Rich innervation of deep infiltrating endometriosis

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BACKGROUND: Deep infiltrating endometriosis (DIE) is a specific type of endometriosis, which can be associated with more severe pelvic pain than other forms of endometriotic lesions. However, the mechanisms by which pain is generated are not well understood.

METHODS: DIE (n = 31) and peritoneal endometriotic (n = 40) lesions were sectioned and stained immunohistochemically with antibodies against protein gene product 9.5, neurofilament, nerve growth factor (NGF), NGF receptors tyrosine kinase receptor-A (Trk-A) and p75, substance P, calcitonin gene-related peptide, vesicular acetylcholine transporter, neuropeptide Y, vasoactive intestinal peptide and tyrosine hydroxylase to demonstrate myelinated, unmyelinated, sensory and autonomic nerve fibres.

RESULTS: There were significantly more nerve fibres in DIE (67.6 ± 65.1/mm²) than in peritoneal endometriotic lesions (16.3 ± 10.0/mm²) (P < 0.01). DIE was innervated abundantly by sensory Aδ, sensory C, cholinergic and adrenergic nerve fibres; NGF, Trk-A and p75 were strongly expressed in endometriotic glands and stroma of DIE.

CONCLUSIONS: The rich innervation of DIE may help to explain why patients with this type of lesion have severe pelvic pain.

Key words: deep infiltrating endometriosis / immunohistochemistry / nerve fibres / pain

Introduction

Endometriosis is defined as the presence of tissue which somewhat resembles endometrial glands and stroma outside the uterus. It is thought to occur in 10–15% of women of reproductive age worldwide. Endometriosis is a chronic, benign, estrogen-dependent multifactorial, gynaecological disease, with pain being the most common and specific symptom. Although all types of endometriosis can often produce pain, the specific lesion called deep infiltrating endometriosis (DIE) is commonly associated with severe pain and discomfort, and it can severely alter a patient’s quality of life (Koninckx et al., 1991, 1994).

DIE is defined as endometriotic lesions penetrating into the retroperitoneal space or the wall of the pelvic organs for a distance of 5 mm or more (Koninckx et al., 1991). Patients with deep pelvic infiltrating nodules usually have severe pain, dysmenorrhea, dyspareunia, dyschezia, urinary symptoms and infertility (Koninckx et al., 1991). DIE was found in about one-third of the laparoscopies performed because of pelvic pain symptoms (Koninckx and Martin, 1994). This type of endometriosis may be very different from other forms of endometriosis. A previous study of retrospective data showed that the various specific anatomic locations of DIE were broadly related to specific pain symptoms (Fauconnier et al., 2002). Chapron et al. (2005) reported that they could predict DIE by evaluating painful symptoms before laparoscopic surgery for pelvic pain symptoms, and the sensitivity and specificity of this model for diagnosing posterior DIE were 74.5% and 68.7%, respectively. The strong association between DIE and severe pelvic pain was also invariably noted in observational studies, and a logistic regression showed deep endometriosis to be the strongest predictor of severe pelvic pain (Koninckx and Martin, 1994; Vercellini et al., 1996).

The pathophysiology of the association between endometriosis and pain is poorly understood. Gynaecologists widely believe that inflammation is a major cause of pain in endometriosis (Vercellini et al., 2008). However, the pathophysiology of severe pain associated with deep endometriosis may be different from that caused by other forms of endometriosis, and the underlying mechanism may be different.

Some researchers have identified nerve fibres in endometriotic lesions in women with endometriosis (Tamburro et al., 2003; Tokushige et al., 2006a, 2007). Berkley et al. (2004, 2005) reported that ectopic endometriotic implants developed a sensory and sympathetic nerve supply both in rats and in women, similar to that of the healthy rat uterus. We have also demonstrated the presence of multiple, small
unmyelinated nerve fibres in peritoneal endometriotic lesions in women with confirmed endometriosis, and the density of nerve fibres in peritoneal endometriotic lesions was much greater than that in the peritoneum of women without endometriosis (Tokushige et al., 2006b). For deep endometriosis, the association between pain and endometriosis is much stronger than in other types of endometriosis (Konincx et al., 1991; Anaf et al., 2006; Vercellini et al., 2008). Women with deep endometriosis often have severe pain close to maximum on a Biberoglu and Behrmann pain scale (Biberoglu and Behrmann, 1981).

