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Angiogenesis: a new theory for endometriosis

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Excessive endometrial angiogenesis is proposed as an important mechanism in the pathogenesis of endometriosis. Evidence is reviewed for the hypothesis that the endometrium of women with endometriosis has an increased capacity to proliferate, implant and grow in the peritoneal cavity. Data is summarized indicating that the endometrium of patients with endometriosis shows enhanced endothelial cell proliferation. Results are also reviewed indicating that the cell adhesion molecule integrin $\alpha_v\beta_3$ is expressed in more blood vessels in the endometrium of women with endometriosis when compared with normal women. Taken together, these results provide evidence for increased endometrial angiogenesis in women with endometriosis when compared with normal subjects. Endometriosis is one of the family of angiogenic diseases. Other angiogenic diseases include solid tumours, rheumatoid arthritis, psoriasis and diabetic retanopathy. Excessive endometrial angiogenesis suggests novel new medical treatments for endometriosis aimed at the inhibition of angiogenesis.

Key words: angiogenesis/endometriosis/endothelial cell proliferation/integrins

Introduction

The most widely accepted hypothesis for the development of endometriosis is retrograde menstruation (Sampson, 1927). However, this phenomenon has been demonstrated in 90% of all women undergoing laparoscopy during menses (Halme *et al.*, 1984), suggesting that retrograde menstruation facilitates transport of endometrial tissue to the peritoneal cavity but that some other factor renders certain women susceptible to the implantation and growth of this ectopic endometrium. Theories proposed to account for this susceptibility include genetic predisposition (Lamb *et al.*, 1986), a greater amount of retrograde menstruation (Cramer *et al.*, 1986), an altered peritoneal environment (Ramey and Archer, 1993), or an immunological susceptibility (Oosterlynck *et al.*, 1991).

Surprisingly little attention has focussed on the possible role of the intrauterine endometrium in the pathogenesis of endometriosis. We propose that women develop endometriosis because of abnormalities inherent in their ectopic or intrauterine endometrium. Endometrium is unique among adult tissues because it undergoes intense proliferation, secretion, regression, and regeneration during each menstrual cycle. It is plausible that subtle alterations in this complex series of events could lead to pathological proliferation of endometrial tissue. Our hypothesis is that the endometrium of women with endometriosis has an increased capacity to proliferate and, therefore, implant and grow in the peritoneal cavity.

Central to the complex changes occurring in the endometrium throughout the menstrual cycle is angiogenesis or new blood vessel growth. Three separate episodes of angiogenesis have been described in primate endometrium (Rogers *et al.*, 1992). These comprise post-menstrual repair during the early proliferative phase, mid-proliferative vessel growth under the influence of oestrogen, and finally growth of coiled arterioles under the influence of progesterone in the secretory phase.

There is evidence that angiogenesis may be important in the pathophysiology of endometriosis. It is obvious at laparoscopy that most endometriotic lesions are surrounded by peritoneal blood vessels. The peritoneal fluid (PF) of women with endometriosis contains more angiogenic factors than that of women without the disease (Oosterlynck *et al.*, 1993). Histological studies and animal experiments

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have shown that endometriotic deposits derive their blood supply from the surrounding microvasculature (Nieminen, 1962), that larger deposits grow in areas with a rich blood supply (Vernon and Wilson, 1985), and that the more vascular endometriotic deposits are more active (Vernon and Wilson, 1985).

Integrin $\alpha_v\beta_3$ is found on many cell types including endothelial cells (EC), and is known to bind at least seven different ligands; vitronectin, fibrinogen, von Willibrand factor, fibronectin, thrombospondin, laminin and thrombin (Dejana *et al.*, 1993; Luscinskas and Fawler, 1994). Recent studies have shown that the ligation of integrin $\alpha_v\beta_3$ was required for the survival and maturation of newly formed blood vessels, with expression increasing four-fold during angiogenesis in chick chorionallantoic membrane (Brooks *et al*., 1994a). The growth of new blood vessels induced by basic fibroblast growth factor (bFGF), tumour necrosis factor-α (TNF-α) and human melanoma fragments was blocked by monoclonal antibody (mAb) to a $\alpha_v\beta_3$, but the mAb had no effect on pre-existing vessels (Brooks *et al*., 1994b; Friedlander *et al*., 1995). Other studies also support a role for integrin $\alpha_v\beta_3$ as an angiogenic marker. Sepp *et al*. (1994) showed that the expression of $\alpha_v\beta_3$ integrin on human microvascular EC could be increased by bFGF. Enenstein *et al.* (1994) documented that α_v subunit concentration, which was normally low in the microvasculature, was increased on angiogenic vascular sprouts. A recent study has provided evidence that integrin $\alpha_v\beta_3$ co-localizes with matrix metalloproteinase (MMP)-2, a family of zinc-requiring matrix-degrading enzymes, on angiogenic blood vessels *in vivo* (Brooks *et al*., 1996).

