Nerve fibres in ovarian endometriotic lesions in women with ovarian endometriosis

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BACKGROUND: Although nerve fibres are present in eutopic and ectopic endometrium, it is unclear whether they appear in ovarian endometriotic lesions. We investigated the presence of nerve fibres in ovarian endometriotic lesions and its correlation with clinical parameters in women with ovarian endometriosis.

METHODS: Histological sections of ovarian endometriotic lesions from 61 women with ovarian endometriosis (Stages II–IV) who underwent laparoscopic endometrioma cystectomy were stained immunohistochemically using a specific polyclonal rabbit anti-protein gene product 9.5 (PGP9.5) antibody to demonstrate myelinated and unmyelinated nerve fibres.

RESULTS: Nerve fibres stained with PGP9.5 were detected in ovarian endometriotic lesions in 31.1% of women, and most appeared in fibrotic interstitium of ovarian endometriotic lesions. The density of PGP9.5-immunoactive fibres in ovarian endometriotic lesions in women with pain symptoms (n = 35) was higher than in women with no pain symptoms (n = 26, P = 0.039), although the percentage (positive cases/total) of PGP9.5-positive fibres did not differ. In women with pain symptoms, PGP9.5-positive fibres appeared in 40.0% of cases and the density of PGP9.5-immunoactive fibres in ovarian endometriotic lesions was correlated with severity of pain symptoms (r = 0.466, P = 0.005). In women with no pain, PGP9.5-positive fibres were detected in only 5 (19.2%) women. Both the percentage and the density of PGP9.5-positive fibres in ovarian endometriotic lesions were associated with pelvic adhesions (χ² = 6.833, P = 0.009; Z = 2.442, P = 0.015, respectively) but not with disease severity.

CONCLUSIONS: PGP9.5-immunoactive nerve fibres in ovarian endometriotic lesions may be involved in the pathophysiology of pain generation and pelvic adhesion formation in women with ovarian endometriosis.

Key words: ovarian endometriosis / nerve fibres / pain / immunohistochemistry / adhesion

Introduction

Endometriosis is a chronic, benign, estrogen-dependent multifactorial, gynaecological disease, with pain being the most common and specific symptom. But the pathophysiology of the association between endometriosis and pain is poorly understood (Wang et al., 2009). Recent studies have found that peritoneal or deep infiltrating endometriotic lesions are innervated by protein gene product (PGP) 9.5-immunoactive nerve fibres, and that the density of nerve fibres stained with PGP9.5 in peritoneal or deep infiltrating endometriotic lesions is significantly correlated with the severity of pain in women with endometriosis (Mechsner et al., 2008; Wang et al., 2009). In a rat model of surgically induced endometriosis, PGP9.5-immunoactive nerve fibres can be detected in ovarian endometriotic lesions (Berkley et al., 2004), but no study has demonstrated PGP9.5-immunoactive nerve fibres in human ovarian endometriotic lesions. Moreover, no neurofilament protein (NF)-immunoactive nerve fibres have been shown in human ovarian endometriotic lesions in women with endometriosis (Al-Fozan et al., 2004).

To date, the mechanism of pain generation for ovarian endometriosis associated pelvic pain remains unclear. Recent evidence has revealed that nerve growth factor (NGF), a cytokine produced by Schwann cells, keratinocytes, fibroblasts, T lymphocytes and macrophages, and involved in the extension and maintenance of sympathetic and primary sensory nerves, is expressed in ovarian endometriotic lesions although its expression is weaker than in deeply infiltrating endometriotic (DIE) lesions (Klein, 1994; Anaf et al., 2002, 2006). Furthermore, S-100 protein, a neurotrophic factor, has also been demonstrated in ovarian endometriotic lesions (Anaf et al., 2002, 2006; Kleindienst and Ross Bullock, 2006). Moreover, in a rat model of...
surgically induced endometriosis, ovarian endometriotic lesions are innervated by autonomic and sensory nerve fibres (Berkley et al., 2004). These previous studies suggest that the innervation of ovarian endometriotic lesions may play a key role in the mechanism of pain generation in women with ovarian endometriosis (Evans et al., 2007; Odagiri et al., 2008).

