Smooth muscles are frequent components of endometriotic lesions

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Deep infiltrating endometriosis (deeper than 5 mm under the peritoneum) often takes the form of a nodular lesion (or ‘adenomyotic nodule’) consisting of smooth muscles and fibrosis with active glands and scanty stroma. Thus, among endometriotic lesions, a certain distinction is drawn between musculo-glandular lesions and glandular lesions composed of endometrial-like epithelium surrounded by a cell-producing (cytogenous) stroma. The aim of this study was to detect by immunohistochemistry, with a monoclonal antibody against muscle-specific actin, the presence of smooth muscles in 54 endometriotic lesions originating from four different pelvic locations (peritoneum, ovary, rectovaginal septum and uterosacral ligaments) and to quantify the smooth muscle content. Smooth muscles were frequent components of endometriotic lesions in pelvic locations. In addition, smooth muscles were significantly (P < 0.001) more abundant in endometriotic lesions than in their respective unaffected sites. This finding supports, at least partially, the occurrence of a metaplastic phenomenon in the pathogenesis of endometriotic lesions. The definition of distinct endometriotic entities based on the difference in the tissue composition of the lesions (endometriotic nodules versus adenomyotic nodules) is inconsistent with the very frequent presence of smooth muscle cells in endometriosis irrespective of its localization.

Key words: adenomyosis/endometriosis/rectovaginal endometriotic nodule/smooth muscle metaplasia

Introduction

Endometriosis is histologically defined as the presence of endometrial-like glands and/or stroma outside the uterus. Deep infiltrating endometriosis is defined as the presence of endometriotic lesions more than 5 mm under the peritoneum (Cornillie et al., 1990) and is strongly associated with chronic pelvic pain. The uterosacral ligaments are one of the most frequent locations of deep endometriosis followed by the rectum and the bladder (Cornillie et al., 1990). Deep endometriosis is a nodular lesion which is histologically characterized by dense tissue composed of fibrous and smooth muscle cells with islands or strands of glands and stroma. The major component of the nodular lesion is not endometrial tissue but fibromuscular tissue with sparse finger-like extensions of glandular and stromal tissue. It is not surprising that, on descriptive grounds, the lesion was initially called adenomyosis. Recently it was proposed that peritoneal, ovarian and rectovaginal endometriosis represent separate entities with different pathogeneses (Nisolle and Donnez, 1997). Red peritoneal endometriosis represents early implantation of endometrial cells and stroma while typical black lesions and white opacifications represent distinctive steps in the evolutionary process of the disease. Ovarian endometriomas result from the metaplasia of the invaginated ovarian mesothelium into the ovarian cortex. Rectovaginal endometriosis must be considered as an endometiotic nodule whose histopathogenesis is related to the metaplasia of Müllarian remnants located in the rectovaginal septum (Nisolle and Donnez, 1997). Whether deep endometriosis actually represents adenomyosis or not, a certain distinction is drawn between musculo-glandular lesions (deep infiltrating endometriosis) and glandular lesions surrounded by a cytogenic stroma (peritoneal and ovarian endometriosis). In order to clarify this issue, we investigated the presence and the abundance of smooth muscles in 54 cases of endometriosis originating from four different pelvic locations and in unaffected tissues from the same pelvic locations.

Materials and methods

Fifty-four endometriotic lesions were obtained from 54 patients who underwent laparoscopy (n = 49) or laparotomy (n = 5) for sterility (n = 22), pelvic pain (n = 16; laparoscopy n = 11; laparotomy n = 5) or both conditions (n = 16). In five patients, laparotomy was performed for chronic pelvic pain because of previous major abdominal surgery (ileal resection: n = 3; total sigmoidectomy: n = 1; partial hepatectomy for traumatic partial hepatic rupture: n = 1). All patients were non-menopausal caucasian women. The mean age of the patients was 28 years old (range: 20–43). None of the patients had taken any hormonal treatment for at least 2 months before surgery. All the lesions were excised in toto, immediately fixed in 10% phosphate-buffered formalin for 12 h and then embedded in paraffin. Serial sections of 4 µm thick were cut and some were stained with haematoxylin and eosin; other sections were immunostained with the monoclonal antibody against muscle-specific actin.

