Vascular endothelial growth factor (VEGF) concentrations are elevated in peritoneal fluid of women with endometriosis

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Active endometriosis is characterized by hypervascularization both within and surrounding the implant; therefore the presence of angiogenic factors in the peritoneal environment would be of great importance. Vascular endothelial growth factor (VEGF) is a potent angiogenic factor involved in both physiological and pathological angiogenesis. We sought to determine if VEGF was present in the peritoneal fluid of women with and without endometriosis, and to establish if differences exist between these groups. VEGF was present in all patients sampled. The fluid from patients with endometriosis contained significantly greater amounts of VEGF than controls. Cyclic variations in VEGF concentration were seen in fluid from patients with endometriosis, the VEGF concentration in proliferative phase being significantly higher than in the secretory phase. The concentration of VEGF in this fluid was also significantly higher than that found in the proliferative and secretory phases of women without endometriosis. No cyclic variations in VEGF were seen in the control group. We suggest that elevated levels of VEGF in the peritoneal fluid of patients with endometriosis may be critical in the pathogenesis of endometriosis.

Key words: angiogenesis/ELISA/endometriosis/human peritoneal fluid/VEGF

Introduction

Endometriosis is one of the most common benign gynaecological conditions and is present in about 10% of women of reproductive age, in the UK and USA (Strathy et al., 1982). Endometriosis has a multitude of appearances, ranging from isolated peritoneal deposits to dense pelvic adhesions (Shaw, 1993). It has recently been suggested that mild, asymptomatic endometriosis should not be considered as a disease but rather a dynamic physiological condition, which occurs intermittently in some if not most women. Only in symptomatic cases where the implants do not regress but develop into progressive infiltrating or cystic lesions should they be considered as having 'endometriosis' (Brosens, 1994; Koninckx, 1994). Current opinion is, however, divided on this suggestion. Although the basic aetiology of the disease is unknown it is generally accepted that endometriosis is a result of the implantation of exfoliated endometrium, deposited in the peritoneal cavity following retrograde menstruation. Patients with endometriosis are characterized by the ability of the endometrium to implant and by the peritoneal response to this tissue. This response takes the form of a local inflammatory reaction with alterations in the peritoneal mesothelium, increase in the number and activation status of macrophages (Haney et al., 1981; Halme et al., 1983) elevation of cytokine expression (Fakih et al., 1987; Halme, 1989) and enhanced neovascularization within the peritoneal cavity (Nisolle et al., 1993). This neovascularization both within and surrounding the endometriotic implant suggests that angiogenesis may be important in the establishment and maintenance of pelvic endometriosis.

The peritoneal environment, most notably the contribution of the peritoneal macrophages, is of critical importance in the aetiology and pathogenesis of endometriosis (Ramey and Archer, 1993; Halme et al., 1987; Hellema, 1994). Peritoneal fluid contains soluble growth and angiogenic factors (Ramey and Archer, 1993), with a range of these being elevated in endometriosis (Oosterlynck et al., 1993). Vascular endothelial growth factor (VEGF) is a heparin-binding growth factor of 30–46 kDa which is active as a disulphide linked homodimer (Ferrara et al., 1992). It is a potent mitogen, morphogen and chemoattractant for endothelial cells (Connolly et al., 1989; Ferrara et al., 1992). In vivo it is a powerful mediator for vessel permeability (Keck et al., 1989). VEGF is strongly implicated in the initiation and development of angiogenesis in the developing embryo (Millauer et al., 1993) and in adult tissue undergoing profound angiogenesis such as cycling endometrium (Charnock-Jones et al., 1993) and the luteinizing follicle (Ravindarnath et al., 1991). In addition to its physiological role, VEGF is implicated as a critical angiogenic factor in the development of tumour vascularization (Kim et al., 1993) and the excessive neovascularization seen in conditions such as rheumatoid arthritis (Koch et al., 1994). Therefore VEGF would be a prime candidate for involvement in the vascularization seen in endometriosis.

