The Endometriotic Tissue Lining the Internal Surface of Endometrioma: Hormonal, Genetic, Epigenetic Status, and Gene Expression Profile

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Abstract

Ovarian endometriomas are found in a consistent proportion of patients with endometriosis and are associated with a more severe form of the disease. The endometriotic tissue lining the inside of the endometrioma has been extensively studied over the years mostly for the need to compare the molecular and cellular characteristics of eutopic and ectopic endometria. Several aspects of hormonal regulation, response to local inflammation, carcinogenesis, and modifications of the local environment have been investigated in order to characterize also the processes associated with peritoneal endometriosis. In this review, we have summarized the current knowledge of pathophysiology of endometrioma, with a particular focus on the cellular components lining the internal surface of the cyst in order to provide a comprehensive overview of the hormonal, genetic, epigenetic, and gene expression profiles of this essential part of the cyst.

Keywords

endometrioma, gene expression, oxidative status

Introduction

Endometriomas are a common feature of endometriosis as they are found in 17% to 44% of patients with endometriosis.¹ Interestingly, their pathogenesis has been suggested to be different from other forms of endometriosis such as peritoneal implants or rectovaginal nodules. Regardless of the theory proposed for their histogenesis,^{2,3} the cyst is known to be surrounded by a distinctive wall and to contain a typical tarry, thick fluid thought to be formed by accumulation of menstrual debris from shedding and bleeding of active endometriotic implants inside the cyst.⁴ This fluid contains proteolytic enzymes, reactive oxygen species, and inflammatory molecules in concentration much higher than those present in other types of cysts.⁵ The cyst wall is composed of either ovarian cortex itself or fibroreactive tissue, and the internal surface is covered by an endometrial lining in 60% of the entire area with a range from 10% to 98%.⁵

The endometriotic tissue lining the inside of the endometrioma is the part that has received the greatest deal of attention over the years, mostly for the need to compare the molecular and the cellular characteristics of eutopic and ectopic endometria. Indeed, given the limited understanding of the pathologic processes that are involved in endometriosis, conventional cellular and molecular biology techniques have been used to

study the protein and gene expression patterns of the endometriotic cyst in an attempt to better understand the pathology of the disease. Moreover, since an association between ovarian endometriosis and ovarian cancer has been suggested based on epidemiological studies, the tissue inside the cyst has been evaluated for the presence of genetic changes able to induce a malignant transformation.⁶ Aspects of cellular function including cell motility, adhesion, invasion, angiogenesis, and immunological factors have also been investigated in order to characterize certain processes of carcinogenesis and metastasis already associated with endometriosis.

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In light of the heterogeneity and complexity of the disease,⁷ we have herein reviewed the current literature describing the molecular specificities of the endometriotic tissue lining the inside of the ovarian cyst compared with the eutopic endometrium. The data deriving from this analysis may help not only in clarifying some aspects of the pathophysiology of endometrioma but also in revealing potential weaknesses of the previous investigations in this area.

Hormone Biosynthesis and Receptivity

Endometriotic tissue at ectopic locations such as the peritoneum or the ovary seems to be quite different from eutopic endometrium in terms of estrogen biosynthesis/metabolism and clinical response to hormonal regulation. Indeed, molecular differences with regard to estrogen and progesterone receptors (PRs) have been described between normal endometrium and eutopic and ectopic tissues from women with endometriosis.⁸ Herein, we will focus on endometriotic tissues and cells lining the endometrioma wall in order to verify the ability of the cyst microenvironment to modify hormonal receptivity and hormone biosynthesis of these cells.

Although eutopic endometrium showed predominantly higher levels of estrogen receptor (ER)- α than ER- β , a strong upregulation of ER- β messenger RNA (mRNA) in the inner lining cells of ovarian endometriosis has been demonstrated by various authors.⁸⁻¹² These findings could not be confirmed by Matsuzaki and colleagues who found a similar expression of ER- β mRNA in endometrioma inner layer and eutopic endometrium using a conventional quantitative Taq-Man real-time polymerase chain reaction. Moreover, they detected ER- β mRNA expression only in half of the ovarian samples using an *in situ* hybridization technique.¹²

Data on PR expression are more controversial. Misao et al have reported significantly higher levels of PR-B mRNA in ovarian endometrioma inner lining samples compared to eutopic endometrium.¹³ According to Bukulmez et al, endometrioma lining tissue samples demonstrated a significantly lower PR-A and significantly higher PR-B mRNA expression than the eutopic endometrium samples. Immunoblotting demonstrated that the endometrioma capsule manifested robust immunoreactivity for PR-B, whereas PR-A immunoreactivity was barely detectable. Similarly, immunostaining showed absence of PR-A while PR-B was detectable in both stroma and epithelium.¹¹