We studied the presence of different types of nerve fibres in endometriotic lesions from women with biopsy-proven deep disease by immunohistochemistry using highly specific markers. We used protein gene product 9.5 (PGP9.5), neurofilament (NF), nerve growth factor (NGF), NGF receptor p75 (NGFRp75), tyrosine kinase receptor-A (Trk-A), substance P (SP), calcitonin gene-related peptide (CGRP), neuropeptide Y (NPY), vasoactive intestinal peptide (VIP), vesicular acetylcholine transporter (VACHT), and tyrosine hydroxylase (TH) to differentiate myelinated, unmyelinated, sensory Aδ, sensory C, adrenergic and cholinergic nerve fibres. At the same time, we also used CD34 in order to study the relationship between nerve fibres and blood vessels.

**Materials and Methods**

**Collection of tissue samples**

This study was approved by the Human Ethics Committees of the Sydney South West Area Health Service and the University of Sydney, and all women gave their informed consent for participation.

DIE tissue was collected from 31 patients with surgically and histologically confirmed DIE (mean age 34.6 years, range 23–48 years): uterosacral ligaments (n=10); cul de sac (n=10); peritoneal sidewall (n=7) and rectum (n=7). Three patients had more than two DIE lesions in different sites. Of the 31 patients, 29 (93.5%) complained of painful symptoms at the time of their hospital admission, whereas the remaining two (6.5%) patients complained of both pain symptoms and infertility. In all patients, the time of operation with respect to menstrual cycle (proliferative or secretory phase) and hormonal treatment was documented. The extent of endometriosis was assessed according to the revised American Fertility Society (AFS) score (Revised American Fertility Society classification of endometriosis, 1985). All women gave informed consent for their data to be used for scientific purposes. None of the women had received medical therapy for endometriosis before laparotomy or laparoscopy excision of endometriotic lesions. A total of 21 patients were found to have Stage III, 5 had Stage IV (revised AFS score ≤70) and 5 had advanced Stage IV (revised AFS score >70). The size of the nodules varied from 0.1 to 3.5 cm.

Slides from the paraffin-embedded specimens of all the patients who underwent surgery for DIE were reviewed by an experienced gynaecological pathologist. A full medical history was obtained in all patients, including a questionnaire on dysmenorrhoea, deep dyspareunia and chronic pelvic pain before surgery. The severity profile score of symptoms of dysmenorrhoea, deep dyspareunia and chronic pelvic pain was assessed by means of a system consisting of a subjective rating scale (0, none; 1, mild; 2, moderate and 3, severe) based on the patients’ self-assessment of pain according to Biberoglu and Behrmann.

**Immunohistochemistry**

After surgical removal, all the specimens were immediately fixed in 10% neutral buffered formalin for ~18–24 h, processed and embedded in paraffin according to a standard protocol. Each section was cut at 4 μm and routinely stained with haematoxylin and eosin. Serial sections, cut at 4 μm, were immunostained using antibodies for polyclonal rabbit anti-PGP9.5 (dilution 1:1400), a highly specific pan-neuronal marker, which recognizes all types of nerve fibres; monoclonal mouse anti-human NF (dilution 1:800), a highly specific marker for myelinated nerve fibres; polyclonal rabbit anti-human NGF (dilution 1:500); monoclonal mouse anti-human NGFRp75 (dilution 1:1500); polyclonal rabbit anti-human TrkA (dilution 1:500); polyclonal rabbit anti-human vasoactive intestinal polypeptide (dilution 1:3000), which is a specific marker for parasympathetic neurons; polyclonal rabbit anti-human NPY (dilution 1:5000), which is a specific marker for sympathetic neurons; polyclonal rabbit anti-human SP (dilution 1:7000), CGRP (dilution 1:150), SP and CGRP are sensory fibre markers, and they can be present in both Aδ and C nerve fibres; monoclonal anti-TH (dilution 1:1500), which is a specific adrenergic marker, is present in sympathetic postganglionic neurons; polyclonal rabbit anti-human VACHT (dilution 1:7000), which is a specific marker for cholinergic fibres, can be present in parasympathetic neurons. Antigen retrieval techniques for these markers were used as described previously (Tokushige et al., 2006a, b, 2007).