In this study we review immunohistochemical techniques which have compared EC proliferation, chemotaxis and the expression of integrin $\alpha_v\beta_3$ in the endometrium from women with and without endometriosis.

Endometrial biopsy and immunohistochemical studies

To study endothelial cell proliferation in the endometrium of women with and without endometriosis, endometrium was sampled at dilation and curettage with 30 women with endometriosis and 27 normal controls. Endometriosis was diagnosed at concurrent laparoscopy and staged according to the revised American Fertility Society score (AFS, 1985). In all, 10 women had stage I disease; 12 had stage II; six had stage III, and two had stage IV. The overall laparoscopic appearance of endometriosis also was described as predominantly red lesions (n = 8), predominantly black lesions (n = 10), combined red and black (n = 8), or deep (lesions infiltrating deeply into the vagina or rectovaginal septum, n = 4). Controls were women with a normal pelvis at laparoscopy performed for tubal sterilization or for infertility where the sole cause of infertility was a male factor. All women had normal menstrual cycles and had not received any hormonal therapy, used an intrauterine device, been pregnant, or lactated during the previous 2 months. Institutional ethical approval was obtained and all subjects gave informed consent.

Endometrial biopsies were fixed in 10% buffered formalin for 4–6 h, washed in phosphate-buffered saline, and processed through increasing concentrations of ethanol to Safsolvent (Ajax Chemicals, Auburn, New South Wales, Australia) and finally to wax (Paraplast X-TRA; Oxford Labware, St. Louis, MO, USA). Serial 5 μm sections were prepared for: (i) immunohistochemistry; and (ii) haematoxylin and eosin (H&E) staining. Sections were dated by a gynaecological pathologist according to the histological criteria of Noyes *et al.* (1950) and classified as menstrual, proliferative or secretory.

Samples incorporating the basalis layer of endometrium were excluded from the analyses because of known differences in cell proliferation between the functionalis and basalis layers of the endometrium (Ferenczy *et al.*, 1979).

Immunohistochemistry involved a double staining technique. Endothelial cells were stained using a mouse monoclonal antibody against CD34 antigen, a glycoprotein expressed on the luminal surface of EC (Traweek *et al.*, 1991) (clone QBEND/10; Serotex, Oxford, UK). All proliferating nuclei in the section (i.e. stromal and epithelial cells in addition to EC) were stained with a mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA) (Clone PC10; Novocastra Laboratories, Newcastle upon Tyne, UK). PCNA is a nuclear protein whose expression peaks during the S phase of the cell cycle and which has been used previously to identify proliferating cells (Goodger and Rogers, 1994). The staining protocol has been described in detail elsewhere. Briefly, a Histostain-DS Kit (Zymed Laboratories Inc, San Francisco, CA, USA) was used. This involves a peroxidase and biotin–streptavidin detection system. PCNA is stained red with aminoethylcarbazole chromogen and CD34 is stained blue with alkaline phosphatase blue. A separate section was stained with CD34 (in this case stained red) and H&E to identify the total number of EC nuclei. In each run, a control section (early proliferative to mid-proliferative phase endometrium) of known immunostaining intensity was included as a positive interassay control. Negative controls of mouse immunoglobulin of the same immunoglobulin class and concentration as the primary antibody were used instead of the primary antibody.

Endothelial cell proliferation was quantified by calculating an EC proliferation index for each section, i.e. the number of proliferating EC per mm^2 divided by the total number of ECs per mm^2, expressed as a percentage. Cells were counted with the aid of a microscope at×400 linked via a colour CCD video camera (MW-FI5e, Panasonic; Matsushita Electrical Industrial Co Ltd, Osaka, Japan) to a personal computer (Amiga model 2000; Commodore, Lane Cove, New South Wales, Australia) with stereology software (Grid version 2. 1; Graffiti Data, Silkeborg, Denmark).