Both PR-B and PR-A transcripts and proteins were found to be downregulated in tissue samples from endometrioma capsule compared to eutopic endometrium by Hayashi and colleagues.¹⁴ In line, Xue et al showed that stromal cells isolated from the cyst wall had significantly lower total PR and PR-B mRNA levels compared to endometrium.¹⁵ No difference in the expression of PR-B isoform at both mRNA and protein level was found by Smuc and colleagues.¹⁶

It should be considered that none of the cited articles have devoted much effort to isolate clean ectopic endometriotic tissue samples or pure ectopic endometriotic cells from the

internal lining of the cyst wall. The cyst wall contains normal ovarian stroma and fibroreactive tissue that is probably not distinguishable from ectopic stroma. This could be the reason for the controversial findings of the various studies as different degree of ovarian tissue and cell contamination cannot be excluded. This is particularly important considering that the isoform ER- β is the dominant steroid receptor in mammalian ovary in reproductive period,¹⁷ while expression of ovarian PR isoforms varies greatly according to the cellular component and is highly regulated upon stimulation.¹⁸ Since most of the studies failed to detail the procedures of endometrioma tissue selective isolation, it is very difficult to compare the results obtained under different conditions. However, since contamination by a specific tissue is likely to occur when extracting mRNA from endometrioma capsule, results from studies that have visually analyzed glands and stroma of the ectopic lesion should be taken into higher consideration.^{11,12} Data derived from these analyses were not always in line with results from studies that have only measured extracted mRNA from tissues or cells.^{13,15} Laser capture microdissection (LCM) that permits to obtain pure populations of cells from a heterogeneous tissue may represent a solution to this problem of the analysis of an mRNA abundance from a mixture of cell types like those present in endometriotic tissues as some studies have indeed suggested.^{19,20}

The endometrial stromal cells are thought to uniquely express the full complex of genes in the steroidogenic cascade which is sufficient to convert cholesterol to estradiol.¹⁵ The biosynthesis and bioavailability of estrogens, depending on the balance between the production of active estrogens and their inactivation, are determined by several enzymes mediating various processes. Most of these processes are present in the human endometrium and some of them appear to be aberrantly regulated in the ectopic endometrium. Aromatase, converting androstenedione to estrone, 17 β -hydroxysteroid dehydrogenases (17 β -HSD), regulating the balance between 17 β -estradiol and estrone, and steroid sulfatase, converting sulfated estrogens into active nonconjugated estrogens have all been shown to be dysregulated in the ectopic tissue, albeit non-consistently.^{21,22} Reasons for these discrepancies may again reflect technical differences in ectopic sampling which were mostly based on mRNA and total protein analysis. On the other hand, Huhtinen and coworkers have recently measured the local 17 β -estradiol and estrone concentrations in different types of endometriotic lesions using a liquid chromatography–tandem mass spectrometry.²³ The ovarian samples contained 17 β -estradiol and estrone at far higher levels than eutopic endometrium or peritoneal and deep endometriosis. Median 17 β -estradiol levels were 3440 pg/mL in proliferative ovarian samples, 112 pg/mL in proliferative deep endometriosis, and 649 pg/mL in proliferative eutopic endometrium. Median estrone levels were 1380 pg/mL in proliferative ovarian samples, 74.3 pg/mL in proliferative deep endometriosis, and 57.3 pg/mL in proliferative eutopic endometrium. The authors suggested a high local estrogen synthesis within the cyst but could not exclude a contribution by the ovarian 17 β -estradiol production

provided in a paracrine manner. Also, in this study, contamination from ovarian tissue cannot be completely excluded.²³

Gene Expression Profile

The discovery of the differentially expressed genes in ectopic endometrium localized at the ovarian site versus eutopic endometrium has been the object of several studies. These studies differ for cell type or tissue type used for RNA extraction, data analysis, and patients enrolled. Until recently, these investigations were limited by the inability to study more than 1 gene at a time. As a matter of fact, the literature has consistently provided a great amount of data supporting the idea that genes involved in several cell functions—adhesion (ie, integrin β 2 and integrin β 7),²⁴ proliferation (ie, platelet-derived growth factor receptor- α and protein kinase C- β 1),²⁵ invasion (ie, matrix metalloproteinases and relaxin),^{26–28} immune recognition (ie, β -defensin-2),²⁹ inflammatory response, (ie, tumor necrosis factor α [TNF- α] and interleukin [IL] 1 β),^{29,30} steroid biosynthesis and response, and angiogenesis (ie, vascular endothelial growth factor, angiopoietin-1, and -2)²⁷—are aberrantly expressed in ectopic endometrium lining the inside of the cyst.