For double staining, serial sections were immunostained with CD34 and PGP9.5, the immunostaining method was the same as described previously (Tokushige et al., 2006b). All immunostaining was carried out on a Dako Autostainer Model S3400 (Dako, Carpinteria, CA, USA). Images of the sections were captured using an Olympus microscope BX51 and digital camera DP70 (Olympus, Tokyo, Japan). We used normal skin as a positive control as it reliably contains myelinated and unmyelinated nerve fibres expressing PGP9.5, NF, NGF, NGFRp75, Trk-A, SP, CGRP, TH, VACHT, VIP and NPY. We used the functional layer of eutopic endometrium from women without endometriosis as a negative control because it does not contain any nerve fibres.

**Statistical analysis**

The images were captured using Olympus microscope BX51 and digital camera DP70, and an assessment of nerve fibre density was performed by Image Pro Plus Discovery (MediaCybernetics, MD, USA). Once the image features were acquired, an orthogonal grid was sketched over the original images. The sections of the grid were 250 μm per side. Once the grid was in position, nerve fibres stained with PGP9.5, NF, NGF, NGFRp75, Trk-A, SP, CGRP, TH, VACHT, VIP and NPY within the squares of the DIE lesions were counted. The total number of nerve fibres was divided by the total number of squares covering the DIE lesions to obtain an average of nerve fibres per square (each square of 250 x 250 μm). The results were expressed as the mean (±SD) number of nerve fibres per millimetre square. The counting procedure was carried out twice by two independent observers (each blinded to the other) without any knowledge of the clinical parameters or other prognostic factors. The concordance rate was more than 95% between the observers. Nerve fibre densities between DIE lesions reported in this study and peritoneal endometriotic lesions reported previously using identical techniques (Tokushige et al., 2006b) and between different sites of DIE were compared using the Mann–Whitney test. Differences were considered to be significant at P < 0.05.

**Results**

This study clearly showed there was much greater density of nerve fibres stained with PGP9.5 in the DIE (n=31) (Fig. 1A) than in peritoneal endometriotic lesions (n=40) (Tokushige et al., 2006b). The density of nerve fibres in DIE (mean ± SD) was 67.6 ± 65.1/mm² (Fig. 2), whereas the density of nerve fibres in peritoneal...
endometriotic lesions was 16.3 ± 10.0/mm² (Tokushige et al., 2006b) (P < 0.01) (Fig. 2).

There were also more myelinated nerve fibres stained with NF in the deep infiltrating lesions than in peritoneal endometriotic lesions (Fig. 1B). The density of myelinated nerve fibres in DIE was 17.5 ± 12.8/mm² (Fig. 2), and in peritoneal endometriotic lesions was 6.7 ± 4.7/mm² (P < 0.01) (Fig. 2) (Tokushige et al., 2006b). Those nerve fibres stained with PGP9.5 and NF were often seen near endometriotic glands and blood vessels, and they often formed obvious thick nerve trunks in the adjacent fibromuscular tissue (Fig. 1A, B and L).

NGF and NGF high-affinity receptor (Trk-A) were strongly expressed in endometriotic glands and stroma of DIE, but NGF low-affinity receptor (NGFRp75) was only strongly expressed in endometriotic glands (Fig. 1C–E).

The densities of nerve fibres stained with PGP9.5 and NF in deep uterosacral ligament lesions DIE (n = 10) were 31.0 ± 27.6/mm² (range 3.7–133/mm²) and 6.3 ± 2.7/mm² (range 1.6–11/mm²), respectively; the densities of nerve fibres in deep cul de sac lesions (n = 10) were 7.8 ± 6.5/mm² (range 2.7–24/mm²) and 5.0 ± 4.0/mm² (range 2.1–18.7/mm²), respectively; the densities of nerve fibres in deep peritoneal side wall lesions (n = 7) were 31.3 ± 30.0/mm² (range 1.6–160/mm²) and 8.5 ± 7.5/mm² (range 1.6–26.7/mm²), respectively; the densities of nerve fibres in rectal lesions (n = 7) were 165.7 ± 95.4/mm² (range 5.3–320/mm²) and 48.0 ± 28.7/mm² (range 53.3–106.7/mm²) respectively. The densities of nerve fibres were highest in rectal lesions (*P < 0.01; **P < 0.001) (Fig. 3).