Nuclear proliferation in glandular and luminal epithelial cells and in stromal cells (excluding EC) was scored semiquantitatively. For each tissue compartment, the fraction of positively stained cells was expressed as 0 (no staining), 1 (fewer than one out of 250 cells stained), 2 (fewer than one out of 50), 3 (fewer than one out of 10) or 4 (more than one out of 10 cells stained) and the intensity of staining was graded as 0 (none), I (mild), 2 (moderate), or 3 (intense). These two scores were then multiplied to derive a total immunostaining score (Bergqvist *et al.*, 1993). The author who scored these sections and who counted the ECs as described above was blinded as to which sections were from women with endometriosis and which were from control women.

For integrin studies, anti-integrin ($\alpha_v\beta_3$ clone LM609; Chemicon, CA USA) was applied at 0.8 μg/ml at 4°C (Hii and Rogers, 1997). All vessel profiles expressing integrin $\alpha_v\beta_3$ were counted in 10 fields per section at ×400 magnification of each endometrial tissue. Each field had an area of 0.019 mm^2. Endothelial expression of integrin $\alpha_v\beta_3$ was further confirmed at ×1000 magnification under oil immersion. Total vessel number was quantified from a CD34 antibody-stained serial section. Results were expressed as percentage of vessels expressing integrin $\alpha_v\beta_3$. The final result for each tissue was the mean of all 10 fields. Integrin $\alpha_v\beta_3$ results for each subject were correlated with EC proliferation.

Data were analysed using Excel (version 5.0; Microsoft Corporation, Redmond, WS, USA) and Minitab (Minitab Incorporated, University Park, PA, USA) software. Comparisons were performed using the Mann–Whitney *U*-test for non-parametric data. Correlations were performed using regression analysis. $P \leq 0.05$ was considered to be significant.

Proliferative endometrium in endometriosis

The mean EC proliferative index was significantly greater in those women with endometriosis compared with controls (mean ± SEM, 12.9 ± 2.6 versus 4.0 ± 3.2, P = 0.0014). However there was wide interpatient variability particularly among women with endometriosis. In this group, 11 out of 30 women had proliferative indices exceeding the mean value for the group (12.9). Only one control endometrium had a proliferative index exceeding this value. If a proliferative index of ≥12 were used as a cut-off point to predict endometriosis, it would have a specificity of 96% and a sensitivity of 40%.

When EC proliferative indices were analysed according to menstrual cycle stage, proliferative phase values showed a significant difference between endometriosis patients and controls. Secretory phase proliferation indices were similar in each group and there were insufficient samples in the menstrual phase to achieve statistically meaningful results.

Epithelial (glandular and luminal) and stromal cells demonstrated significantly higher immunostaining scores in endometriosis patients compared with controls. As with ECs the difference was statistically significant in the proliferative phase of the cycle. Using regression analysis, EC proliferation correlated with glandular proliferation in the endometriosis group (Pearson $r = 0.51$, $P = 0.004$) but not in control endometrium (Pearson $r = 0.01$, $P = 0.98$). There was no correlation between EC proliferation and either stromal or luminal proliferation.

Endothelial cell proliferation showed no significant variation throughout the menstrual cycle in either endometriosis or control endometrium. In endometrium from endometriosis patients, however, mean proliferation scores were significantly higher in the proliferative phase compared with the secretory phase in glandular epithelial cells (mean ± SEM, 8.7 ± 0.8 versus 3.6 ± 1.0, P = 0.002), luminal epithelial cells (6.7 ± 1.0 versus 0.9 ± 0.2, P = 0.0002), and stromal cells (4.2 ± 0.6 versus 2.5 ± 0.4, P = 0.04).

Proliferation scores in the endometriosis group were analysed according to various clinical characteristics. None of the endometrial parameters studied (EC proliferative indices and immunostaining scores for glandular and luminal epithelial and stromal cells) showed any significant difference between women with endometriosis who presented with pain or asymptomatic infertility or between those with revised AFS stages I–IV of endometriosis. When endometrial data were analysed according to general laparoscopic appearance of endometriosis (i.e. red, black, mixed, or deep), three of four women with deeply infiltrating disease had very high EC proliferative indices (>32) in their uterine endometrium, but this was not significantly different from the other groups, possibly because of small numbers.

