High density of small nerve fibres in the functional layer of the endometrium in women with endometriosis

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BACKGROUND: Endometriosis is a common gynaecological disease and is frequently associated with recurrent and serious pelvic pain such as dysmenorrhoea and dyspareunia, but the mechanisms by which these symptoms are generated are not well understood. METHODS: Histological sections of endometrial tissue were prepared from endometrial curettings and hysterectomies performed on women with endometriosis (n=25 and n=10, respectively) and without endometriosis (n=47 and n=35, respectively). These were stained immunohistochemically for the highly specific polyclonal rabbit anti-protein gene product 9.5 (PGP9.5) and monoclonal mouse anti-neurofilament protein (NF) to demonstrate both myelinated and unmyelinated nerve fibres. RESULTS: Small nerve fibres were identified throughout the basal and functional layers of the endometrium in all endometriosis patients, but were not seen in the functional layer of the endometrium in any of the women without endometriosis (P<0.001). NF-immunoreactive nerve fibres were present in the basal layer in all endometriosis patients but not in non-endometriosis patients, with one exception (P<0.001). CONCLUSIONS: Small nerve fibres detected in the functional layer in all women with endometriosis may have important implications for understanding the generation of pain in these patients. The presence of nerve fibres in an endometrial biopsy may be a novel surrogate marker of clinical endometriosis.

Key words: endometriosis/endometrium/immunohistochemistry/nerve fibres/pain

Introduction

Endometriosis, defined by the presence of focal endometrium-like tissue in sites outside the uterus (Binkovitz et al., 1991), is a gynaecological condition which is being increasingly recognized in most societies, especially in developed countries. Frequently it is associated with recurrent and disabling symptoms, primarily pelvic pain, yet little is understood about the mechanisms by which symptoms are generated (Anaf et al., 2000). Investigators have studied the presence of nerve fibres in endometriotic plaques and, although there is some evidence for the ingrowth of small nerve fibres into endometriotic plaques (Tamburro et al., 2003; Berkley et al., 2004), no neurofilament protein (NF)-immunoreactive nerve fibres could be identified in the capsule of ovarian endometriomas (Al-Frozan et al., 2004).

The multiplicity of molecular and cellular abnormalities demonstrated in the eutopic endometrium of women with endometriosis (Lessey et al., 1994; Ota and Tanaka, 1997; Healy et al., 1998) suggests that primary abnormalities in this tissue may have a role in the genesis of endometriosis and perhaps even in the genesis of symptoms. We speculated that this might include local growth factors or cytokines for nerve fibres which, if also present in the ectopic endometrial-type tissue of patients with endometriosis, could induce the growth of nerve fibres into the endometriotic foci and thus provide a mechanism for lesion-specific pain. We therefore studied the presence of nerve fibres by immunohistochemistry in the basal and functional layers of the endometrium from women with and without visually and biopsy-proven endometriosis using highly specific markers polyclonal rabbit anti-protein gene product 9.5 (PGP9.5) (Lundberg et al., 1988) and NF (Schlaepfer, 1987) for both myelinated and unmyelinated nerve fibres. PGP9.5 is a highly specific pan-neuronal marker and it stains Aα, Aβ, Aγ, Aδ, B and C fibres. NF is a highly specific marker for myelinated nerve fibres. Aδ fibres are small myelinated fibres and transmit sharp, pricking localized pain to the central nervous system. C fibres are small unmyelinated fibres and transmit dull, aching, burning poorly localized pain.

Materials and methods

Collection of tissue

This study was approved by the Human Ethics Committees of the Central Sydney Area Health Service and the University of Sydney, and all women gave their informed consent for participation. Endometrial tissue (uterine curettage) was collected from 25 women with endometriosis (mean age, 27.5 years; range, 20–35) and
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47 women without endometriosis (mean age 32.7 years; range, 28–38) who underwent laparoscopy combined with hysteroscopy and diagnostic curettage. The endometriosis patients all complained of dysmenorrhea and a range of related pain symptoms. The 47 endometrial curette tissue samples collected from women without laparoscopic evidence of endometriosis were from patients undergoing tubal sterilization, assessment prior to tubal reanastomosis or investigation of infertility. Full-thickness uterine blocks, which included the endometrium and myometrium, were selected from 10 women with endometriosis (mean age 44.0 years; range, 42–46) and 35 women without endometriosis (mean age 46.0 years; range, 38–54) who had undergone hysterectomy. The indications for hysterectomy in the 35 women without endometriosis included multiple small uterine fibroids, uterine prolapse, abdominal adhesions or abnormally heavy menstrual bleeding.