Endometriotic lesions

Four different endometriotic lesions were studied: peritoneal endometriosis (n = 21), ovarian endometriomas (n = 13), uterosacral endometriotic nodules (n = 8) and rectovaginal septum endometriotic nodules (n = 12).

Peritoneal endometriosis

Fourteen lesions were typical black implants and seven were red lesions. The surface areas of the biopsies were between 0.5 and 1 cm². The peritoneal lesions were excised in toto with the help of
scissors or a CO₂ laser. The lower limit of the biopsies was the subperitoneal fat. In order to avoid sampling smooth muscles from an organ lying under the endometriotic lesion, no biopsies were from the uterine serosa, tubes or round ligaments.

**Ovarian endometriosis**

Ovarian endometriotic lesions were ovarian endometriotic cysts of >3 cm diameter (n = 9) and ovaries containing endometriomas of the same diameter (n = 3).

**Uterosacral and rectovaginal nodules**

Uterosacral and rectovaginal lesions were nodular lesions felt at physical examination and located deep in the uterosacral ligaments and rectovaginal septum. These lesions were excised by laparoscopy with a CO₂ laser until no residual induration was felt in the surrounding tissues.

**Controls**

Controls were obtained the eutopic endometrium (n = 10) of patients with and without laparoscopically proven endometriosis. In both groups, actin stainings were performed on endometrial tissues of the early proliferative (n = 2) and secretory (n = 2) phases, mid proliferative (n = 2) and secretory (n = 2) phases and premenstrual phase (n = 2). Controls were also performed on the normal pelvic peritoneum (n = 10), ovaries (n = 4) and uterosacral ligaments (n = 6). To investigate the normal rectovaginal septum, actin stainings were performed on the rectovaginal septum of three female fetuses of more than 30 weeks (pregnancy interruption for lethal fetal cytomegalovirus infection) and on the rectovaginal septum of pelvectomy specimens (n = 2).

**Immunohistochemistry**

After deparaffinization in xylene and rehydration through graded concentrations of alcohol, the endogenous peroxidase activity was blocked in 0.3% H₂O₂ in methanol for 30 min. Then normal serum was applied in order to minimize non-specific reactivities. The sections were then incubated at 4°C overnight with the specific monoclonal primary antibody against muscle-specific actin (clone HHF 35, dilution 1/50; Biogenex, San Ramon, CA, USA) able to recognize actin isoforms alpha and gamma of smooth muscle cells. This antibody does not recognize other muscle filament proteins and it is non-reactive for other mesenchymal or epithelial cells except myoepithelial cells. After rinsing with Tris-buffered saline (TBS), biotinylated anti-mouse immunoglobulin (IgG) was applied for 30 min at room temperature. After rinsing again with TBS, preformed avidin and biotinylated horseradish peroxidase macromolecular complex (Vectastain Elite ABC kit; Vector Laboratories, Inc., Burlingame, CA, USA) was applied for 30 min at room temperature. The antigen–antibody reaction was visualized using diaminobenzidine. The sections were then slightly counterstained with Mayer’s haematoxylin, dehydrated and coverslipped. Negative control sections were processed by omitting the primary antibody. Positive controls consisted in uterine leiomyomas and normal myometrium.

**Quantification of the smooth muscle content in endometriotic lesions and in unaffected tissues**

Using a microscope grid (at ×400) linked via a colour CCD video camera (MW-F15-e, Panasonic; Matsushita Electrical Industrial Co. Ltd, Osaka, Japan) to a large screen monitor (Panasonic Quintrix; Matsushita Electrical Co., Pentwyn, Cardiff, UK), we measured the smooth muscle content in the four different endometriotic locations and in their respective unaffected tissue by measuring the relative area occupied by smooth muscle cells (stained area/area occupied by unaffected tissue or by endometriotic lesion). Because the mean number of grid squares only partially filled (by actin positive tissue or by pathological tissue) did not exceed 0.9% per studied field (in the four different endometriotic locations for actin positive area evaluation and total lesion area evaluation as well as in the controls), only the entirely filled grid squares were counted. It must be emphasized that at ×400 high power field, the area of a myocyte is larger than that of a grid square, which means that partially filled squares represent less than the area of a smooth muscle cell. In order to perform precise measurements, the areas of the intersects on the grid were measured on the monitor screen and taken into account in the evaluation of the area of actin-positive tissues, endometriotic lesions and the controls. The relative area occupied by smooth muscles was measured in at least 10 non-overlapping randomly selected high power fields (×400) in each endometriotic lesion on each slide and in each biopsy of the unaffected same pelvic location. Results are expressed as percentages (mean ± SD) representing the relative areas occupied by smooth muscles in unaffected tissues and in endometriotic lesions.