PGP9.5 is a highly specific pan-neuronal marker for both myelinated and unmyelinated nerve fibres, including Aβ, Aγ, Aδ, B and C fibres, whereas NF is a highly specific marker for myelinated nerve fibres, including Aβ, Aγ, Aδ and B fibres (Schlaepfer, 1987; Lundberg et al., 1988). Immunohistochemistry studies have demonstrated the wide distribution of PGP9.5 and NF in the central and peripheral nervous systems (Dalsgaard et al., 1984; Stjernholm et al., 1999), and the localization of PGP9.5 in all neurons and nerve fibres at all levels of the central and peripheral nervous system as well as in cells of the diffuse neuroendocrine system (Stjernholm et al., 1999). In fact, PGP9.5 has recently been considered as a good diagnostic marker when using endometrial biopsy for investigating endometriosis (AI-jefout et al., 2009; Bokor et al., 2009).

In this study, we hypothesized that nerve fibres stained by PGP9.5 could be detected in ovarian endometriotic lesions in women with ovarian endometriosis. The objective of this study was to investigate the presence of nerve fibres stained by PGP9.5 in ovarian endometriotic lesions, and the correlation of nerve fibres in ovarian endometriotic lesions and clinical parameters in women with ovarian endometriosis who underwent laparoscopic surgery.

Materials and Methods

Patients

Between November 2008 and June 2009, a total of 61 women (mean age: 35.3 years; range: 24–45) underwent conservative laparoscopic surgery because of ovarian endometriotic cysts and/or pain symptoms. Of all the 61 women, 29 (47.5%) had ovarian endometriotic cysts alone, 32 (52.5%) had ovarian endometriotic cysts with DIE lesions and/or peritoneal endometriotic cysts. For the 32 women with ovarian endometriotic cysts who had DIE and/or peritoneal endometriotic lesions, 18 (56.3%) had peritoneal and ovarian endometriotic cysts, 9 (28.1%) DIE and ovarian endometriotic lesions and 5 (15.6%) DIE, peritoneal and ovarian endometriotic lesions.

During surgery, the presence, localization, and extent of typical powder-burn and subtle lesions, adhesions and deep infiltrating implants were recorded. Disease was classified according to the revised American Fertility Society (rAFS) score (American Society for Reproductive Medicine, 1996), and in our patients ranged from II to IV (II = 2, III = 19, IV = 40). Of all the 61 women, 34 (55.7%) had pelvic adhesions, 27 (44.3%) had no pelvic adhesions. For the 29 women with ovarian endometriotic cysts alone, 12 (41.4%) had pelvic adhesions, 17 (58.6%) had no pelvic adhesions. After adhesiolysis, all lesions were treated as follows: complete removal of ovarian endometriomata by stripping, excision of DIE and peritoneal implants. Specimens underwent thorough histologic analysis. The women with DIE and/or ovarian endometriotic lesions were all confirmed by histological diagnosis. However, for the 23 women with peritoneal endometriotic lesions, only 18 (78.3%) were confirmed by histology. None of the patients had received medical therapy for endometriosis before laparoscopic excision of endometriotic lesions. The study was approved by the Human Ethics Committees of Women’s Hospital, School of Medicine, Zhejiang University, and all women gave their informed consent for participation.

Pain evaluation

The severity of endometriosis-associated pain symptoms was documented before surgery by using a standardized questionnaire with a visual analogue scale. The pain scale was subdivided into 10 grades. ‘No pain’ was indicated at the left side of the scale and ‘the maximum pain you could imagine’ at the right side of the scale (Vercellini et al., 2006). Of all the 61 women, 35 (57.4%) complained of dysmenorrhea and a range of related pain symptoms, and 26 (42.6%) had no pain symptoms. For the 29 women with ovarian endometriotic cysts alone, 17 (58.6%) had pain symptoms and 12 (41.4%) had no pain symptoms.