The percentage of endometrial vessels expressing integrin $\alpha_v\beta_3$ in endometriosis subjects was significantly increased above controls (53 versus 36%; $P = 0.0001$). Statistical analysis showed that the difference was more marked in the secretory phase ($P = 0.016$).

Taken together, these results support the hypothesis that the endometrium of some women with endometriosis is significantly different from that of women without the disease. Using immunohistochemistry, we have demonstrated increased numbers of proliferating ECs as well as increased expression of cell adhesion molecule integrin $\alpha_v\beta_3$ in the endometrium of these women. These findings suggest that the intrauterine endometrium may be central to the pathogenesis of endometriosis.

Conclusions

There is growing evidence that the endometrium of women with endometriosis is not 'normal'. Endometrium from patients with minimal endometriosis has been shown to secrete more complement-3 than that of patients without endometriosis or those with severe disease (Isaacson *et al.*, 1990). Others have demonstrated an increased presence of complement receptor type-3-positive macrophages infiltrating the proliferative endometrial stroma of patients with mild-to-moderate endometriosis (Isaacson *et al.*, 1990). These authors also showed that endometrial extracts from patients with endometriosis have enhanced chemotactic activity for neutrophils and macrophages in the proliferative phase of the cycle compared with controls. Oosterlynck *et al.* (1991) demonstrated that the endometrium of women with endometriosis is more resistant to the cytotoxic effect of heterologous lymphocytes than that of control women. Another study has shown significant reduction in glycogen content and glycogen synthetase in the endometrium of women with endometriosis (Ishihara *et al.*, 1991). Menstrual fluid CA-125 values were almost three times greater in women with endometriosis than in controls (Takahashi *et al.*, 1990), while a recent study employing endometrial cell culture has demonstrated two to four times more in-vitro CA-125 production by endometrium from women with endometriosis compared with that from control women (McBean and Brunsted, 1993).

This review suggests that the endometrium in women with endometriosis has enhanced proliferation and increased ability to implant and survive in ectopic locations. The concept of enhanced endothelial proliferation is attractive since angiogenesis is essential if endometrium is to survive outside the uterus. Excessive angiogenesis is seen not only in endometriosis but in a variety of other diseases (Table I).

Table I. Angiogenic diseases

Disease
Endometriosis
Solid tumours
Rheumatoid arthritis
Psoriasis
Diabetic retinopathy

This concept of a group of angiogenic diseases indicates novel approaches to the medical management of these various conditions. In particular, the number of anti-angiogenic agents have been identified *in vitro* and may in some cases, are now entering phase I clinical trials. These potential medicines include various fragments of known proteins. For example, angiostatin is a 38 kDa fragment of plasminogen. It selectively inhibits endothelial proliferation. This appears to induce dormancy in murine tumours (O'Reilly *et al.*, 1997). Endostatin is a related 20 kDa fragment of collagen 18 which also appears to be a potent anti-angiogenic substance. Prolactin is synthesized and released from decidualized human endometrium and the 16 kDa and terminal prolactin inhibits endothelial cell migration. It has also be demonstrated that 16 kDa, in human prolactin also increases plasminogen activator inhibitor-1 expression in endothelial cells (Bentzien *et al*, 1997; Healy *et al.*, 1977; Lee and Weiner, 1997).

One potent inhibitor of angiogenesis currently being evaluated is combretastatin. This agent is purified tree bark of a native Australian tree, the Kakadu plum. A final anti-angiogenic agent which may prove clinically useful are synthetic analogues of Fumagillin (TNP-470/AGM-1470). This agent is currently undergoing Phase I clinical trials and appears to inhibit angiogenesis by inhibiting DNA synthesis of vascular smooth muscle cells (Parangi *et al.*, 1996).

If any of the above potential medicines for endometriosis are worthy of further study, stained release micro delivery systems delivered via laparoscopy to areas of endometriosis would seem possible. The angiogenesis theory in endometriosis seems worthwhile since this hypothesis could be tested once safe and effective anti-angiogenic medicines become available.

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