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ORIGINAL ARTICLE

Heterogeneity of estrogen receptor α and progesterone receptor distribution in lesions of deep infiltrating endometriosis of untreated women or during exposure to various hormonal treatments

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ABSTRACT

Deep infiltrating endometriosis (DIE) responds variably to hormonal therapy. Mutations in cancer driver genes have been identified in a fraction of the ectopic endometrial epithelial cells, suggesting a functional heterogeneity of these lesions. To evaluate the phenotype heterogeneity of cells in DIE, we measured the expression of estrogen receptor α (ERα) and of progesterone receptor (PR) in DIE of untreated women or under various treatments. We analyzed the luminal epithelial height (LEH), immunoreactive epithelial staining (IRS) and stromal staining intensity (SSI) of ERα and PR. We observed a high variability in the same gland, among distinct glands in the same sample and among distinct patients receiving the same treatment. LEH variability was primarily due to epithelial cells heterogeneity in a gland, secondarily to the glands randomly evaluated on the same section, and tertiary to the patient category. Variability in IRS and SSI scores was primarily the consequence of their heterogeneity in the same woman and to a lesser extent to variability among patients. LEH and SSI were not modified according to treatment. IRS for PR was lower in treated patients. This heterogeneity of ERα and PR distribution could explain why endocrine treatments are unable to cure this condition.

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Introduction

Endometriosis is characterized by the development of stromal and epithelial endometrial cells outside the uterine cavity [1]. This condition affects 6–10% of the female population of reproductive age [2]. We provided evidence that ovarian, peritoneal and deep infiltrating endometriosis (DIE) must be considered as three distinct entities of the same disease [3]. DIE is defined as the presence of endometriotic tissue 5 mm beneath the peritoneal surface [4]. It presents with histological characteristics different from peritoneal or ovarian endometriosis, and is locally invasive and surrounded by fibrosis including smooth muscle actin cells [5].

DIE has great clinical relevance as it is frequently associated with chronic pelvic pain (CPP) symptoms, dysmenorrhea, dyschezia, dyspareunia and infertility [6]. DIE lesions are considered to be benign inflammatory lesions but have cancer-like features such as local invasion and resistance to apoptosis [7]. They harbor somatic cancer driver mutations in 26% patients [7]. All mutations are confined to the epithelial compartment and present in only a fraction of clustered glandular epithelial cells. The coexistence of epithelial cells with variable genomic and phenotypic activities is also evident from studies evaluating a partial loss of BAF250a expression, used as a surrogate marker for ARID1A mutations. BAF250a negative epithelial cell clusters coexist with neighboring cell groups with homogeneous positivity for BAF250a. This clonal loss of BAF250a expression in epithelial cells is indicative of epithelial cells heterogeneity in DIE [8].

Estrogen receptor α (ERα) and progesterone receptor (PR) are present in the epithelium and stromal cell nuclei. They play essential roles in participating to the control of endometrial cells proliferation, differentiation and apoptosis. In normal endometrium, their expression is tightly controlled and finely tuned according to the day of cycle. In a given sample, their distribution is homogenous in glandular epithelial cells of the same gland and identical between epithelial cells of distinct glands in the endometrium [9–13]. Since only a fraction of endometriotic epithelial cells in DIE exhibit cancer driver gene mutations, we might anticipate that these genomic alterations are associated with the onset of phenotypic heterogeneity between epithelial cells of the same glands or between cells of distinct glands in the same patient. To better evaluate the phenotypic heterogeneity of cells in DIE, we evaluated the distribution of the two key regulatory proteins, ERα and PR, in DIE lesions of untreated women or during various types of hormonal treatments.

Material and methods

Patients

Patients with stage III–IV DIE [14] undergoing laparoscopy for CPP or infertility were included. The study was approved by the

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Supplemental data for this article can be accessed here.

Institutional Ethics Committee of the University Hospital of Liège (N° B70720083955). Written consent for use of pathology specimens and access to medical chart has been obtained from all patients.