Histology and immunohistochemistry

After surgical removal, all samples of ovarian endometriotic lesions were fixed in 10% neutral buffered formalin for ~18–24 h, processed, and embedded in paraffin wax according to a standard protocol. Generally, we obtained two sections (cut at 4 μm) from each lesion: one section was used for hematoxylin and eosin staining, and another for immunohistochemical staining. Immunohistochemical staining using a highly specific marker, polyclonal rabbit anti-PGP9.5, to determine the innervation of ovarian endometriotic lesions was performed as previously described by Zhang et al. (2009b). Briefly, after retrieving antigen for PGP9.5, serial sections, cut at 4 μm, were immunostained using the polyclonal rabbit anti-PGP9.5 antibody (dilution 1:500, Z5116; Dako Cytomation, Denmark A/S) for 60 min at room temperature. Sections were washed in phosphate-buffered saline (PBS) and incubated with Envision-labelled polymer-alkaline phosphatase mouse/rabbit (EnVision+/-HRP/Mo, GK400105; EnVision+/-HRP/Rb, GK400305/15; Novocastra, UK) for 60 min. The antigen–antibody reaction was visualized using diaminobenzidine as chromogen (DAB, GK346810; Novoceastra, UK). After washing, the sections were counterstained with Mayer’s hematoxylin, then dehydrated and mounted on a mounting medium. Normal vulval skin was used as a positive control, and PBS was used as a negative control. The concentrations were matched with the concentrations of the PGP9.5 antibody. An experienced gynaecological pathologist who was blinded to the sample background performed the evaluation of histopathology and immunostaining. If no immunoactive nerve fibres appeared on the slides, we defined them as a negative case, otherwise we designated them as a positive case and counted percentage (positive cases/total cases) and positive nerve fibre.

Quantification of nerve fibre density

We used the technique of microvascular density quantification described by Weidner et al. (1995) to count the number of nerve fibres identified by PGP9.5 staining in ovarian endometriotic lesions. Following immunostaining, the entire section was scanned at low power (× 100) (Microscope system, MZ16; Leica, Germany) to identify hot spots, which represent the areas of highest innervation. Individual nerve fibres were then counted under high power (× 400) to obtain a nerve count in a defined area. The total number of nerve fibres was divided by the total number of hot spots on each section to obtain an average of nerve fibres per hot spot (each hot spot with a square of 1 × 1 mm²). The results were expressed as the mean (± SD) number of nerve fibres per mm² in each specimen from all ovarian endometriotic lesion samples. The average nerve count in five hot spots was calculated because there were no significant differences in the total number of hot spots between study groups, and most slides did not show more nerve fibre hot spots. A single observer blinded to the sample background counted the number of PGP9.5-positive nerve fibres.
Statistical analysis

We used the Statistical Package for the Social Sciences Version 11.0 to perform statistical analyses. The results were expressed as the mean (±SD) number of nerve fibres per mm² in each specimen from all ovarian endometriotic lesion sections, although the measured values of the variables were not normally distributed. The Mann–Whitney U-test was used to compare the differences in density of PGP9.5-immunoactive nerve fibres in ovarian endometriotic lesions between groups. The χ² test was used to compare the differences in percentage of PGP9.5-positive nerve fibres in ovarian endometriotic lesions between groups. The Spearman analysis was used to analyse the correlation between density of PGP9.5-immunoactive nerve fibres in ovarian endometriotic lesions and the severity of pain symptoms. Differences were considered to be significant at \( P < 0.05 \).