A computerized stereological method was not used in this study because there was not enough contrast between the haematoxylin-stained tissue and the actin-stained tissue.

**Statistical analysis**

Comparison of the data was performed using the Student’s t-test (two-tailed). Statistical significance was defined as P < 0.001.

**Special stainings**

In order to differentiate smooth muscle cells from myofibroblasts, we performed silver stainings in the four different endometriotic locations. Silver stainings are able to reveal the external lamina basalis, composed of glycoproteins and collagen type 4, which is present in smooth muscle cells but not in myofibroblasts (Stevens and Lowe, 1997).

**Results**

**Controls**

Eutopic endometrium from endometria and non-endometriotic patients were negative (n = 10; 100%) for actin staining irrespective of the cycle phase (early proliferative and secretory phase, mid-proliferative and secretory phase or premenstrual period). Actin stainings were negative both in the non-endometriotic peritoneum (n = 12) and in the rectovaginal septum (n = 5). The smooth muscle content was estimated at 2 ± 1% in the normal ovary, essentially located in the ovarian stroma, and at 36 ± 5.2% in the normal uterosacral ligament. There was a significant difference (P < 0.001) in smooth muscle content between the normal uterosacral ligament and the ovary (Table I).

**Endometriotic lesions**

All endometriotic lesions (100%) were positive to various degrees for actin staining (Figures 1, 2 and 3). The smooth muscle content of all endometriotic lesions was significantly greater (P < 0.001) than in their respective unaffected pelvic locations. Comparisons between the different locations are illustrated in Table I. The smooth muscle content of typical black peritoneal lesions was higher than in red lesions but this difference was not found to be significant (Table II).
Table I. Smooth muscle (SM) content of controls and endometriotic lesions in the four different locations

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Lesions</th>
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<tr>
<td></td>
<td>P (n = 12)</td>
<td>OV (n = 7)</td>
</tr>
<tr>
<td>Actin +</td>
<td>0/12 (0)</td>
<td>7/7 (100)</td>
</tr>
<tr>
<td>SM content (%)</td>
<td>0 ± 0</td>
<td>2 ± 1</td>
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|                | PE (n = 21) | OVE (n = 13) | USLE (n = 8) | RVSE (n = 12) |
| Actin +        | 21/21 (100) | 13/13 (100)  | 8/8 (100)    | 12/12 (100)   |
| SM content (%) | 75.7 ± 5.7  | 23 ± 6      | 73.3 ± 10.6  | 78.7 ± 7.1    |

*Significantly different (P < 0.001) when compared with the unaffected pelvic location.

Values in parentheses are percentages.

Actin +: positive immunostaining with smooth muscle actin antibody.

SM content is expressed as mean percentage area of lesions occupied by smooth muscle cells.

P = peritoneum; OV = ovary; USL = uterosacral ligament; RVS = rectovaginal septum; PE = peritoneal endometriosis; OVE = ovarian endometriosis; USLE = uterosacral ligament endometriosis; RVSE = rectovaginal septum endometriosis.

Figure 1. Peritoneal endometriosis: presence of smooth muscle cells (stained brown) at the periphery of the stroma. Muscle-specific actin immunohistochemistry. Original magnification ×100.

Figure 2. Section through the wall of an ovarian endometriotic cyst. Presence of smooth muscle cells (stained brown) in the deep layers of the stroma and at the periphery of the stroma. Muscle-specific actin immunohistochemistry. Original magnification ×400.

Figure 3. Section through a rectovaginal endometriotic nodule. Presence of abundant smooth muscle cells around the lesion. Muscle-specific actin immunohistochemistry. Original magnification ×10.

Table II. Comparison of the smooth muscle (SM) content of red and black peritoneal lesions

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 12)</th>
<th>Red lesions (n = 7)</th>
<th>Black lesions (n = 14)</th>
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<tbody>
<tr>
<td>SM content (%)</td>
<td>0</td>
<td>73.4 ± 5.5</td>
<td>78 ± 5.9*</td>
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</table>

*Not significant when compared with the smooth muscle content of red lesions.