Treatments

Eighteen patients did not receive any hormonal treatment for 3 months before surgery. Twenty-three patients were treated for 3 months at the time of surgery with either combined oral contraceptive (COC) ($n = 8$), progestins (PRO) ($n = 9$) or GnRH agonist (GnRHa) ($n = 6$) (Supplemental Table S1).

Immunohistochemistry

Tissues were fixed in 4% formalin for 24 h and embedded in paraffin. Six micrometers thick tissue sections were prepared, mounted on glass slides and dried overnight at 37 °C. The tissue slides were randomly selected from the entirely sectioned lesions in order to avoid selector bias. Sections were dewaxed in xylol, rehydrated through ethanol bath to deionized water and incubated in ethylenediaminetetraacetic acid buffer during 11 min at 126 °C at 1,4 Bar (DAKO S2367, Santa Clara, CA, USA). Cooled sections were then incubated 20 min at room temperature (RT) in H_2O_2 3% to quench endogenous peroxidase activity. To block non-specific binding, sections were incubated in Dako Protein Block (DAKO X0909) for 10 min at RT, and then overnight at 4 °C with anti-ERα antibody (ROCHE ventana 790–4325 Era-SP1) or 1 h at 4 °C with anti-PR antibody (ROCHE ventana 790–4296, Basel, Switzerland). The secondary antibody, Envision goat anti-rabbit HRP, was applied for 30 min at RT (DAKO K4003). Peroxidase activity was revealed by 3, 3′-diaminobenzidine tetrahydrochloride substrate (DAKO). The slides were counterstained with hematoxylin, dehydrated and coverslipped.

Evaluation of luminal epithelial height, immunoreactivity score and stromal staining intensity

After picture acquisition of the entire slide with a digital scanner (Nano Zoomer 2.0HT HAMAMATSU©), luminal epithelial height (LEH) was measured from the basal membrane to the apical surface in three randomly selected areas of an average of 20 glands per slide.

The immunoreactivity score (IRS) was measured by two independent reviewers blinded to the case as previously described [9]. The slides were divided into four groups depending on the percentage of positive stained nuclei (PP): group 1 (low) < 25%, group 2 (moderate) 25–50% stained, group 3 (high) 50–90% stained, group 4 (very high) > 90% stained. The intensity of staining (IS) was scored from 1 = low (+) to 4 = very high (++++). IRS was obtained by the formula PP (1–4) × IS (1–4). Finally, the stromal staining intensity (SSI) was evaluated as described above and slides were divided in four groups: 1 = low (+), 2 = mild (++), 3 = high (+++), and 4 = very high (++++).

Statistics

Results were expressed as mean and standard deviation (SD) for each variable. Experimental data were analyzed by linear mixed models with receptor and treatment as fixed factor and with patient, gland and area on gland (for LEH) as random factors. The same data were also used to compute variance components, hence assessing the relative proportions of the total variability due to area (LEH), gland and patient. Results were considered significant at the 5% critical level ($p < .05$). All calculations were performed with SAS (version 9.4 for Windows) statistical software.

Results

The distributions of IRS, SSI and LEH according to type of receptors (ERα, PR) and treatment (none, COC, PRO, and GnRHa) are displayed in Table 1. The effect of type of receptor and treatment was assessed by linear mixed modeling.

Effect of receptor and treatment

Overall, IRS scores were significantly higher in PR than in ERα stained lesions (9.38 ± 4.43 vs. 7.53 ± 3.92, $p < .0001$) and similarly for SSI values (2.12 ± 1.11 vs. 1.45 ± 0.89, $p < .0001$; Table 1). By contrast, luminal epithelial heights were comparable for both receptors (12.2 ± 5.92 vs. 12.5 ± 5.85, $p = .18$). Some columnar ERα positive cells coexisted in the same gland with flattened ERα negative cells. In general, the ERα positive cells were higher than the ERα negative cells (Figure 1). No significant difference was found between epithelial heights recorded in the four treatment groups, neither for ERα nor for PR ($p = .25$). For IRS, however, the linear mixed model evidenced a significant interaction between treatment and receptor ($p < .0001$) (Table 1). Specifically, although IRS scores were similar for each treatment group in ERα stained lesions, they differed markedly for PR stained lesions, being systematically lower in treated patients compared to non-treated ones. Mean values were 8.56 ± 3.97 for COC, 9.42 ± 4.87 for PRO and 7.50 ± 4.90 for GnRHa, respectively, while the mean score was 10.6 ± 4.90 for untreated patients. Findings were similar for SSI. A significant interaction was noted between treatment and receptor ($p = .0024$). As seen in Table 1, SSI values were systematically higher in PR than in the ERα stained lesions but treatment differences were not comparable for the two receptors. While the relative difference (%) in SSI scores for COC and GnRHa treated patients compared to untreated patients was negative and similar for ERα and PR receptors, respectively, −27% vs. −24% (COC) and −32% vs. −40%