In this study we investigated the presence of VEGF in peritoneal fluid throughout the cycle and determined if differences in the concentration of VEGF existed between women with and without endometriosis. In addition, we investigated the possibility of a correlation between the
**Materials and methods**

Women aged between 25–40 years, who were not on medication and who were undergoing either diagnostic laparoscopy for dysmenorrhoea, or elective laparoscopy for infertility were used for this study. The study was approved by the ethical committee of the Cambridge Health Authority, and informed consent was obtained from each patient. Patients were diagnosed as normal or endometriotic following laparoscopic investigation and staged according to the revised American Fertility Society Classification. Suspected endometriotic tissue was biopsied and diagnosis confirmed histologically. Normal subjects were women in whom no visible evidence of pelvic endometriosis was evident. In all, 19 women without pelvic endometriosis and 24 women with endometriosis were included in this study. The phase of the menstrual cycle was determined by histological dating of eutopic endometrium samples taken simultaneously with the peritoneal fluid samples. Dating was carried out by an independent pathologist.

All visible peritoneal fluid was aspirated via a Verres needle from the Pouch of Douglas immediately after insertion of the laparoscope. The volumes were recorded and the samples were clarified by centrifugation at 1500 g for 10 min; the supernatants were isolated and stored at −70°C until assayed.

Peritoneal fluid VEGF concentrations were measured, in triplicate, using a sandwich enzyme linked immunosorbent assay (ELISA) system developed in our laboratory. Ninety-six-well plates were coated with a rabbit polyclonal anti-VEGF antibody (100 μg/ml) raised against complete human rVEGF165 and incubated overnight at 4°C and then blocked with 3% bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 2 h at room temperature. Human rVEGF165 (R & D Systems, Abingdon, UK) (between 1 and 128 ng/ml) or fluid samples were added to the coated well and incubated for 2 h at room temperature. Plates were then incubated with a biotinylated rabbit polyclonal anti-VEGF antibody raised against complete human rVEGF165 in our laboratory. Substrate solution was added to plates and the colour developed. The reaction was stopped by the addition of 2.25 M sulphuric acid and absorbance at 409 nm was determined on a plate reader. The plate was washed between each step with TBS containing 0.01% Tween 20. The ELISA system was validated and optimized using rVEGF165 spiked standards and irrelevant growth factors. Inter-assay variability was between 10 and 15%, whilst intra-assay variability was between 5 and 8%. Using this system the limit of sensitivity was 4 ng/ml and a standard curve was generated by plotting absorbance versus log of human VEGF concentration which was linear over a concentration range of 4–64 ng/ml.

The PF VEGF concentrations were not normally distributed. Log transformation of the data resulted in their normal distribution and statistical analysis was performed using unpaired Student’s t-test. Statistical significance was accepted at \( P < 0.05 \).

**Results**

VEGF was detected in the peritoneal fluid of all women tested, with a significantly higher \( (P < 0.014) \) peritoneal fluid VEGF concentration seen in endometriosis \((24.05 \pm 15 \text{ ng/ml})\) compared to normals \((13.25 \pm 7.2 \text{ ng/ml})\) (Table I). In patients with endometriosis a cyclical specific pattern was observed, with the concentration of VEGF in the proliferative phase \((33 \pm 13 \text{ ng/ml})\) being significantly greater \( (P < 0.01) \) than in the secretory phase \((10.72 \pm 5 \text{ ng/ml})\). This cyclic variation was not evident in fluid from normal patients (Figure 1). Comparing fluid from the two patient groups, it was seen that the VEGF concentration in proliferative phase fluid from

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Table I. Clinical characteristics and peritoneal fluid vascular endothelial growth factor (VEGF) concentrations in normal and endometriotic patient groups. Values are means ± SD

<table>
<thead>
<tr>
<th></th>
<th>Normal patient group, phase of cycle</th>
<th>Endometriosis patient group, phase of cycle</th>
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<tbody>
<tr>
<td></td>
<td>Proliferative</td>
<td>Secretory</td>
</tr>
<tr>
<td>Age (years)</td>
<td>31 ± 6</td>
<td>35 ± 5</td>
</tr>
<tr>
<td>Peritoneal fluid vol. (ml)</td>
<td>15.1 ± 12.9</td>
<td>16.6 ± 9.2</td>
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<tr>
<td>VEGF concentration (ng/ml)</td>
<td>13.25 ± 7.2</td>
<td>11.3 ± 3</td>
</tr>
<tr>
<td>Cycle specific VEGF concentration (ng/ml)</td>
<td>10.86 ± 6.5</td>
<td>13.1 ± 8</td>
</tr>
<tr>
<td>Patient no.</td>
<td>9</td>
<td>10</td>
</tr>
</tbody>
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\(^{a}\)Significantly different from normals \((P < 0.014)\).