Special stainings

Silver stainings showed the presence of the external lamina basalis in the peristromal actin positive areas in red and black peritoneal endometriosis, ovarian endometriomas, uterosacral endometriotic nodules and in rectovaginal endometriotic nodules.
Discussion
This is the first study on pelvic endometriosis with a monoclonal antibody against smooth muscle-specific actin. One of the most intriguing findings of this study is the presence of smooth muscles in peritoneal lesions and their absence in the unaffected peritoneum and in the eutopic endometrium of women with and without pelvic endometriosis. This absence of muscular tissue in the unaffected peritoneum and in the eutopic endometrium suggests a metaplastic phenomenon. This metaplastic phenomenon refers to the induction theory (Levander, 1955; reviewed by Ridley, 1968, and van der Linden, 1996). It has been demonstrated that smooth muscle metaplasia can occur in the peritoneum. According to some authors, metaplasia of the subcoelomic mesenchyme in the direction of smooth muscles underlies the pathogenesis of leiomyomatosis peritonealis disseminata (Fujii et al., 1980, 1981; Parmley et al., 1987). The term ‘secondary Müllerian system’ has been applied to the pelvic and lower abdomen mesothelium and underlying mesenchyme of females, on the basis of its close embryological relationship with the Müllerian ducts (Lauchlan, 1972). The potentiality of this tissue is manifested by the existence in the peritoneal cavity and most often in the pelvic region of a large variety of metaplastic and neoplastic lesions that are analogous in all regards to those more commonly found in the ovary, uterus, or other organs of the female genital tract (Thor et al., 1991). In Rosai’s classification of peritoneal lesions, endometriosis, endosalpingiosis, endocervicosis, ectopic decidual reaction and leiomyomatosis peritonealis disseminata are considered as metaplastic lesions of the secondary Müllerian system (Rosai, 1996). The smooth muscle component of endometriosis may thus result from the totipotential capacity of the secondary Müllerian system to differentiate not only into endometrial glands and stroma but also into smooth muscles.

Another possibility is that peristromal smooth muscles result from the differentiation of the stromal cells into smooth muscle cells. Red peritoneal lesions are often considered as early implantation of endometrial tissue while typical black lesions and white opacifications represent distinctive steps in the evolutionary process of the disease (Nisolle and Donnez, 1997). If this is correct, we would expect to find some smooth muscle differentiation in the eutopic endometrium of women with endometriosis, at least in the premenstrual tissue. However, actin stainings performed in the eutopic endometrium were all negative. In fact, smooth muscle metaplasia of the endometrial stroma is a rare event. Indeed, in the eutopic endometrium, smooth muscle metaplasia occasionally represent small foci of typical benign smooth muscles within the endometrium called ‘intra-endometrial leiomyomatosis’. In addition, only 1–2% of endometrial polyps show the presence of smooth muscle in their stroma (Silverberg and Kurman, 1991).

According to Leyendecker, endometriosis is a disease of the endometrial–subendometrial unit also called archimetra (Leyendecker, 1998). This model postulates that endometriosis results from hyperperistalsis, dysperistalsis of the archimetra and increased intruterine pressure causing increased transabdominal transport. In agreement with Leyendecker’s model but also Sampson’s transplantation theory (Sampson, 1927), the absence of actin positive cells in the eutopic endometrium of patients with and without laparoscopically proven endometriosis and its very frequent presence in endometriotic lesions suggests that regurgitated endometrium undergoes some smooth muscle metaplasia under the influence of peritoneal fluid or that implanted endometrium causes a metaplastic response in the underlying tissue. There are numerous arguments to consider that the endometrium of patients with endometriosis differs from that of disease-free patients. These range from gross morphological changes such as the polyoid appearance of the endometrium at hysterosalpingography (McBean et al., 1996), to changes in immunocytochemistry such as aberrant integrin expression (Lessey et al., 1994), and biochemical changes such as overexpression of plasminogen activator receptor (Sillem et al., 1997) and abnormal endothelial, epithelial and stromal proliferation (Wingfield et al., 1995). P.450 aromatase, which converts androgens into oestrone and oestradiol, is not expressed in the endometrium of patients without endometriosis (Prefontaine et al., 1990; Noble et al., 1996; Kitawaki et al., 1997) but its expression has been demonstrated in the eutopic endometrium of patients with endometriosis and in the stroma of endometriotic lesions (Yamamoto et al., 1993; Noble et al., 1996, 1997; Kitawaki et al., 1997). This may result in an increased local concentration of oestrogens in the endometriotic lesion. Although actin positive cells were absent in the endometrium of patients with endometriosis in this study, such differences between the endometrium of patients with endometriosis and disease-free patients may result in microenvironmental conditions that could favour smooth muscle metaplasia within the regurgitated tissue or within the underlying tissue.

