on several cellular proteins [29]. Caspases' activation occurs through two different signaling pathways: extrinsic and intrinsic, depending on whether the activating signal arises. There are two main classes of caspases: caspase-9 homologues, knows as "initiators," and "effector caspases," such as caspases-3 which are activated downstream of caspase-9. In mammals, the apoptosome, a complex consisting of CED-4/Apaf-1, procaspase-9 and cytochrome c, provides the activation of procaspase-9 (initiator) which subsequently triggers the downstream cascade of effector caspase and, so, the degradation of the cellular components [30]. The apoptosome formation is tightly regulated by several mechanisms [31]: on the one hand, the B-cell lymphoma protein 2 (Bcl-2) family can facilitate or prevent the cytochrome c release from mitochondria into the cytoplasm; on the other hand, the inhibitor of apoptosis proteins (IAPs) may prevent the activation on the intrinsic pathway. As widely evidenced [28], several intracellular factors such as the CD40 ligand, viral genes and anti-apoptotic members of the Bcl-2 family play a detrimental role suppressing apoptosis. It may be triggered through the extrinsic pathway when the interaction of Fas ligand (FasL/CD95L) [32], tumor necrosis factor α (TNFα) [33], transforming growth factor β (TGFβ) and cytokines shifts the balance toward pro-apoptotic signaling. In particular, the extrinsic pathway is triggered after the activation of cell-surface death receptors (DRs), such as Fas/CD95 and TNFα receptor. The related ligand (FasL, TNFα) binds with great affinity to its DR and can activate apoptosis directly through the activation of caspases, or indirectly, by amplifying the death signal through the activation of the intrinsic/mitochondrial pathway. Activated death receptors bind the adaptor molecule Fas-associated death domain (FADD) via the death domain (DD), and FADD recruits the initiator procaspase-8 and procaspase-10 into a complex, the death-inducing signaling complex (DISC), through the death effector domain (DED), which is present both in FADD and

in the procaspase. The recruitment of procaspase-8 and procaspase-10 into the DISC complex leads to the autoproteolytic cleavage and activation of these caspases, with subsequent activation of the effector caspases [34]. In a well-studied model, the anti-apoptotic multidomain members, such as Bcl-2, bind to and neutralize the pro-apoptotic members (Bax) in non-apoptotic cells. The pro-apoptotic Bcl-2 family proteins then oligomerize, creating pores in the mitochondrial outer membrane and allowing the release of cytochrome c into the cytoplasm, which leads to caspase activation and cell death. Overexpression or inappropriate expression in time of c-MYC has been found to promote apoptosis. In mammalian cells, p53 is a major regulator of cell cycle arrest and apoptosis. Deregulated MYC upregulates ARF (acute renal failure protein), which in turn activates p53 to regulate a group of target genes that activate apoptosis and cell cycle arrest [35]. Furthermore, p53 activation is dependent on the type of cell and nature of the cellular stress. One of the important target genes transcriptionally activated by p53 is the cyclin-dependent kinase (CDK) inhibitor p21 [36]. This fine regulation permits a transient arrest in the G1 phase of the cell cycle under mild DNA damage or stress and the survival of cells until optimal cellular conditions are restored. Finally, the NFkB canonical pathway is activated in response to injury, inflammation, infection and other stress conditions. In this condition, some of the target genes activated by the NFkB pathway include anti-apoptotic factors of the Bcl-2 family [37].

In vitro models

Emerging data suggested that dysregulation of microRNA (miRNA) expression might play a role in the development of endometriosis [38]. Functional analysis of miR-183 revealed that this miRNA plays a role in induction of apoptosis in endometrial stromal cells. Factors such as IL-6, estradiol and progesterone can decrease the expression of miR-183, suggesting that downregulation of miR-183 expression during endometriosis can block the apoptosis and, subsequently, support the progression of the disease [39]. If these results are confirmed, it would support the epigenetic mechanisms of endometriosis. A role of miRNA in apoptosis was confirmed in additional studies. miR-191 inhibits TNFα-induced apoptosis in tissue samples from both endometriosis and endometrioid cancer. Also, miR-191 was found to target DAPK1, which is a positive mediator of apoptosis. Therefore, miR-191–DAPK1 axis might be involved not only in development of endometriosis, but also in malignant transformation of endometriosis [40]. Another study [41] on miRs focused on miR-29c family. The results showed that miR-29 was differently expressed in eutopic and ectopic samples. In vitro experiments suggested that miR-29c exerted its effects by suppressing cell proliferation and invasion, and by stimulating apoptosis. These effects probably occurred via targeting c-Jun. If confirmed, these data might be used in the development of a new therapeutic target.