Laparoscopic biopsies were collected from suspicious areas on the peritoneum, and curetage samples of the functional layer of the endometrium were carefully collected in single long strips allowing orientation during paraffin embedding. The basal layer of the endometrium was defined as 300μm above the endometrial–myometrial interface (±300μm) (Gambino et al., 2002) and the functional layer of the endometrium was defined as the area from the epithelial surface to the basal layer. The basal and functional layers were further divided into two sublayers, namely superficial and deeper portions.

The severity of pain was not assessed systematically and prospectively in this preliminary observational study, but detailed clinical information was recorded in a standard format. All endometriosis patients were staged according to the revised American Fertility Society (AFS) score (American Society for Reproductive Medicine, 1996), which ranged from 1 to 1IV. Eighty-six percent of the endometriosis subjects had peritoneal endometriosis alone, while the remainder had ovarian endometriosis with or without endometriosis in other sites.

Menstrual cycle staging was determined on all samples of endometrium, by a single gynaecological histopathologist (P.R.), and all phases of the cycle were represented: menstrual (days 1–7 of the cycle), proliferative (days 8–14) and secretory (days 15–28) phases of a normalized 28 day cycle. Of the 35 women with endometriosis, four were assessed as being in the menstrual phase, 11 were in the proliferative phase and 20 were in the secretory phase, whereas of the 82 women without endometriosis, 12 were assessed as being in the menstrual phase, 36 were in the proliferative phase and 34 were in the secretory phase of the cycle. None of the women had received medical therapy for endometriosis in the 3 months prior to endometrial or hysterectomy sampling.

**Immunohistochemistry**

After resection, the specimens were immediately fixed in 10% neutral buffered formalin for 18–24h, processed and embedded in paraffin wax according to a standard protocol. Each section was cut at 4μm and routinely stained with haematoxylin and eosin (H&E). Antigen retrieval techniques for PGP9.5 and NF were used. Serial sections, also cut at 4μm, were immunostained using an antibody for polyclonal rabbit anti-PGP9.5 (dilution 1:700; Dako Cytomation, Sydney, Australia), a highly specific pan-neuronal marker (Lundberg et al., 1988), and monoclonal mouse anti-human NF (dilution 1:200; Dako Cytomation), a highly specific marker for myelinated nerve fibres (Schlaepfer, 1987), for 30min at room temperature. Sections were washed in Tris buffer, incubated with Envision-labelled polymer-AP mouse/rabbit for 30min (Dako Cytomation) and stained with permanent fast red chromogen (Dako Cytomation) for 10 min. All immunostaining was carried out on a Dako Autostainer Model S3400 (Dako Cytomation Inc., Carpinteria, CA). Images of the sections were captured using an Olympus BX51 digital camera (Olympus, Tokyo, Japan). We used normal skin as a positive control as it reliably contains myelinated and unmyelinated nerve fibres expressing both PGP9.5 and NF. Rabbit immunoglobulin fraction was used as a negative control, the concentration being matched with the concentration of the PGP9.5 and NF antibodies.

**Statistical analysis**

The images were captured by using an Olympus BX51 digital camera, and an assessment of nerve fibre density was performed by Image Pro Plus Discovery (MediaCybernetics, Silver Springs, MD). Once the features of the images were controlled, an orthogonal grid mask was sketched above the original images. The sections of the grid were 250μm a side. Once the grid was in position, nerve fibres in the endometrium and myometrium, and the total number of squares covering the sections of endometrium and myometrium were counted. The total number of nerve fibres was divided by the total number of squares covering the endometrium to obtain an average of nerve fibres per square (each square of 250×250μm). The results were expressed as the mean (±SD) number of nerve fibres per mm² in each specimen from all endometrial and myometrial samples, stained with the polyclonal antibody against PGP9.5 and monoclonal antibody against NF. Nerve fibre density between eutopic endometrium from endometriosis patients and normal women was compared using the Mann–Whitney U-test. Differences were considered to be significant at P<0.05.

**Results**

Multiple small unmyelinated nerve fibres stained for PGP9.5 were present in the functional layer of the endometrium in all endometriosis patients (Figure 1A). Most of the nerve fibres were in the deeper portion of the functional layer; however, some nerve fibres in the superficial portion were very close (50–180μm) to the endometrial epithelial surface and they often accompanied blood vessels and endometrial glands. In patients without endometriosis, no nerve fibres were detected in the functional layer of the endometrium (Figure 1B; Table I; P<0.001).