Among these differences, angiogenic factors and in particular vascular endothelial growth factor (VEGF) seem to play an important role in the evolution of the lesion after the first stage of implantation (Donnez et al., 1998; Healy et al., 1998). It has been shown that the VEGF content is higher in red peritoneal lesions than in black peritoneal lesions. Lower VEGF concentrations in black lesions may explain the decrease in both stromal and epithelial vascularization, followed by fibrosis and inactivation of the implant (Donnez et al., 1998). The smooth muscle content of peritoneal lesions does not seem to be correlated with the age of the lesion. Indeed, as we have shown here, black lesions contained a similar amount of muscle to red lesions. Nevertheless, it has been demonstrated that the amount of fibrotic tissue was more important in the stroma of black than in red lesions (Matsuzaki et al., 1999).

Interestingly, a recent immunohistological study on pleuro-pulmonary endometriosis and pulmonary ectopic deciduosis also showed the presence of smooth muscle actin positive cells in most stromal cells of all endometriotic and ectopic decidual lesions (Flieder et al., 1998). It is postulated that viable endometrial tissue regurgitated through the Fallopian tubes enters the thorax through right diaphragmatic fenestrations (Sampson, 1927). However, it must also be emphasized that the pleural and peritoneal cavity are both of mesodermal embryologic origin and that factors able to induce metaplasia could also pass through these diaphragmatic defects and
induce plural endometriosis by means of coelomic metaplasia (Gruenwald, 1942).

Ovarian, uterosacral and rectovaginal lesions also contain significantly more smooth muscle cells than their respective unaffected sites \( (P < 0.001) \). In the normal ovary, the actin-stained cells are essentially located in the stroma while those of the endometriotic cysts are situated in the cortex (Figure 2). This observation is consistent with the hypothesis that ovarian endometriomas result from the metaplasia of the invaginated ovarian mesothelium into the ovarian cortex (Nisolle and Donnez, 1997).

Uterosacral ligament and rectovaginal endometriotic nodules are considered as myoproliferative lesions (Brosens, 1994). Both these lesions are very similar: they are nodular and essentially composed of a similar amount of smooth muscle.

In addition to the above-mentioned smooth muscle metaplasia mechanisms, we cannot exclude that hyperplasia of the pre-existing smooth muscle cells occurs when endometriosis is located within or near abundant smooth muscle layers. Although there were no actin positive cells in the unaffected rectovaginal septum, this virtual space separates two important muscular zones: the anterior rectal muscularis and the posterior vaginal wall. Some authors found the presence of abundant smooth muscle cells in this area (Donnez et al., 1995). However they did not perform anti-actin immunohistochemistry. We believe their finding can be explained by the taking of very deep biopsies involving partly the anterior rectal or posterior vaginal muscularis (Donnez et al., 1995). Concerning the significant difference between the smooth muscle content of ovarian endometriomas and uterosacral or rectovaginal endometriosis, there is no obvious explanation. Both deep infiltrating endometriosis and ovarian endometriotic cysts have escaped from the predominant influence of the peritoneal fluid. However, deep infiltrating lesions are mainly influenced by plasma sex steroid hormone concentrations whereas ovarian cysts will probably be influenced by the ovarian microenvironment with much higher steroid concentrations.

The growth of endometriotic lesions is not only under the control of sex steroids. Paracrine, autocrine and microenvironmental factors must also be considered. Further studies will be necessary to determine which microenvironmental factors – cytokines, angiogenic factors, growth factors and specific proteins – promote or inhibit the development of endometriotic lesions and their respective components.

References


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