Subsequent studies showed that this expression positively correlated with upregulation of Bcl-2 and activated ERK signaling. Manipulation of CD147 function resulted in the decrease of Bcl-2 expression, blocked ERK signaling and accumulation of apoptotic factors such as cleaved caspases 3 and 9 and cleaved poly ADP-ribose polymerase [42]. As human endometriosis is characterized by insufficient apoptosis, it is possible to speculate that abnormal upregulation of CD147 results in subsequent abnormal activation of ERAK-Bcl-2 signaling contributing to the development of endometriosis.

Additional studies found positive expression of Bcl-2 in endometrium with strong differences between cystic endometriotic and endometriotic stromal cells. In general, there is a large body of evidence about the role of Bcl-2 in apoptosis regulation in endometriosis. Even when proteins such as Bax or Bcl-1 are also involved in apoptosis, their role in endometriosis has been neglected with the exception of a study [43] showing increased association of a pro-apoptotic gene Bcl-x_s in eutopic endometrium. It was important, therefore, that similar findings were found for Bax and Mcl-1 expression, suggesting the role of these proteins in apoptosis regulation [44]. An excellent summary of apoptosis-related genes such as Bcl-2/Bax and Fas/FasL can be found in older reviews [45–47].

mTOR activity is abnormally high in endometriotic lesions, which might contribute to the changes in endometrial cell autophagy, subsequently affecting apoptosis of these cells. A study [48] testing this hypothesis showed that autophagy of endometrial cells was increased by mTOR inhibition as the menstrual cycle progressed in the normal endometrium, and that this process is correlated with apoptosis. On the other hand, in tissues from endometriotic cysts, autophagy, mTOR activity and apoptosis remained constant during the whole menstrual cycle. These results suggest that a constant level of autophagy is maintained by disinhibition of mTOR and is related to decreased apoptosis. Use of an mTOR inhibitor and/or autophagy inhibitor suggested that mTOR inhibition promoted endometrial cell apoptosis via autophagy reduction.

Cell cycle regulating protein $p27^{kip1}$ is involved in numerous cell differentiation processes including controlling the G1 to S phase transition. In a cancer model, gene therapy with this protein resulted in VEGF inhibition [49], leading to suggestions that this protein might play a role in endometriosis. Genetic manipulation resulting in overexpression of $p27^{kip1}$ in primary cultures of endometrium from patients with endometriosis showed strong inhibition of cell

proliferation in the endometriosis group. However, significant modulation of proteins involved in cell cycle (p16, p21, p27, and p53) and increase of apoptotic cell occurred. Also, the treatment led to a downregulation of VEGF expression, suggesting a link between apoptosis and cell cycle control proteins [50]. Elevated expression of CD147 has been found to be involved in endometrial cell apoptosis. Immunodepletion of CD147 induced apoptosis in uterine epithelial cells, suggesting that abnormal expression of CD147 in endometriotic lesions enhances cell survival by reducing apoptosis [51]. A study on histone acetylation, CYP19 gene and endometriosis revealed that valproic acid significantly promoted histone acetylation in the endometrial stromal cells and, subsequently, suppressed the expression of CYP19 gene. The same cells showed development of apoptosis [52]. Synthetic progestin norethisterone and progesterone were found to inhibit proliferation of human endometriotic stromal cells; the addition of estradiol had no effects. When the authors studied apoptosis, only the progestin norethisterone has any effect [53].

The search for a possible treatment for endometriosis led to evaluation of DLBS1422, which is a bioactive fraction of *Phaleria macrocarpa*. Using a human endometrial cell line RL95-2, Tandrasasmita et al. [54] showed increased apoptosis after exposure. Also, downregulation of estrogen receptor level and inhibition of eicosanoid signaling pathway were observed, making this molecule a strong candidate for potential treatment of endometriosis.