We also counted the densities of nerve fibres stained with PGP9.5 and NF in deep rectal lesions (Fig. 2) (Tokushige et al., 2006b). Those nerve fibres were highest in rectal lesions. There was no difference in nerve fibre density between cul de sac, uterosacral ligament and pelvic side wall lesions. The densities of nerve fibres stained with SP, VIP, TH and NPY showed little or no difference in all these tissues. These nerve fibres were expressed in all sites. However, VACHT was mainly expressed in rectal lesions, while lesions in other sites contained weak or no nerve fibres which expressed VACHT.

Women with endometriosis, especially those with DIE, typically experience severe pelvic pain (Cornillie et al., 1990; Koninckx et al., 1991, 1994; Anaf et al., 2004). They may also experience hyperalgesia. Hyperalgesia is defined as an exacerbation of pain or initiation of painful sensation when a non-painful stimulus is applied. This pain sensation is associated with a lower threshold of pain perception and increased sensitivity of nociceptors (Anaf et al., 2002). Hyperalgesia is a major characteristic of ‘neuropathic pain’, and this phenomenon frequently occurs in DIE where nerve invasion into endometriotic stromal cells is observed (Anaf et al., 2000, 2006). It therefore seems that central neural mechanisms could also be involved in the pain experiences with DIE (Karen et al., 2001).

The histological findings of DIE lesions are mainly characterized by fibromuscular hyperplasia that surrounds the foci of endometriosis. The endometrial glands and stroma infiltrate the adjacent fibromuscular tissue and elicit smooth muscle proliferation and fibrous reaction, resulting in solid nodule formation and the histology is similar to an adenomyoma (Koninckx et al., 1991; Vercellini et al., 2004). Therefore, DIE may be different from other forms of endometriosis, in terms of its aetiology and pathophysiology. However, we have shown that there was a high density of nerve fibres in these deep infiltrating lesions, whereas we have not found nerve fibres within nodules of typical uterine adenomyosis (Al-Jefout et al., unpublished data).

The pathophysiology of the association between endometriosis and pelvic pain is not well understood, especially the reason why the specific entity of deep infiltrating nodules may be associated with severe symptoms. Pelvic pain is frequently ascribed to intraperitoneal bleeding from ectopic endometrium during menstruation. It has also been proposed that endometriosis is a consequence of neurological dysfunction, and that endometriosis is based on a process of denervation and reinnervation (Quinn, 2004). Endometriotic implants may continuously release signal molecules that can act on nociceptor peripheral terminals to produce either a depolarization sufficient to initiate action potentials or a reduction in threshold levels such that innocuous stimulus now activate what have been high threshold nociceptors. So following a peripheral nerve injury in women with endometriosis, there may be a new growth and/or sprouting of central terminals of the low thresholdafferent into the zone normally occupied exclusively by the nociceptors terminals which may cause hyperalgesia (Lundeberg and Lund, 2008).

Nociceptors are present in most viscera, including reproductive organs (Berkley et al., 1988, 1990; Tong et al., 2006). Some studies have demonstrated changes in populations of uterine nociceptors in ectopic endometriotic lesions (Berkley et al., 2004, 2005; Tokushige et al., 2006a, b). Women with endometriosis have many small unmyelinated nerve fibres in the functional layer of the endometrium (Tokushige et al., 2006a), which were found to be nociceptors in experimental and clinical studies (Berkley et al., 1988, 1990, 2004). This has been interpreted as an abnormal sprouting of nociceptors in the endometrium and ectopic endometriotic lesions (Lundeberg et al., 1990, 1994, 1995a, 1995b).