\(^{b}\)Significantly different from secretory normals \((P < 0.01)\).

\(^{c}\)Significantly different from proliferative normals \((P < 0.01)\).

\(^{d}\)Significantly different from secretory normals \((P < 0.01)\).
patients with endometriosis (33 ± 13 ng/ml) was significantly greater ($P < 0.01$) than that detected in both proliferative (11.3 ± 3 ng/ml) and secretory phase (13.1 ± 8 ng/ml) fluid from the normal patient group (Table I). The majority of patients with endometriosis were scored as having minimal to mild forms of the disease, and we were unable to determine if any correlation existed between the severity of the disease and the concentration of VEGF in the peritoneal fluid.

**Discussion**

This study shows for the first time that immuno-reactive VEGF is present in human peritoneal fluid and that significant differences in fluid VEGF concentrations exist between women with and without endometriosis in the proliferative but not the secretory phase of the cycle.

The high concentrations of this potent angiogenic factor, within the peritoneal cavity, have important implications for endometriosis, which is dependent on angiogenesis. It will stimulate and maintain the neovascularization both within and surrounding the endometriotic tissue, facilitating its establishment and proliferation. The cyclic variation in peritoneal fluid VEGF levels in endometriosis may have two important consequences. Firstly, it may be an important aetiological factor leading to favourable implantation. The high concentrations of VEGF seen during the proliferative phase may lead to the vascularization of the peritoneum just at the time when it is exposed to retrograde menstrual effluent. Secondly, active endometriosis can undergo cycling and high concentrations of VEGF during the proliferative phase may help the implant to re-vascularize and proliferate.

There is increasing evidence to suggest that many of the growth/angiogenic factors found within the peritoneal fluid originate from peritoneal macrophages (Olive et al., 1991; Eischen et al., 1994; Chien Chao et al., 1993) and that activated macrophages and macrophage-derived factors play a critical role in the induction of neovascularization (Sunderkotter et al., 1994). Macrophages, as well as being an important cell in cellular immunity, can be viewed as a dispersal secretory organ capable of synthesizing and secreting a large number of products (Auger and Ross, 1992). It is well established that in endometriosis there are increases in the number and activation/secretory activity of peritoneal macrophages (Halm et al., 1983). It could be that these cells are the principle source of VEGF and that differences in the function of these cells may account for differences in the concentrations of VEGF; therefore the activation status of the macrophages may account for the differences seen in the concentration of VEGF in the peritoneal fluid of women with endometriosis compared to fluid from normal women. Other cytokines and growth factors have been found in the peritoneal fluid, including IL-1 (Ueki et al., 1994), IL-5, IL-6 (Koyama et al., 1993), Rantes (Khorram et al., 1993) and IL-8 (Ryan et al., 1995). All except IL-1 are elevated in patients with endometriosis, and so far no cyclic variations in the expression of any of these factors have been documented.

Another possible source of this factor is from uterine endometrium. We know from previous work in our laboratory that mRNA encoding for VEGF is upregulated during menstruation in normal endometrium (Charnock-Jones et al., 1993). In addition, it is known that retrograde menstruation occurs in the vast majority of women (Blumenkrantz et al., 1961); therefore it is possible that VEGF may gain entry to the peritoneal cavity via this route and that eutopic endometrium in endometriosis may produce more VEGF. It is also possible that established active endometriotic tissue itself may secrete VEGF. Since endometriotic tissue contains functionally active oestrogen/progesterone receptors (Berquist et al., 1991; Prentice et al., 1992) and has been shown to secrete a number of factors (Sharpe et al., 1993; Akoum et al., 1995), the presence of these receptors may explain the cycle specific pattern of VEGF seen in endometriosis.

In conclusion, VEGF is present in increased amounts in the peritoneal fluid of women with endometriosis. The consequence of the increased peritoneal fluid VEGF may be the promotion of neovascularization within the peritoneal environment. This may be critical in the pathogenesis of endometriosis through successful implantation and survival of the endometriotic tissue.

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**References**


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