Results

PGP9.5-immunoactive nerve fibres could be detected in ovarian endometriotic lesions in 19 out of all the 61 (31.1%) women with ovarian endometriosis. Immunoactive nerve fibres stained with PGP9.5 mostly appeared in the fibrotic interstitium of ovarian endometriotic lesions although endometrial stroma in ovarian endometriotic lesions also showed some PGP9.5-positive nerve fibres (Fig. 1). The controls provided the expected positive or negative results (not shown). Of the 35 women with ovarian endometriosis who had pain symptoms, PGP9.5-positive nerve fibres in ovarian endometriotic lesions appeared in 14 (40.0%) women, although in the 26 women with ovarian endometriosis who had no pain symptoms, PGP9.5-positive nerve fibres could be detected in ovarian endometriotic lesions in only 5 (19.2%) women (Table I). Although the percentage (women with PGP9.5-positive nerve fibres/total women) of PGP9.5-positive nerve fibres in ovarian endometriotic lesions was higher in women with ovarian endometriosis who had pain symptoms (40.0%) than in women with ovarian endometriosis who had no pain symptoms (19.2%), the difference did not reach significance (\( \chi^2 = 3.001, P = 0.083 \)).

The mean (±SD) density of PGP9.5-immunoactive nerve fibres in ovarian endometriotic lesions in women with ovarian endometriosis with and without pain symptoms was 0.72 ± 1.36/mm² and 0.10 ± 0.25/mm², respectively (Table I). There were more PGP9.5-immunoactive nerve fibres in ovarian endometriotic lesions in women with ovarian endometriosis who had pain symptoms when compared with women with ovarian endometriosis who had no pain symptoms (\( Z = 2.062, P = 0.039 \)). Moreover, the density of PGP9.5-immunoactive nerve fibres in ovarian endometriotic lesions was significantly correlated with the severity of pain symptoms in women with ovarian endometriosis who had pain symptoms (\( r = 0.466, P = 0.005 \); Fig. 2).

The percentage and the density of PGP9.5-positive nerve fibres in ovarian endometriotic lesions were 44.1% (15/34) and 0.69 ± 1.34/mm² in women with ovarian endometriosis who had pelvic adhesions, and 14.8% (4/27) and 0.16 ± 0.53/mm² in women with ovarian endometriosis who had no pelvic adhesions, respectively (Table I). The percentage and the density of PGP9.5-positive nerve fibres in ovarian endometriotic lesions were both significantly higher in women with ovarian endometriosis who had pelvic adhesions than those in women with ovarian endometriosis who had no pelvic adhesions (\( \chi^2 = 6.833, P = 0.009; Z = 2.442, P = 0.015 \), respectively). In turn, the percentage and the density of PGP9.5-positive nerve fibres in ovarian endometriotic lesions were 37.9% (11/29) and 0.47 ± 0.89/mm² in women with ovarian endometriosis alone, and 25.0% (8/32) and 0.43 ± 1.21/mm² in women with ovarian and other endometriosis, respectively (Table I). There were no significant differences of the percentage or the density of PGP9.5-positive nerve fibres in ovarian endometriotic lesions between the women who had ovarian endometriosis alone and the women who had ovarian and other endometriosis (\( \chi^2 = 1.186, P = 0.276; Z = -0.283, P = 1.074 \)). Moreover, the percentage or the density of PGP9.5-positive nerve fibres in ovarian endometriotic lesions was not statistically associated with the disease severity in women with ovarian endometriosis (\( \chi^2 = 0.099 P = 0.753; Z = 0.816, P = 0.414 \); Table I).

Figure 1  Nerve fibres in ovarian endometriotic lesions. (A) Ovarian endometriotic lesions from a woman with ovarian endometriosis who had pain symptoms stained for protein gene product (PGP) 9.5 (Magnification ×200). Arrows denote PGP9.5-positive nerve fibres in the fibrotic interstitial of ovarian endometriotic lesions. (B) Ovarian endometriotic lesions from a woman with ovarian endometriosis who had no pain symptoms stained for PGP9.5 (Magnification ×200). Arrows denote PGP9.5 positive nerve fibres in the fibrotic interstitial of ovarian endometriotic lesions. Scale bars represent 50 mm in (A) and (B).
Nerve fibres in ovarian endometriotic lesions