To gain insight into the potential differences in the gene expression profile between eutopic and ectopic endometria inside the cyst, we have decided to focus our attention on microarray analysis.

Table 1 lists the characteristics of 10 published gene expression studies comparing endometrial and ovarian endometriotic cyst tissue. We have specifically focused on studies that have considered only endometriomas as ectopic tissue. Moreover, for the sake of standardization of data presentation, we have taken into consideration only the lists of differentially expressed genes published in articles as publicly, data sets are only available from a minority of studies. Different studies have found common gene expression patterns, typical of various cellular pathways and processes—(ie, cell cycle, steroid hormone metabolism, cytoskeleton remodelling, immune response, chemotaxis, homeobox (HOX) gene system, Wnt pathway)—that have been proposed to be altered in ectopic tissue. Among the specific genes found in common among the various studies are the following^{31–40}:

1. the hydroxysteroid 11 β -dehydrogenase (HSD11B1), a gene encoding for an enzyme that catalyzes the conversion of cortisone to active cortisol, was about 7-fold upregulated in ectopic tissue in 3 of the studies. Genes involved in the response to glucocorticoids have also been demonstrated to be upregulated in peripheral blood mononuclear cells of patients affected by endometriosis as shown by the Functional Gene Ontology analysis of data derived from oligonucleotide microarrays.³⁴ In line with these findings, Lima et al have demonstrated that serum cortisol levels were significantly higher in infertile women with stage III and IV endometriosis (20.1 ± 1.3 ng/mL) than in controls (10.5 ± 1.4 ng/mL). Conversely, levels of cortisol in

follicular fluid and peritoneal fluid did not significantly differ between the groups.⁴¹ Contrary to these findings, Smith and colleagues found reduced levels of cortisol concentration in the follicular fluid of women with minimal–mild endometriosis compared to women with tubal factor of infertility.⁴² The relationship between cortisol and endometriosis needs further investigation.

2. Phospholipase A2 group II (PLA2G2) and phospholipase A2 group V (PLA2G5), which encode the enzyme responsible for the production of the prostaglandin precursor, arachidonic acid, were more than 10-fold upregulated in ectopic tissue in 3 of the studies. The PLA2G2 gene expression was as well found to be increased in peritoneal macrophages of patients with endometriosis compared to those from controls.⁴³ Overproduction of prostaglandins by an increase in cyclooxygenase 2 (COX-2) activity is considered a key factor in the development of endometriosis.⁴⁴ Increased concentrations of prostaglandins have been found in peritoneal fluid of patients and ectopic endometrium and elevated expression of COX-2 in endometrial and endometriotic tissues of women with endometriosis has also been described.⁴⁴ The important role of prostaglandins as inflammatory mediators suggest them as possible therapeutic targets for the disease-associated pain and inflammation.
3. Apolipoprotein E (APOE), whose protein is primarily produced by macrophages in peripheral tissues, was 3-fold upregulated in ectopic tissues. It is well known that the expression of APOE is regulated by TNF- α , the expression of which is increased in the peritoneal fluid of patients with endometriosis.^{45,46} Apolipoprotein E acts through cell signaling pathways that are also activated by estrogen and other growth factors, leading to cell proliferation and survival.⁴⁷ However, its expression levels were found to be decreased in eutopic endometrium of women with endometriosis compared to eutopic endometrium of healthy women.²⁴
4. Peroxisome proliferator-activated receptor γ (PPARG), whose activation was shown to be involved in an in vitro model of early endometriotic lesion,⁴⁸ was about 2-fold upregulated in ectopic tissue. In other contexts, this transcription factor mediates inflammatory reactions by controlling cytokine gene transcription.^{49,50} More recently, it has been found that PPARG protein is expressed in epithelial and stromal cells in endometriotic lesions regardless of their location. A significant positive correlation was observed between pain and expression of PPARG only in the peritoneal endometriotic lesions but not in rectovaginal septum/ovarian lesions.⁵¹ From a therapeutic point of view, a thiazolidinedione agonist of PPARG has been tested in both in vitro and in vivo studies. In a prospective randomized placebo-controlled study using a model of induced endometriosis in baboons, Lebovic and colleagues showed that pioglitazone was able to reduce the surface area and volume of endometriotic lesions, thus limiting

Table 1. Characteristics of the Microarray Studies That Evaluated the Differentially Expressed Genes Between Ectopic Endometrium From Ovarian Cysts Compared to Eutopic Endometrium.^a