Table 1. Characteristics of epithelial immunoreactivity score (IRS), stromal staining intensity (SSI), and luminal epithelial height (LEH), globally and according to type of receptors and treatment.

		ERα stained lesions		PR stained lesions	
Parameter	Treatment	*N*	Mean ± SD	*N*	Mean ± SD
IRS	Globally	871	7.53 ± 3.92	877	9.38 ± 4.43
	Untreated	331	7.10 ± 3.91	336	10.6 ± 3.99
	COC	255	7.82 ± 3.84	256	8.56 ± 3.97
	PRO	174	8.18 ± 4.06	174	9.42 ± 4.87
	GnRHa	111	7.14 ± 3.79	111	7.50 ± 4.90
SSI	Globally	867	1.45 ± 0.89	877	2.12 ± 1.11
	Untreated	327	1.55 ± 0.82	336	2.36 ± 1.07
	COC	255	1.13 ± 0.62	256	1.79 ± 0.90
	PRO	174	1.99 ± 0.91	174	2.56 ± 1.05
	GnRHa	111	1.06 ± 1.10	111	1.42 ± 1.20
LEH	Globally	2625	12.5 ± 5.85	2631	12.2 ± 5.92
	Untreated	1005	12.8 ± 6.13	1008	12.6 ± 5.91
	COC	765	12.9 ± 5.24	768	12.8 ± 5.85
	PRO	522	12.2 ± 6.21	522	11.9 ± 6.39
	GnRHa	333	10.7 ± 5.18	333	9.71 ± 4.52

(GnRHa), it was positive and notably higher for PRO in ERα compared to PR stained lesions (+28% vs. + 9%).

Heterogeneity

Variance component analysis (Table 2) revealed that 70% of the overall heterogeneity of IRS scores resulted from glands and 30% from patients for ERα stained lesions. The corresponding proportions for PR stained lesions were almost similar, 67% and 33%, respectively. When considering these proportions according to treatment, a much higher patient effect was noted for PRO, 54% (ERα) and 53.3% (PR), respectively. By contrast, the patient effect (9.1%) was much lower for COC but only in PR. For SSI, the greatest contribution to the total variability of results was attributed to the stroma surrounding the gland, respectively, 64.3% for ERα and 64.8% for PR, about one third resulting from patients. These proportions remained fairly stable when considering each treatment separately, except for COC in ERα stained lesions where the patient contribution was only 9.3%. As far as