Drug-induced apoptosis was found to be markedly attenuated in endometriotic stromal cells, which might be associated with abnormal survival in ectopic sites in an environment that is probably unfavorable [55]. At the same time, the susceptibility to the drug-induced apoptosis varied widely among individual patients without direct comparison to the stage of the disease.

Using morphology, TUNEL reaction technique and electron microscopy, Rad's group demonstrated that endometrial cells in samples from women with endometriosis have higher potential to survive due to significantly lower occurrence of apoptotic cells [56]. A detailed study detecting apoptosis in samples of ovarian biopsies revealed that the reduction of apoptosis in unaffected tissue in women with endometriosis is probably caused by the fact that they might be predisposed to develop endometriosis [57].

Animal models

An in vivo murine model using implanted human endometriotic cells into SCID mice was used for evaluation of possible effects of a combination of *N*-acetyl cysteine, alpha-lipoic acid and bromelain. The data showed high anti-inflammatory effects and activation of the pro-inflammatory stimulus TNFα. Also, in mice obtaining endometrial cells, the treatment induced strong apoptosis. In mice implanted with cells from healthy uterus, no apoptosis was found [58]. This treatment must be first compared with standard therapies before any suggestions about possible clinical use can be reached. Another animal model employed Balb/c mice with induced peritoneal endometriosis. This study tested curcumin as possible anti-endometriotic agent. Results showed that the implanted cells developed strong MMP-3 activity [59]. The authors speculate that this activity might be involved in the Fas-mediated apoptosis. Curcumin showed strong effects on regression of the disease. These effects were mediated via mitochondrial pathway. The whole study is focused, however, on different aspects of apoptosis. The results suggested that instead of its involvement in protection of endometrial tissue, the MMP-3 activation results in accelerating of apoptosis in local phagocytes clearing way for seeding tissue.

A mouse model of peritoneal endometriosis using human endometrial fragments engineered to express the fluorescent protein mCherry showed that double-stranded RNA mimic complexed with polycations caused significant increase in apoptosis and a decrease in neovascularization. However, the overall size of implants was not changed [60]. In a study using cells isolated from chocolate cysts and eutopic endometrial tissue, the authors evaluated the role of apoptosis proteins. Four different proteins (c-IAPI, c-IAP2, XIAP, and survivin) were expressed in endometriotic tissue, suggesting their possible role in progression of this disease. When used in a mouse model, all four proteins were expressed again, which means that these cells may possess innate anti-apoptotic properties. Using an inhibitor of these apoptosis proteins, the proliferation of cells in endometriotic lesions was decreased [61], suggesting that inhibition of apoptosis proteins might be used as a therapeutic target. Multikinase inhibitor Sorafenib was tested for possible control of growth of endometriosis lesions. In vitro experiments showed that Sorafenib abrogated the phosphorylation of signal-regulated kinase in stromal cells isolated from women with endometriosis, but not in cells obtained from healthy women. In the mouse model, Sorafenib regulated the endometriosis activity by targeting endometriosis-related proliferation and inflammation [62]. As these inhibitors work both in vivo and in vitro, its effects on and suppression of phosphorylated VEGFR-2/VEGFR-2 ratio might be a more promising agent than most of the agents mentioned in this review.

ENMD-1086 is another potential inhibitor of endometriosis' progression. Since protease-activated receptor 2 plays a significant role in the pathogenesis of

endometriosis, it is not surprising that its antagonist ENMD-1086 has been evaluated for inhibition of endometriosis. Using a mouse model, five daily injections resulted in dose-dependent inhibition of endometriotic lesions development. In addition, the treatment also inhibited cell proliferation, IL-6 and NFkB expression and increased percentage of apoptotic cells [63]. Less often, rat model of endometriosis was used to characterize apoptosis occurrence and its effects on surgical induction of endometriosis. Significant differences were found in the apoptotic index for the glandular epithelium, increasing the chance for development of endometriosis [64]. Nevertheless, author's suggestions about transferring their methodology into human investigations seem to be rather premature.

An interesting report tested the hypothesis that some molecules specifically expressed on the endometrial surface could be used for targeting via injected drugs. Using a phage display library, a specific 9-mer z13 peptide specifically binding to glandular epithelial cells of endometriosis was found. In addition, its specific receptor CNGB3 was also found [65]. This protein can be linked with an apoptosis-inducing peptide. This animal model showed that cells in lesions selectively underwent apoptosis after administration of z13 peptide together with an apoptosis-inducing peptide.