Microarray type	Eyster et al ³¹	Arimoto et al ³²	Wu et al ³³	Hever et al ³⁴	Mettler et al ³⁵	Borghese et al ³⁶	Zafrakas et al ³⁷	Khan et al ³⁸	Monsivais et al ³⁹	Laudanski et al ⁴⁰
Human Genes GenFilters (Research Genetics, Huntsville, Alabama) 4133	Human Genes GenFilters (Research Genetics, Huntsville, Alabama) 4133	Microarray Spotter Generation III (Amersham Biosciences)	cDNA on glass slide	Affymetrix human genome (HG-U133 plus2; Basel, Switzerland)	Atlas human 1.2 array (Clontech, Mountain View, California) 1176	NimbleGen platform (Reykjavik, Iceland)	Affymetrix human genome (HG-U133SET; Santa Clara, California)	Agilent whole human genome (Santa Clara, California)	Affymetrix human genome (HG- U133A)	PCR array of Sabiociences (Prospekta, Poland)
Genes/transcripts		23 040	41 472	47 000		?	40 000	29 412	47 000	84
Menstrual phase information	Follicular	9 proliferative, 14 secretory	9 proliferative, 1 secretory, 1 inactive	8 luteal, 2 follicular	Proliferative	Luteal	Proliferative	13 proliferative, 5 secretory	Follicular	14 proliferative
Sample size (ectopic-/eutopic- matched samples)	3	23	11	10	5	6	14 ectopic/4 eutopic	18	8	14
Criteria for identification of DE genes	not available	2-fold difference	Structural modeling taking phase and location into account	$P < .05$	2-fold difference	2-fold difference	2-fold difference	3-fold difference ($P < .01$)	2-fold difference ($P < .05$)	2-fold difference ($P < .01$)
Number of genes reported to be DE	8	352	388	not available	18	8000	not available	unknown	1222	25
Number and list of genes found in common with at least another study	2; ACTB, ACTA2	11; ACTB, BF, CIR, CEBPD, DLX6, DPYSL3;HLA- DRA, HLA-DRB1, HLA-DQB1 RNASEI, TRH	7; IGFBP5, PLA2G5, PRELP, PLSCR1, RNASEI, SEPP1, SPTBN1	20; APOE, BF, C3, C7, DPYSL3, GPX3, HLA-DRA, HLA- DRB1, HLA-DQB1, HSD11B1, KLF2, IGL@/IGLC2, LTBP2, PLA2G2A PLSCR1, PNOC, PPARG, RARRES1, STAR, THBS1	3; CIR, HLA-C, SPTBN1	13; C7, DLX6, IGLC2, IGKC MSX2 , MYH11, PLA2G5, PNOC, PRELP, STAR, TRH , UGT8 , WISP2	11; ACTA2, CEBPD, GPX3, HSD11B1 ID1, IGKC, LTBP2, MYH11, PLA2G2A, RARRES1, THBS1	8; APOE, HLA-C, KLF2, MSX2 , SEPP1, STAR, UGT8 , WISP2	4; HSD11B1, PPARG, PLA2G2A, PLA2G5	1; ID1

Abbreviations: ACT, actin; APOE, apolipoprotein E; cDNA, complementary DNA; DE, differentially expressed between eutopic and ectopic endometrium derived from ovarian cysts reported in published studies; HLA, human leukocyte antigen; HSD, hydroxysteroid dehydrogenases; MYH11, myosin 11; PPARG, peroxisome proliferator-activated receptor γ ; STAR, steroidogenic acute regulatory protein.

^a In gray: genes that are downregulated in ectopic versus eutopic endometrium. In normal: genes that are upregulated in ectopic versus eutopic endometrium.

the initiation of the disease.⁵² In human endometriotic cells, the same authors demonstrated that ciglitazone exerted an antiproliferative effect, decreased prostaglandin E₂ receptor expression, and inhibited expression and function of aromatase.⁵³

5. Genes encoding several complement components including C1R, C3, and C7 were described to be upregulated in endometriosis. In particular, C3 plays an important role in the activation of the classic and alternative pathways of the complement cascade and thus in inflammation. Tao et al have previously demonstrated an increased production of C3 by endometriotic tissue that correlated with elevated C3 protein levels in the peritoneal fluid of patients with endometriosis.⁵⁴ The upregulation of complement genes in the ectopic tissue can contribute to the immunological dysfunction associated with the disease.³¹
6. Genes encoding for molecules of the major histocompatibility complex (MHC) human leukocyte antigen (HLA)-C, HLA-DRA1, HLA-DRB1, and HLA-DQB1 have been reported to be upregulated in the ectopic endometrium.