There were also NF-immunoreactive myelinated nerve fibres in the deeper portion of the basal endometrium in all endometriosis patients, while in patients without endometriosis no myelinated nerve fibres were detected within the basal layer of the endometrium, with one exception (Table II; P<0.001). This exceptional patient did not have endometriosis, but she had pelvic pain, menorrhagia, uterine fibroids and chronic inflammation in the cervix. No NF-immunoreactive myelinated nerve fibres were detected in the functional layer of the endometrium in patients with or without endometriosis.

There were many more small unmyelinated nerve fibres present throughout the basal layer (both deeper and superficial) of the endometrium in women with endometriosis (Figure 1C) than without endometriosis. Occasionally, small unmyelinated nerve fibres were noted in women without endometriosis but only in the deeper portion of the basal layer (four women out of 35; Figure 1D; Table II; P<0.001). In women with endometriosis, these nerve fibres often formed obvious thick trunks in the basal layer or at the endometrial–myometrial interface (Figure 1C). These trunks were never seen in women without endometriosis. Thirty-one women without endometriosis showed no nerve fibres throughout the basal layer of the endometrium. All four women without endometriosis who showed nerve fibres in the deeper portion of the basal endometrial layer had uterine fibroids.

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Small and large nerve fibres including thick trunks stained with PGP9.5 were present in myometrium in women with and without endometriosis. There were more nerve fibres stained with PGP9.5 in the myometrium in women with endometriosis than in women without (Table III; \( P < 0.001 \)). A small number of nerve fibres stained with NF was seen throughout the myometrium of all women with endometriosis (Table III), but only 16 out of 35 women (46%) without endometriosis showed a few nerve fibres stained with NF throughout in the myometrium (\( P > 0.097 \)).

There appeared to be a difference in the patterns of PGP 9.5 in the endometrium at different stages of the menstrual cycle. There was a greater density of nerve fibres in the functional and basal layers of the endometrium in the secretory phase than either the menstrual or proliferative phases. The distribution of nerve fibres was reasonably uniform throughout the functional layer and basal endometrial layers in women with endometriosis (Figure 1).

There was also a greater density of nerve fibres stained with PGP9.5 in the functional and basal layers of the endometrium than the myometrium in women with endometriosis (Tables I–III; \( P < 0.001 \)).

Discussion
This study has demonstrated multiple small unmyelinated nerve fibres in the functional layer of the endometrium in 35 women with confirmed endometriosis but in none of 82 women without endometriosis. There were also many more nerve fibres in the basal layer of the endometrium in women with endometriosis compared with women with no endometriosis. Some nerve fibres in the basal layer of the endometrium in women with endometriosis were NF immunoreactive and myelinated, but no NF-immunoreactive nerve fibres were present in the functional layer of the endometrium in women with or without endometriosis.

Table 1. Small unmyelinated nerve fibre density in the functional layer of endometrium (per mm\(^2\), mean±SD) in women with and without endometriosis

<table>
<thead>
<tr>
<th>Stages</th>
<th>n</th>
<th>Mean±SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hysterectomy specimens: endometriosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Menstrual</td>
<td>1</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Proliferative</td>
<td>2</td>
<td>6</td>
<td>5–6</td>
</tr>
<tr>
<td>Secretory</td>
<td>7</td>
<td>15±5</td>
<td>9–23</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>11±5</td>
<td></td>
</tr>
<tr>
<td>Hysterectomy specimens: no endometriosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Menstrual</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Proliferative</td>
<td>16</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Secretory</td>
<td>14</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>35</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Curettage specimens: endometriosis</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Menstrual</td>
<td>3</td>
<td>7±1</td>
<td>6–8</td>
</tr>
<tr>
<td>Proliferative</td>
<td>9</td>
<td>5±1</td>
<td>3–6</td>
</tr>
<tr>
<td>Secretory</td>
<td>13</td>
<td>13±6</td>
<td>7–30</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>10±7</td>
<td></td>
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<td>Curettage specimens: no endometriosis</td>
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<tr>
<td>Menstrual</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Proliferative</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Secretory</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>47</td>
<td>0</td>
<td>0</td>
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</table>

Nerve fibres were stained with PGP9.5. \( P < 0.001 \) for endometriosis versus no endometriosis.