**Discussion**

This study has demonstrated that there were significantly more nerve fibres in DIE (mean, 67.6/mm²) than in superficial peritoneal endometriotic lesions (mean, 16.3/mm²) (P < 0.01) (Tokushige et al., 2006b). DIE was innervated abundantly by sensory Aδ, sensory C, cholinergic and adrenergic nerve fibres. NGF and Trk-A were strongly expressed in endometriotic glands and stroma of DIE, but NGFRp75 was only strongly expressed in endometriotic glands. The densities of nerve fibres were highest in rectal lesions. There was no difference in nerve fibre density between cul de sac, uterosacral ligament and pelvic side wall lesions. The densities of nerve fibres stained with SP, VIP, TH and NPY showed little or no difference in all these tissues. These nerve fibres were expressed in all sites. However, VACHT was mainly expressed in rectal lesions, while lesions in other sites contained weak or no nerve fibres which expressed VACHT.
and Lund, 2008). Peritoneal sympathetic (as well as sensory) nerve fibres are involved in hyperalgesia (Zhang et al., 2008), and peripheral nerve injury can generate sympathetic sprouting surrounding primary afferent neurons in the periphery and dorsal root ganglia (Mclachlan et al., 1993; Ramer and Bisby, 1997; Grelik et al., 2005; Yen et al., 2006). This leads to the chemical interaction coupling between sympathetic and afferent neurons that can sensitize and/or activate primary afferent nociceptors (Zhang et al., 2008). Persistent

**Figure 1** Nerve fibres in DIE.

Permanent fast red chromogen has been used throughout unless noted. (A) A rectal lesion stained with PGP9.5, thick nerve trunks in the adjacent fibromuscular tissue (magnification × 100). (B) A rectal lesion stained with NF (magnification × 200). (C) A uterosacral ligament lesion stained with NGF (magnification × 200). (D) A rectal lesion stained with NGFRp75 (magnification × 200). (E) A peritoneal sidewall lesion stained with Trk-A (magnification × 200). (F) A peritoneal sidewall lesion stained with CGRP (magnification × 200). (G) A cul de sac lesion stained with SP (magnification × 200). (H) A rectal lesion stained with VACHT (magnification × 200). (I) A cul de sac lesion stained with VIP (magnification × 200). (J) A peritoneal sidewall lesion stained with NPY (magnification × 100). (K) A rectal lesion stained with PGP9.5, high densities of nerve fibres in rectal lesion (magnification × 200). (L) A uterosacral ligament lesion stained with PGP9.5 and CD34 (magnification × 200). The nerve fibres were intensely stained red (permanent fast red chromogen) and blood vessels were stained brown (DAB chromogen). Scale bars represent 100 μm in A, J and K; and 50 μm in B–I, L. Black arrows represent nerve fibres; deep orange arrows represent endometriotic glands; yellow arrows represent rectal mucosa epithelia; green arrows represent blood vessels.
nociceptive input from endometriotic tissue in women might lead to central sensitization and might result in increased responsiveness of dorsal horn neurons processing input from the affected viscera and somatic tissue. This persistent nociceptive input could increase the afferent barrage from the reproductive organs towards the central nervous system leading to increased excitability of viscero-visceral convergent neurons to the spinal cord (Bajaj et al., 2003), and these can cause persistent neuropathic pain and hyperalgesia.

A relationship has been demonstrated between the severity of the chronic pelvic pain symptoms and infiltration of nerves in the recto-vaginal space in DIE lesions (Anaf et al., 2000). Patients with the highest pain scores displayed significantly more neural invasion into endometriosis than those with lower pain scores (Anaf et al., 2006).

Coosemans et al. (2008) have demonstrated that all innervated deep endometriotic nodules contain Wilms’ tumour gene 1 (WT1)-positive nerves, and WT1 gene is suggested to play a role in neuronal pathology. This innervation might partly explain why patients with DIE feel more severe pain. It is possible that inflammation may be a more important mechanism in the generation of pelvic pain in superficial peritoneal endometriosis, whereas nerve distortion, compression or damage may be more important in DIE (Anaf et al., 2000).

Some researchers have postulated that DIE is also associated with significant inflammatory cell invasion (Cornillie et al., 1990). DIE lesions contain a significantly greater number of mast cells and of activated mast cells than peritoneal lesions (Anaf et al., 2006). Mast cells are located closer to nerves than in peritoneal and ovarian lesions, and
there are also more degranulating mast cells in deep infiltrating than peritoneal lesions (Anaf et al., 2006). Tamburro et al. (2003) have also showed greater TGFβ1 to be expressed in deep endometriotic foci than normal peritoneum. Proinflammatory cytokines have been shown to contribute to the development of inflammatory pain and hyperalgesia (Clatworthy et al., 1995; Watkins et al., 1995; Woolf et al., 1997) and proinflammatory cytokines may be involved in endometriosis pathophysiology. Endometriotic lesions can produce both inflammatory and pain mediators, including prostaglandin F, kinins, bradykinin and TGFβ1, (Vernon et al., 1986; Vercellini et al., 1991; Muzii et al., 1997; Nap et al., 2004), and they may induce local algesic stimulation in nerve fibres (Tulandi et al., 2001). The high concentration of cytokines and prostaglandins found in the peritoneal fluid of patients with endometriosis may contribute to an increase in nociceptive stimuli on nerve fibres (Karck et al., 1996; Koninckx et al., 1999).