Human leukocyte antigen C belongs to the MHC class I heavy-chain receptor family and it has a relatively limited polymorphism. The HLA-C molecules are expressed at the cell surface at about 10% the levels of HLA-A and HLA-B. Since expression of HLA-C antigens is characteristically low, their physiological relevance with regard to antigen presentation to CD8⁺ T cells has been questioned, as only a minority of cytotoxic CD8⁺ T cell responses are restricted by HLA-C. On the other hand, HLA-C molecules clearly play a role in natural killer (NK) cell activation through binding to killer immunoglobulin receptors.⁵⁵ Therefore, the overexpression of HLA-C in the endometriotic epithelial cells could have a role either in the recognition and in the presentation to macrophages, with the triggering of an autoimmune reaction or for the modulation of NK cell-mediated response.

Human leukocyte antigen DR and HLA-DQ belongs to the MHC class II family. The HLA-DR is involved in autoimmune reactions, disease susceptibility, and resistance. Both HLA-DR and DQ are typically expressed by professional antigen-presenting cells such as macrophages, B cells, and dendritic cells.⁵⁶ Therefore, in studies reporting their expression in ectopic tissue, contamination with cells of the immune system cannot be excluded.

7. Genes encoding for proteins involved in cytoskeleton remodeling such as actin $\alpha 2$ (ACTA2) and myosin 11 were described to be upregulated in tissue lining the inside of the cyst. It is known that smooth muscle metaplasia has been associated with ovarian endometriosis.⁵ Based upon several histological findings, the submesothelial mesenchymal cells undergo a prominent smooth muscle metaplasia and strongly express ACTA2.⁵⁷

Among the various gene expression studies performed comparing eutopic endometrium and ectopic tissue derived

from cysts, a well-conducted study was from Wu et al.³³ Epithelial cells have been precisely isolated from endometriotic lesions by LCM in order to exclude contaminating cells from the analysis. Using a complementary DNA microarray with 9600 genes, Wu et al identified signaling pathways that might be differentially expressed between eutopic endometrium and ovarian or nonovarian endometriotic epithelial cells. A replication assay has also been performed to reduce the variability associated with microarray output. Based on the statistical analysis that took into account lesion location and menstrual cycle, the genes identified include some ILs and their receptors (ie, IL-8, IL-15, and IL-15R), growth factors (platelet-derived growth factor β), and a member of the transforming growth factor (TGF) family (TGF- $\beta 3$).³³

Limits of the Gene Expression Studies

Important issues regarding gene expression studies analyzing cells lining the internal surface of the cyst should be considered. The purity of endometriotic cells isolated from endometriomas for subsequent mRNA extraction was usually checked by immunohistochemistry for specific vimentin expression on stromal cells and for cytokeratin expression on epithelial cells. However, the fibrous stroma of the ovarian cortex always stains intensely with antivimentin antibodies.⁵⁸ Thus, it is unlikely that the ovarian stroma can be distinguished from the endometriotic stromal cells obtained by a conventional tissue collection, and the isolation of a pure endometriotic cell component from the endometriotic cyst appears to be particularly complicated. Some studies have also added immunohistochemistry for CD10, a marker of normal endometrial stroma⁵⁹ that is also focally positive in a variable proportion of supportive stromal cells as well as in endothelial or muscular cells of various vascular channels.⁶⁰ Moreover, when a chronic inflammatory reaction is present, a proportion of lymphocytes also express CD10. Therefore, the establishment of the purity of the endometriotic stromal cell population via CD10 staining requires a very detailed analysis of the percentage of positive cells to rule out any contamination from other cell types and the maintenance of the positive staining during the cell passages.⁶¹ In general, characterization of the isolated endometriotic cells from ovarian cysts does not receive the necessary attention that would require a first-line cell characterization.

In addition, the ectopic tissue taken or scrapped from the internal lining of the cyst wall for mRNA extraction may possibly be contaminated by the ovarian components or by the fibroreactive tissue. Thus, information derived from these studies need to be considered with caution and the analysis of the gene expression profile of the endometriotic cells lining the inside of the cyst would require a more scientific rigor for cell or tissue identification.

Another explanation for the controversial results deriving from these studies refers to the different statistical analyses used in the various studies. Softwares used to perform absolute and comparison analysis vary greatly among studies. Although most of the studies simply compared genes increased or

decreased in a certain fold,^{31,32,35} other studies have used elaborate modeling that included the interaction between the tissue type, menstrual phase, and lesion location.³³ Moreover, hierarchical cluster analysis was performed using very different tools.