Figure 1. Nerve fibres in the functional and basal layers of the endometrium from woman with and without endometriosis. (A) Endometrium from the functional layer of a woman with biopsy-confirmed endometriosis stained for PGP9.5 (magnification×400). Arrows denote multiple small nerve fibres in the functional layer, several within 100\( \mu \)m of the epithelial surface. (B) Endometrium from the functional layer of a woman with no visible endometriosis stained for PGP9.5 (magnification×400). No nerve fibres could be identified. (C) Myometrial–endometrial interface from a woman with biopsy-confirmed endometriosis stained for PGP9.5 (magnification×200) showing thick nerve fibre trunks stained red. (D) Myometrial–endometrial interface from a woman with no visible endometriosis and with a small nerve fibre in the deeper portion of the basal layer stained with PGP9.5 (magnification×200). (E) Myometrial–endometrial interface from a woman with biopsy-confirmed endometriosis stained for NF (magnification×400) showing several nerve fibres stained red. Scale bars represent 100\( \mu \)m in (A), (B) and (E); and 200\( \mu \)m in (C) and (D).
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Table I. Small unmyelinated and large myelinated nerve fibre densities in the basal layer of endometrium (per mm², mean±SD) in women with and without endometriosis

<table>
<thead>
<tr>
<th></th>
<th>Endometriosis</th>
<th>No endometriosis</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>PGP9.5</td>
<td>NF</td>
</tr>
<tr>
<td>Menstrual</td>
<td>n 12</td>
<td>Mean±SD 12</td>
</tr>
<tr>
<td>Proliferative</td>
<td>n 2</td>
<td>Mean±SD 16</td>
</tr>
<tr>
<td>Secretory</td>
<td>n 7</td>
<td>Mean±SD 11–80</td>
</tr>
<tr>
<td>Total</td>
<td>n 10</td>
<td>Mean±SD 18±8</td>
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</tbody>
</table>

Unmyelinated and myelinated nerve fibres were stained with PGP9.5; myelinated nerve fibres were stained with NF. P<0.001 for endometriosis versus no endometriosis.

Table III. Small unmyelinated and large myelinated nerve fibre densities in myometrium (per mm², mean±SD) in women with and without endometriosis

<table>
<thead>
<tr>
<th></th>
<th>Endometriosis</th>
<th>No endometriosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PGP9.5</td>
<td>NF</td>
</tr>
<tr>
<td>Menstrual</td>
<td>n 1</td>
<td>Mean±SD 2.28</td>
</tr>
<tr>
<td>Proliferative</td>
<td>n 2</td>
<td>Mean±SD 2.62</td>
</tr>
<tr>
<td>Secretory</td>
<td>n 7</td>
<td>Mean±SD 3.58±1.36</td>
</tr>
<tr>
<td>Total</td>
<td>n 10</td>
<td>Mean±SD 3.26±1.23</td>
</tr>
</tbody>
</table>

Unmyelinated and myelinated nerve fibres were stained with PGP9.5; myelinated nerve fibres were stained with NF. P<0.001 for PGP9.5; P>0.097 for NF.

It has been known for some time that the myometrium, the endometrial–myometrial interface and the deeper portion of the basal endometrium can be innervated by nerve fibres, but that nerve fibres are absent from the superficial two-thirds of the endometrium (the functional layer) in the normal human uterus (Coupland, 1969). The function of these nerve fibres in normal basal endometrium is not well understood. However, some acetylcholinesterase (AChE)-immunoreactive nerve fibres were detected in the basal layer of normal human endometrium (Coupland, 1969). Neuropeptide-Y (NPY), substance-P (SP), vasoactive intestinal peptide- (VIP) and tyrosine hydroxylase (TH)-immunoreactive nerve fibres were also present in normal human endometrium (Heinrich et al., 1986). Yet little is known about the functions of these nerve fibres in human endometrium.

Some researchers have studied nerve fibres in normal endometrium in animals. Calcitonin gene-related peptide (CGRP) (Shew et al., 1990), AChE- and NPY-immunoreactive nerve fibres were detected in rat endometrium (Papka et al., 1985) and many CGRP-immunoreactive nerve fibres were near the surface epithelium (Shew et al., 1990). SP and CGRP were also localized in myometrial nerves in a rat model (Shew et al., 1990).