Our results are in keeping with those of Anaf who have demonstrated that NGF and Trk-A are more strongly expressed in deep endometriotic nodules than in ovarian and peritoneal endometriosis (Anaf et al., 2002). Moreover, NGF is crucial for the development of sympathetic and small diameter sensory neurons, as it stimulates the expression and release of neuropeptides involved in pain transmission, and interacts with cellular and molecular mediators of inflammation (Apfel, 2000). Increased NGF levels in inflamed tissues can lead to hyperalgesia by both peritoneal and central mechanisms (Pezet and McMahon, 2006), and also can directly activate C-fibres contributing to hyperalgesia (Zhang et al., 2008). Additionally, NGF may perform an important role for migration of nerves into postoperative adhesions (Sulaiman et al., 2000).

In this study, the stromal cells of DIE did not express NGFRp75, and stromal cells of superficial peritoneal endometriotic lesions also did not express NGFRp75 (Tokushige et al., 2006b), whereas NGFRp75 was strongly expressed in stromal cells of eutopic endometrium from women with endometriosis (Tokushige et al., 2006a). The eutopic endometrium from women with endometriosis exhibits some fundamental differences compared with eutopic endometrium from women without endometriosis, including structure, proliferation, apoptosis, immune components, cell adhesion molecules, proteolytic enzymes and inhibitors, steroid and cytokine production and responsiveness, and gene expression and protein production (Sharpe-Timms, 2001; Al-Jefout et al., 2008). There are also differences in function between ectopic lesions and eutopic endometrium from women with endometriosis, and it appears probable that there are also differences in function between different types of ectopic lesions.
In our study, there were 10 patients with DIE of the uterosacral ligaments, 10 patients with cul de sac DIE, 7 patients with peritoneal sidewall DIE and 7 patients with rectum DIE. The uterosacral ligaments, cul de sac, peritoneal sidewall and rectum are the most common pelvic sites involved by DIE. The densities of nerve fibres varied among these sites, and the density of nerve fibres in the rectal lesions was the greatest. The VACHT enzyme was richly expressed in nerve fibres in rectal lesions, but weak or absent in other endometriotic lesions. VACHT nerve fibres were not present in eutopic endometrium, but only in myometrium of the colon. However, we found a 6-fold greater concentration of nerve fibres in rectal endometriotic lesions than in normal rectal wall.

Rectovaginal endometriotic nodules often represent the deepest and most severe lesions. A high proportion of the nerves in these lesions appeared to be encapsulated in this nodular fibrosis with a close morphological relationship between nerves and endometriotic foci by means of perineural and endoneurial invasion (Anaf et al., 2000). Why do DIE lesions occur only in some anatomical sites? Why are the densities of nerve fibres highest in rectal lesions? Both these questions remain unanswered in the abundant literature on endometriosis. The rectum is probably more innervated than other areas and rectal DIE may infiltrate nerves to induce pain in a way which does not occur in other more superficial endometriotic lesions. It may also be the case that DIE lesions recreate greater amounts of neurotrophins than other endometriotic lesions and therefore stimulate proliferation of new nerve fibres. It is not known whether such extensive innervation occurs in other gastrointestinal endometriotic lesions.

In summary, we have demonstrated that nerve fibre density in DIE is much greater than in superficial peritoneal endometriotic lesions, and that NGF, NGFPr75 and Trk-A are strongly expressed in DIE. These deep lesions are richly innervated by nerve fibres expressing a wide range of functional markers. Nerve fibre densities in rectal lesions are significantly greater than in other deep lesions. It is probable that the very high density of nerve fibres in these lesions plays an important role in the pathogenesis of pain and tenderness.

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