Epigenetic Alterations

There are some experimental evidence supporting the idea of endometriosis as an epigenetic disease:

1. Hypermethylation of PR-B promoter has been shown in ectopic epithelial cells and suggested to be responsible for the lower expression of PR-B in the ectopic tissue.⁶² More recently, Meyer and colleagues found that the PR-B was methylated exclusively in the endometriotic lesions, specifically deep lesions, when compared to eutopic endometrium obtained from the same patient, while no differences were observed in the DNA methylation for the ER- α and ER- β genes.⁶³
2. HOXA10, a gene important for uterine development and function, has been reported to be hypermethylated at the promoter region in the eutopic endometrium of (1) women with endometriosis compared to that of control women^{64,65}; (2) baboons with induced endometriosis⁶⁶; and (3) mice with induced endometriosis.⁶⁷
3. Deoxyribonucleic acid methyltransferase (DNMT) 1, DNMT3A, and DNMT3B responsible for DNA methylation have been shown to be all overexpressed in ectopic endometrium.⁶⁸

The studies addressing the relationship between endometriosis and epigenetic alterations usually involved samples derived from a pool of peritoneal and ovarian endometriosis.^{63,68} The following few studies have specifically focused on ovarian endometrioma inner lining samples:

- (a) Previous studies have demonstrated that methylations of histone H3 at lysine 9 (H3K9) and lysine 27 (H3K27) are associated with transcriptional repression, whereas methylations at H3K4, H3K36, and H3K79 are associated with transcriptional activation.⁶⁷ Recently, Xiaomeng and coworkers found an aberrant histone code in women with ovarian endometriosis.⁶⁹ A global histone H3K9 hypomethylation in the ectopic ovarian endometrium and a global histone H3K4 hypomethylation in both ectopic and eutopic endometria of women affected have been demonstrated. Among the various chromatin modifier genes, HDAC1, SUV39H1, SUV39H2, and G9a were found to be significantly downregulated in ectopic ovarian lesions.
- (b) Hyperacetylation of histones H3 and H4 is often associated with activated transcription, while hypoacetylation of histones H3 and H4 correlates with transcriptional silencing or repression. Kawano and coworkers have demonstrated that levels of acetylated histones H3 and H4 were significantly lower in human

endometriotic stromal cells derived from the cyst wall in comparison to normal endometrial stromal cells supporting the idea that aberrant histone modifications are present in endometriosis.⁷⁰ More recently, the same group found an accumulation of acetylated histones H3 and H4 in the promoter region of the CCAAT/enhancer-binding protein (C/EBP) α gene in endometriotic stromal cells with a reduction in its expression. This gene is a member of the C/EBP family of transcription factors, which plays a role in cell cycle control, cell differentiation, metabolism, and inflammation.⁷³

- (c) The transcriptional enhancer steroidogenic factor 1 (SF-1) has been shown to be specifically expressed in endometriosis but not in eutopic endometrium due to an epigenetic mechanism that would permit binding of activator versus inhibitor complexes to its promoter. The SF-1 promoter hypomethylation was demonstrated in endometriotic stromal cells derived from cysts.⁷² The SF-1 binds to a specific response element in the promoters of a number of steroidogenic genes in the ovary and mediates the responsiveness to cyclic adenosine monophosphate. In endometriotic stromal cells, prostaglandin E2 induces coordinate binding of SF-1 to the promoters of steroidogenic acute regulatory protein (STAR) and aromatase gene to coactivate their expression.
- (d) Recently, another protein involved in the epigenetic process, the lysine-specific demethylase 1 (LSD-1), has been studied in endometriosis. This protein is a component of several histone deacetylase complexes able to silence genes by functioning as a histone demethylase. The LSD-1 demethylates H3K4me1/me2 and H3K9me1/me2 resulting in gene repression.⁷³ An aberrant expression of LSD-1 has been shown in many types of cancers, being upregulated in bladder, small cell lung, and colorectal clinical cancer tissues when compared with the corresponding nonneoplastic tissues.