A variety of clinical conditions including endometriosis, pelvic inflammatory disease, uterine fibroids, adhesions and uterine contractions can cause pelvic pain. Various investigators have demonstrated nerve fibres in endometriotic plaques, abdominal adhesions and myometrium (Tulandi et al., 1998; Anaf et al., 2000; Sulaiman et al., 2001; Tamburro et al., 2003; Berkley et al., 2004). Endometriotic plaques in a rat model were innervated by SP, CGRP (sensory C and Aδ fibres) and vesicular monoamine transporter (VMAT) fibres (Berkley et al., 2004). Nerve fibres were present in human peritoneal endometriotic plaques, and transforming growth factor (TGF-β1) was expressed in the nerve fibres only in red and white lesions (Tamburro et al., 2003). Rectovaginal endometriotic nodules from women with severe dysmenorrhea and deep dyspareunia showed the presence of nerve fibres staining with the non-specific antibody, S-100 protein (Anaf et al., 2000). Human peritoneal adhesions contained synaptophysin- CGRP-, SP-, vasoactive intestinal peptide- (VIP)- and tyrosine hydroxylase (TH)-immunoreactive nerve fibres (Sulaiman et al., 2001) and 78% of endometriosis-related adhesions showed NF-immunoreactive nerve fibres (Tulandi et al., 1998).

SP- and CGRP-immunoreactive nerve fibres were present in human myometrium (Samuelson et al., 1985) and SP-induced contractions were inhibited by CGRP. It has been reported that women with endometriosis had uterine contractions with higher frequency, amplitude and basal pressure tone during menses (Bulletti et al., 2002); therefore, SP- and CGRP-immunoreactive nerve fibres may play a role in the pain of women with endometriosis.

The pathogenesis of endometriosis is not well understood, but there are numerous theories, including implantation of endometrial fragments transported via the Fallopian tubes by retrograde menstruation (Sampson, 1927, 1940), mechanical transplantation (Allen et al., 1954; Levander and Normann, 1955), lymphatic and vascular metastasis (Halban, 1924; Jubanyik and Comite, 1997), direct extension or invasion (Cullen, 1908), coelomic metaplasia (Ridley, 1968, Vasquez, 1984), embryonic rests (Von Recklinghausen, 1896; Russell, 1899) and a composite theory (Javert, 1949; Nisolle and Donnez, 2000; Sulaiman et al., 2004).
1997). Most researchers support variations of the implantation theory, which postulates that peritoneal endometriotic lesions result from the reflux of viable endometrial tissue fragments or cells through the Fallopian tubes which then implant and grow on the peritoneal surface and pelvic organs.

If the implantation theory is true, the nerve fibres in endometriotic plaques may originate from nerve fibre progenitors in the functional layer of the endometrium or, more probably, from ingrowth of local nerve fibres due to the secretion of nerve growth factors (NGFs) and increased expression of two NGF receptors, namely Trk-A and p75, by the implanting endometrium. It is not yet clear what neural stimuli may initiate the severe pain sensations which some women experience with endometriosis. However, a number of molecules that are capable of stimulating nerve fibres, such as tumour necrosis factor-α (TNF-α) (Bergqvist et al., 2001), prostaglandin E2 (Ylikorkala and Viinikka, 1983), prostaglandin E2 and F2α (De Leon et al., 1988), and NGF (Anaf et al., 2002) can be released from endometriotic plaques. It seems highly probable that the endometrium of women destined to develop endometriosis is functionally abnormal (Lessey et al., 1994; Ota and Tanaka, 1997; Healy et al., 1998). It may also be programmed to secrete large amounts of nerve trophic factors, resulting in the remarkable ingrowth of unmyelinated small nerve fibres demonstrated in this study. This may also be translated into ingrowth of nerve fibres into new endometriotic plaques on the peritoneum or ovary. These nerve fibres may have an important role in the mediation of pain symptoms. No other satisfactory theory has yet been advanced for the genesis of pain symptoms in women with endometriosis.

This demonstration of small nerve fibres in the functional layer of eutopic endometrium of women with endometriosis is so striking in the present study that we believe it could become a relatively simple surrogate marker of this condition using endometriotic biopsies. It is even possible that an endometrial biopsy may demonstrate nerve fibres in women destined to develop the condition later in life. Much further research is required to define possible roles and mechanisms of these nerve fibres in endometriosis, as well as defining the presence of uterine nerve fibres in other gynaecological conditions.

Acknowledgements
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