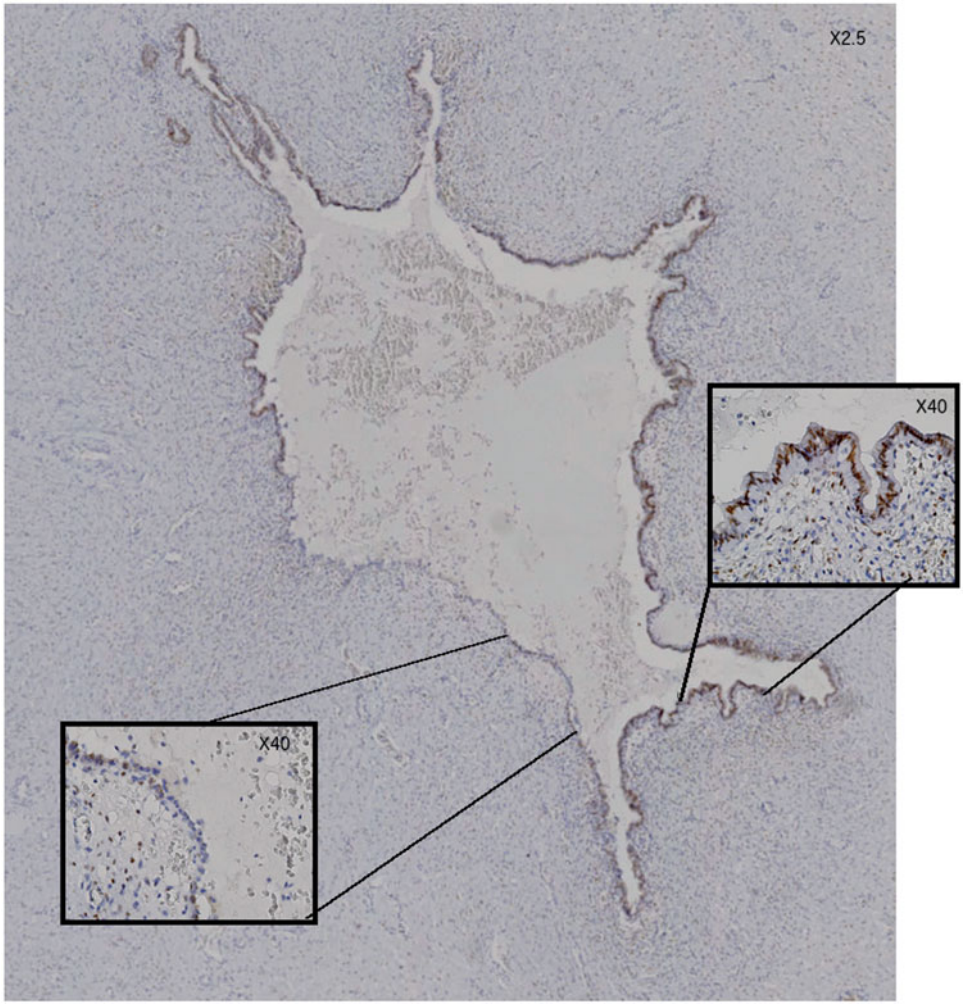

Figure 1. ERα immunostaining in the gland of an untreated patient. Original magnification ×2.5. Two zones are magnified to ×40. LEH was measured in 30 ERα positive cells (mean ± SD: 19.9 ± 4.4 µm) or negative cells (mean ± SD: 9.0 ± 4.3 µm).

Table 2. Variance components of epithelial immunoreactivity score (IRS), stromal staining intensity (SSI), and luminal epithelial height (LEH), globally and according to type of receptors and treatment.

		ERα					PR				
Parameter	Factor	All $N = 41$	None $N = 18$	COC $N = 8$	PRO $N = 9$	GnRHa $N = 6$	All $N = 41$	None $N = 18$	COC $N = 8$	PRO $N = 9$	GnRHa $N = 6$
IRS											
	Gland	70.0	72.5	72.9	46.0	81.9	67.0	65.4	90.9	46.6	73.3
	Patient	30.0	27.5	27.1	54.0	18.1	33.0	34.6	9.1	53.4	26.7
SSI											
	Stroma	64.3	74.3	90.7	55.7	73.6	64.8	68.1	73.6	69.8	79.5
	Patient	35.7	25.7	9.3	44.3	26.4	35.2	31.9	26.4	30.2	20.5
LEH											
	Area	51.3	52.7	52.6	37.9	69.4	44.5	47.0	48.7	33.4	48.3
	Gland	32.0	36.0	36.7	24.6	20.2	32.9	30.5	33.4	37.8	26.2
	Patient	16.7	11.3	10.8	37.5	10.4	22.6	22.5	17.9	28.8	25.5

Results are expressed in %.

LEH was concerned, 51.3% of the overall heterogeneity of data in ERα stained lesions resulted from repeated measurements within glands, while glands within patients and patients themselves added 32% and 16.7%, respectively. These percentages were more homogeneous for PRO and GnRHa treatments in both receptors stained lesions. For PR stained lesions, the corresponding proportions were 44.5% (areas), 32.9% (glands) and 22.6% (patients) but once more, for PRO treated patients, the profile was slightly different (33.4, 37.8, and 28.8%, respectively).