Ding and collaborators have demonstrated that expression of LSD-1 was higher in eutopic and ectopic endometria of patients affected compared to healthy endometrium and levels were higher comparing ectopic endometrium with its homolog eutopic counterpart. The results have been confirmed as well by Western blot analysis at the cellular level. Moreover, they found that in endometriotic stromal cells isolated from the cyst, inhibition of this protein expression was able to reduce cellular proliferation, cell cycle progression, and invasiveness.⁷⁴ This finding is also consistent with the previously reported global H3K4 and H3K9 hypomethylation in endometriosis.⁶⁹

- (e) A more global study about DNA methylation in the pathogenesis of the endometriosis was performed by Borghese and coworkers using the methylated DNA immunoprecipitation technology in combination with hybridization to gene promoter arrays. This represents the first genome-wide analysis of promoter methylation in endometriosis. The analysis was performed

comparing DNA from ectopic endometrial tissue derived from ovarian, superficial, and deeply infiltrating endometriosis versus eutopic endometrium.⁷⁵ Specific methylation patterns were found for each subtype of the disease, although 20 regions were commonly methylated in all subtypes. Interestingly, this study correlated the methylation patterns with previously validated gene expression data.³⁵ In general, the methylation level in the promoter regions was not linked to the expression levels of nearby genes, except for 35 genes. Among genes found hypomethylated and upregulated in ovarian endometrioma compared to eutopic endometrium were scavenger receptor class B, member 1, phospholipase D2, cystathionine- β -synthase, and Fas apoptotic inhibitory molecule 3. Among genes found hypermethylated and downregulated were HOX D11, HOX D10, Solute carrier family 16, member 3, and 1-acylglycerol-3-phosphate O-acyltransferase 3.

- (f) More recently, a genome-wide DNA methylation study was published by Yamagata and collaborators⁷⁶ using eutopic and ectopic endometrial stromal cells. The methylation status of endometrial stromal cells from ectopic endometrium derived from chocolate cyst was compared to endometrial stromal cells from eutopic endometrium from women with and without endometriosis. A transcriptome analysis of 28 869 genes was used to evaluate concordance with the results of the DNA methylation analysis. Only 75 genes were found to have a correlation between DNA methylation and mRNA expression. DNA methyltransferase expressions were not found to be different between the groups. The authors focused their attention on specific steroidogenesis-related genes. High mRNA expression and DNA hypomethylation in the NR5A1-encoding SF-1 and STAR genes were found in ectopic samples, in line with previous findings.⁷² Low expression of STRA6, a receptor for retinol binding protein and DNA hypermethylation, was also found in ectopic samples.

Limits of Epigenetic Studies

Similar to the gene expression studies, epigenetic studies suffer potentially from a poor attention in limiting the ectopic tissue contamination by the ovarian components or by the fibroreactive tissue. It is plausible that ovarian cells have a completely different epigenetic status than endometrial cells and this issue should represent a matter of great alertness for every investigator in this field.

An issue to be consider in evaluating epigenetic studies is that it is difficult to distinguish between the etiologic factor and the alterations occurring with time or under different hormonal and inflammatory conditions. The inflammatory environment, like that present in an endometrioma,⁵ constitutes a trigger for epigenetic reprogramming. Reactive halogen compounds, which are derived by chemical reactions produced by inflammatory processes, can cause DNA methylation changes.

Oxidative stress and proinflammatory mediators have been suggested to influence histone acetylation and phosphorylation via mechanisms dependent on the activation of various signaling pathways such as those of mitogen-activated protein kinase and nuclear factor κ B (NF- κ B).^{77,78} The NF- κ B-dependent transcription, which is highly involved in endometriosis-associated inflammatory processes,⁷⁷⁻⁷⁹ was shown to require multiple coactivators that possess histone acetyltransferase activity.⁸⁰⁻⁸² On the other hand, some epigenetic phenomena may actually be pathogenetic for the disease. An important task for the future would be to distinguish important pathogenetic epigenetic events from processes secondary to the disease establishment. The potential role of epigenetics in determining consistent changes in gene expression cannot be underestimated, given the complex nature of the disease in which environmental or lifestyle factors are well known to be involved.⁸³

Genetic Alterations

The potential for neoplastic degeneration of endometriosis has been long debated from biological, epidemiological, and clinical perspectives.^{81,84} Based on results from epidemiological population-based studies, strength of association between endometriosis and ovarian cancer ranges between 1.3 and 1.9. However, when considering specific histotypes of disease, an association with endometrioid and/or clear cell ovarian carcinoma was documented with odds ratios varying⁸⁴ between 3.7 and 35.4. Based on these observations, malignant transformation of endometriosis has been mostly supposed to be confined to the ovaries.⁸⁵⁻⁸⁷ An ascertainment bias cannot be excluded as pathologists tend to obtain sections from an affected ovary rather than randomly from the peritoneum. However, the idea that cancers associated with endometriosis develop much more frequently in the ovary than at extragonadal sites, despite the fact that endometriosis occurs just as frequently outside the ovary than in the ovary, has supported the extensive investigation of the peculiar features of the local ovarian milieu potentially favoring the malignant transformation.^{88,89} The local factors in the cysts claimed to be responsible for the induction of genetic alterations in endometriotic cells lining the inside of ovarian endometriomas are (1) inflammatory factors and cytokines that, via NF- κ B activation, have been found to promote angiogenesis, proliferation, inhibition of apoptosis, and production of reactive oxygen species that may, in turn, induce DNA damage and mutations; (2) an altered steroid hormone balance or responsiveness as estrogens have been linked to the pathogenesis of gynecological cancers; (3) iron-associated oxidative stress considered currently the most important trigger, being able to attack "fragile" sites in the genome, including the p53 pathway.^{4,90,91}