Table 1 Associations of protein gene product (PGP) 9.5-immunoactive nerve fibres in ovarian endometriotic lesions with pelvic adhesions, pain symptoms and the disease severity in women with ovarian endometriosis (mean ± SD)

<table>
<thead>
<tr>
<th>All women with ovarian endometriosis</th>
<th>Cases (n)</th>
<th>PGP 9.5-positive nerve fibers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percentage*</td>
<td>Density*</td>
</tr>
<tr>
<td>With pain symptoms</td>
<td>35</td>
<td>40.0% (14/35) 0.72 ± 1.36</td>
</tr>
<tr>
<td>Without pain symptoms</td>
<td>26</td>
<td>19.2% (5/26) 0.10 ± 0.25</td>
</tr>
<tr>
<td>With pelvic adhesions</td>
<td>34</td>
<td>44.1% (15/34) 0.69 ± 1.34</td>
</tr>
<tr>
<td>Without pelvic adhesions</td>
<td>27</td>
<td>14.8% (4/27) 0.16 ± 0.53</td>
</tr>
<tr>
<td>With ovarian endometriosis alone</td>
<td>29</td>
<td>37.9% (11/29) 0.47 ± 0.89</td>
</tr>
<tr>
<td>With ovarian and other endometriosis</td>
<td>32</td>
<td>25.0% (8/32) 0.43 ± 1.21</td>
</tr>
<tr>
<td>Stage II + III (rAFS)</td>
<td>21</td>
<td>28.6% (6/21) 0.28 ± 0.63</td>
</tr>
<tr>
<td>Stage IV (rAFS)</td>
<td>40</td>
<td>32.5% (13/40) 0.34 ± 1.05</td>
</tr>
</tbody>
</table>

*Women with PGP9.5-positive nerve fibres/Total women.
*Fibres/mm²
rAFS: revised American Fertility Society.

Figure 2 Correlation of the density of PGP9.5-positive fibres in ovarian endometriotic lesions and the severity of pain symptoms [assessed using a visual analogue scale (VAS) of 1–10] in women with ovarian endometriosis who had pain symptoms (n = 35).

Discussion

Our results showed that PGP9.5-immunoactive nerve fibres could be detected in ovarian endometriotic lesions in women with ovarian endometriosis. The density of PGP9.5-positive nerve fibres in ovarian endometriotic lesions was significantly associated with pain symptoms in women with ovarian endometriosis. Moreover, the density of PGP9.5-positive nerve fibres in ovarian endometriotic lesions was correlated with the severity of pain symptoms in women with ovarian endometriosis who had pain symptoms. These results suggest that PGP9.5-immunoactive nerve fibres in ovarian endometriotic lesions may be involved in the mechanisms of pain generation in women with ovarian endometriosis (Berkley et al., 2004).

PGP 9.5 is a highly specific pan-neuronal marker for both myelinated and unmyelinated nerve fibres (Lundberg et al., 1988). It has been shown that PGP9.5-positive nerve fibres can be detected in peritoneal endometriotic lesions, and are correlated with the severity of pain symptoms in women with peritoneal endometriosis (Mechsner et al., 2008). Moreover, PGP9.5-positive nerve fibres have been demonstrated to appear in endometrium in women with pain symptoms, whether the women have endometriosis, uterine fibroids or adenomyosis, and to correlate with the severity of pain symptoms in these three disorders (Zhang et al., 2009a, b). In women with endometriosis, the density of PGP9.5-positive nerve fibres in endometriotic lesions is significantly higher in deeply infiltrating endometriosis than in peritoneal endometriosis, suggesting a correlation between PGP9.5-positive nerve fibres in endometriotic lesions and pain severity in women with endometriosis (Porpora et al., 1999; Wang et al., 2009).