Human models

Gonadotrophin-releasing hormone agonist (GnRHa) has often been used for the treatment of endometriosis, but the results are variable, most probably depending on the patient's medical history and the receptor–ligand binding affinity [66]. Using biopsy specimens from women with GNRHa therapy, Khan et al. [67] found significant decreases in inflammatory and angiogenic responses and strong increase in apoptotic index, helping in regression of the disease at the tissue level. Also, GnRHa therapy caused similar results in samples from myoma.

Fas/Fas-ligand system is one of the most important mediators of apoptosis. Among other biological processes, this system is also involved in remodeling of the endometrium as a result of hormonal changes [68]. In the proliferative phase of the menstrual cycle, both proteins remain inside the endometrial cells, but they are secreted during the secretory phase [69]. Analysis of expression of these proteins in samples from patients with severe endometriosis showed that staining for FasL in the eutopic endometrium was stronger in the epithelial cells of secretory phase. The epithelial cells of endometriotic lesions demonstrated higher staining independently of the menstrual phase. In the case of Fas, the staining in eutopic endometrium was reduced during the proliferative stage, but strong in the secretory phase. In the ectopic endometrium, the staining for Fas was reduced regardless of the phase, suggesting possible immune privilege of this tissue [70]. This privilege may play a role in inducing of apoptosis in Fas^{+} cells.

Conclusion and future perspective

Apoptosis mechanisms during apoptosis are still far to be fully elucidated, based on our data collection and analysis. Accumulating evidence already suggested that programmed cell death plays a pivotal role in addressing the immune homeostasis in the peritoneal microenvironment. Thus, the result of interaction between peritoneal fluid mononuclear cells and endometriotic cells underlies the progression of the disease and reflects the clinical symptoms and signs. Furthermore, it is widely accepted that the retrograde menstruation occurs in approximately 90 % of the healthy women without causing the disease: in this view, it is crystal clear that several other additional factors may influence the peritoneal microenvironment of women who will develop the disease and allow the implantation and proliferation of endometriotic cells. In normal conditions, endometriotic cells, refluxed through the fallopian tubes into the peritoneal cavity, should be attacked and removed by phagocytes and NK cells. During endometriosis, the breakdown of peritoneal homeostasis causes the failure of scavenging mechanisms, allowing the survival of endometriotic cells. The consequent so-called "immunoescaping" of endometriotic cells could be due, at least in part, to the reduction of apoptotic-mediated pathways previously described. Also, as widely reviewed by Maeda et al. [71], cells expressing human leukocyte antigen (HLA)-G, a ligand of NK receptors, were identified in the peritoneal fluid only in the menstrual phase but not in the proliferative or secretory phases. More recent evidence advocated that HLA-G is expressed by endometriotic glandular epithelium but not by eutopic endometrium under normal conditions, suggesting that peritoneal inflammation or cellular stress may upregulate mechanisms to promote ectopic endometrial survival [72]. In this view, impairment of NK cytotoxicity via HLA-G may allow peritoneal endometrial cell survival and implantation through the reduced immunosurveillance. Similar to HLA-G, also soluble receptor-binding cancer antigen expressed on SiSo cells (sRCAS1) was found high during menstruation and low during the proliferative cycle phase [73]; furthermore, sRCAS1 blood serum concentration level has been shown to increase as ovarian endometriosis progresses, suggesting a possible role in inducing selective suppression of the immune cytotoxic cells [74].

Considering the large amount of evidence retrieved from in vitro as well as in vivo models, the reduced apoptosis of endometriotic cells together with the increased apoptosis of peritoneal fluid mononuclear cells may address the peritoneal homeostasis to a permissive environment for the progression of the disease. Nevertheless, to date, data about target-therapy of endometriotic pathways are still not robust. Despite this point and basing on available (although preliminary) data, the induction of apoptosis of endometriotic cells could be considered a key research field. In this view, we strongly solicit future studies which may shed new light about the possibility of increasing the apoptosis of endometriotic cells, mainly modifying the peritoneal microenvironment addressing the response to avoid their "immunoescaping".

Compliance with ethical standards

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