The major genetic alterations likely to occur in endometriotic cells initiating an endometriosis-associated ovarian carcinogenetic process are the following:

1. Inactivation of phosphatase and tensin homolog (PTEN): by virtue of its phosphatase activity, PTEN (gene located in 10q23.3) is a major negative regulator

Figure 1. Representative genes differentially expressed in the endometriotic cells lining the internal surface of the cyst compared to eutopic endometrial samples.

of the phosphoinositide 3-kinase pathway and has been isolated as a tumor suppressor in a variety of malignancies.⁹² Sato and colleagues found *PTEN* gene missense mutations and deletions in 20.6% solitary ovarian endometriotic cysts, in 20% ovarian endometrioid carcinomas, and in 8.3% clear cell carcinomas.⁹³ These findings have been subsequently confirmed at protein level as PTEN protein expression was demonstrated to be reduced in 15% of endometriosis cases.⁹⁴

2. *ARID1A* mutations: *ARID1A* (located in 1p36.11) is thought to be a tumor suppressor gene and the encoded protein, BAF250a, is part of the large adenosine triphosphate-dependent chromatin-remodeling sucrose nonfermenting/switching (SNF/SWI) complex which is required for transcriptional activation of several genes normally repressed by chromatin. The *ARID1A* may act as a chromatin-remodeling modifier that can lead to cell cycle arrest and cell death in the event of DNA damage. *ARID1A* mutations were found in 46% of ovarian cancers of clear cell histotype, in 30% of endometrioid histotype, and in none of the serous histotype. The *ARID1A* mutation status was also correlated with the loss of expression of BAF250a. More interestingly, by comparing ovarian clear cell carcinomas to their contiguous atypical cystic endometriotic lesions in 2 patients, the same mutations could be detected in the putative precursor lesions and in the tumors but not in a region of distant endometriosis.⁹⁵
3. *Mutation or loss of function of the tumor suppressor gene TP53* (located in 17p13.1): activation of p53 can induce several cell responses including differentiation, senescence, and DNA repair but best understood is the ability of p53 to induce cell cycle arrest and apoptotic cell death.⁹³ Mutations of *TP53* gene are frequently related to allelic loss at 17p13 and overexpression of non-functional p53 protein that accumulates within the nuclei.⁹⁶⁻⁹⁸ The *TP53* gene mutations are present especially in serous tumors and also in about 30% and 10%

of endometrioid and clear cell carcinomas, respectively.⁹⁶ Accumulation of p53 in endometriotic lesions next to carcinomas as a potential sign of a continuum from endometriosis to cancer is debated. Studies describing sporadic occurrence of endometriosis adjacent to malignant carcinomas consistently failed to demonstrate p53 accumulation in the endometriotic areas and found staining for p53 in all malignant tumors.⁶ In contrast, other authors have detected the overexpressed protein in a consistent portion of benign endometriotic areas adjacent to ovarian cancers.⁹⁸ A clear increase in p53 overexpression from typical endometriosis to atypical endometriosis and then to cancer has been observed by Sáinz de la Cuesta and colleagues.⁹⁹

Conclusions

A number of published studies have demonstrated that the endometriotic tissue lining the internal surface of the cyst represents a peculiar site characterized by (1) an altered steroid hormone balance with a strong upregulation of ER- β mRNA compared to the eutopic counterpart; (2) a change in the expression of genes involved in prostaglandin and corticosteroid pathways, in cytoskeleton remodeling, and in the complement cascade compared to eutopic samples (Figure 1); (3) aberrant histone modifications and DNA methylation at specific genes involved in steroidogenesis; (4) an increase in major genetic alterations involved in inactivation of PTEN, p53 overexpression, and *ARID1A* mutation status. The pelvic environment around the cyst and the milieu inside the cyst are now recognized as significant factors influencing the phenotypic expression and the functional behavior of this tissue and further studies are eagerly needed in this context. A better characterization of the ectopic tissue might result in significant improvements in the management of endometriomas and in the prevention of endometriosis-associated ovarian cancer.

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