Discussion

This study analyzed LEH, IRS and SSI of ER and PR in DIE as markers of phenotypic heterogeneity. Salient findings can be underscored from this work.

For PR, IRS was significantly lower in patients treated than in non-treated subjects, whichever treatment considered (COC, PRO, or GnRHa). No treatment effect was observable on LEH. For SSI, no real differences were noted between treated and non-treated patients but a significant interaction effect was evidenced between treatment and receptor. As an illustration, patients treated with GnRHa compared to non-treated subjects had much lower SSI, IRS, and LEH values for PR than for ER.

The contribution of each factor analyzed to the variability of observations, regardless of the type of receptor and treatment received by the patient, has shown that the main source of variability is (a) the heterogeneity of epithelial height measured within the same gland, followed by (b) variability between glands of the same patient and finally (c) to a lesser extent by variability between patients. In other terms, the variation affecting a single individual patient result is primarily due to the heterogeneity of epithelial height in a gland, secondarily to the gland randomly evaluated on the same section, and tertiary to the patient category. Variability due to the patient can be partly explained by the treatment received. However, most of the heterogeneity between ER and PR is the consequence of distinct cellular expression in the glands or stroma of the same patient.

Estradiol (E2) and progesterone (P4) regulate many endometrial cell functions through ER and PR including epithelial and stromal cell proliferation, survival or apoptosis and secretory protein production. The distribution of ERα or PR in glands and stroma of functional endometrium varies according to the day of cycle but remains homogenously distributed between cells in glands or stroma of the same woman [10–13]. ERα and PR expression decrease significantly in the glandular epithelium of the functional layer from the proliferative to the late secretory phase.

Accordingly, a dynamic pattern of homogeneous expression of ERα and PR proteins is seen in both epithelial and stromal compartments of the functional layer of the eutopic endometrium.

We show here that treatment for at least 3 months with PRO, COCs, or GnRHa do not significantly alter neither the pattern nor the intensity of ERα IRS, but decrease PR IRS score. In the stromal compartment of the ectopic endometrium, SSI for PR or ERα is not modified by treatment (Table 1).

Estrogens increase to the same extent the height of the glandular epithelial endometrial cells in eutopic endometrium [15–17]. In contrast, important variations in height of the epithelial cells were measured between ectopic glands of individual women, either untreated or under treatment with PRO, COCs, or GnRHa (Table 1, Figure 1). This is primarily, the consequence of heterogeneity between cell height in the same gland, and to a lesser extent to differences between the epithelial heights measured in distinct glands of the same woman (Table 2). Finally, the type of treatment contributed only to about 20% to the variance of epithelial height.

Altogether our data point to a considerable heterogeneity of ERα and PR expression and of LEH in glands of individual women that are not considerably modified by treatment. No typical pattern of ERα or PR distribution could be delineated in treated or untreated women.

The lack of IRS variation in DIE somewhat resembles the feature of the basal endometrial layer. While the ERα and PR expression in the functional layer of endometrium varies according to the cycle and is modulated by PRO, COC, or GnRHa [18–20], their distribution and staining intensity do not vary in the basal endometrium throughout the cycle [9].

The persistent steroid receptors expression in PRO, COCs, or GnRHa treated groups indicates a differential local regulation of ERα and PR gene expression in DIE that varies from gland to gland in the same woman. It has been previously suggested that this pattern could be the consequence of locally increased conversion of androgenic precursors into estrogen by local aromatase [21]. The recent identification of cancer driver gene mutations could provide an alternative explanation for the cellular heterogeneity observed here and for the persistent steroid receptors expression in treated women [7].

The heterogeneity of the distribution of cancer driver gene mutations in epithelial cells and of ERα and PR distribution the cells of the same glands indicate considerable variability between epithelial cells activities in DIE lesions of the same woman. Such variability probably explains why endocrine treatments alone are unable to cure this condition.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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