In a rat model of experimentally induced endometriosis, nerve fibres stained with PGP9.5 as well as other neuronal markers, including calcitonin gene-related peptide (CGRP), substance P (SP) and vasoactive intestinal peptide (VIP), can be detected in ovarian endometriotic lesions, and are believed to play a significant role in the mechanisms of pain generation in this disorder (Berkley et al., 2004). In women with ovarian endometriosis, neural cell adhesion molecule, NGF and S-100 protein are expressed in ovarian endometriotic lesions, suggesting that human ovarian endometriotic lesions are innervated, and that the innervation of ovarian endometriotic lesions may play a key role in the pathogenesis of pain generation in women with ovarian endometriosis (Anaf et al., 2002, 2006; Odagiri et al., 2008).

There is good agreement that endometriosis is a chronic inflammatory disease associated with pelvic adhesions (Daniel et al., 2000), and that pelvic adhesion reformation occurs even after laparoscopic excision of endometriosis and adhesiolyis (Gurgan et al., 1996; Parker et al., 2005; Luciano et al., 2008). It has been found that the levels of interleukin (IL)-1, IL-6 and tumour necrosis factor (TNF)-α in peritoneal fluid are correlated with the degree of pelvic adhesions present (Cheong et al., 2002a), suggesting the contribution of inflammatory cytokines and growth factors to the formation of pelvic adhesions (Cheong et al., 2002a, b, 2003). In women with endometriosis, pelvic adhesions contain estrogen and progesterone receptors, and produce basic fibroblastic growth factor and vascular endothelial growth factor, implying a regulation of pelvic adhesion formation by steroid hormone (Jirásek et al., 1998; Wiczyn et al., 1998; Schindler, 2004). Moreover, pelvic adhesions are innervated by synaptophysin, CGRP, SP, NF, VIP and tyrosine hydroxylase fibres (Tulandi et al., 1998; Sulaiman et al., 2001), although no NF-immunoactive nerve fibres were present in ovarian endometriotic lesions in women with endometriosis (Al-Fozen et al., 2004). In this study, we found that both the percentage and the density of PGP9.5-positive nerve fibres in ovarian endometriotic lesions were significantly higher in women...
with ovarian endometriosis who had pelvic adhesions than in those women with ovarian endometriosis and no pelvic adhesions. It is suggested that ovarian endometriotic lesions may be innervated through mediating effects of peritoneal inflammatory cytokines and growth factors including IL-1, IL-6 and TNF-α, in women with pelvic adhesions, thus leading to an increase of nerve fibres in ovarian endometriotic lesions in women with ovarian endometriosis (Berkley et al., 2005; Zhang et al., 2007; Binshtok et al., 2008; Schäfers et al., 2008; Temporin et al., 2008a, b; Gavaldà et al., 2009; Shirai et al., 2009).

In summary, our preliminary results showed that PGP9.5-immunoactive nerve fibres mostly appeared in the fibrotic interstitial of ovarian endometriotic lesions, and that the density of PGP9.5-immunoactive nerve fibres in ovarian endometriotic lesions was significantly associated with pelvic adhesions and pain symptoms in women with ovarian endometriosis. These results only suggest that PGP9.5-immunoactive nerve fibres in ovarian endometriotic lesions may be involved in the pathophysiology of pelvic adhesion formation and pain generation in women with ovarian endometriosis. However, in the present study, we did not detect nerve fibres with PGP9.5 in pelvic adhesion tissues, and only observed a low positive rate of PGP9.5-immunoactive nerve fibres in ovarian endometriotic lesions. Moreover, nerve fibres were counted in hot spots and not in randomly selected areas, although the technique of hot-spot quantification has been used to count microvessel density for many years. Therefore, further prospective studies to overcome these limitations are needed.

**Authors’ Roles**

X.Z.: is a chief designer and responsible for this manuscript writing. H.Y.: is responsible for immunostaining and data analysis. X.H.: is responsible for data analysis and experimental design. B.L.: fourth author is responsible for data analysis. H.X.: is responsible for statistical analysis. C.Z.: is responsible for immunostaining.

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