Published in final edited form as:

Reprod Sci. 2009 February ; 16(2): 140–146. doi:10.1177/1933719108324893.

Mechanistic and Therapeutic Implications of Angiogenesis in Endometriosis

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Abstract

Like tumor metastases, endometriotic implants require neovascularization to proliferate and invade into ectopic sites within the host. Endometrial tissue, with its robust stem cell populations and remarkable regenerative capabilities, is a rich source of proangiogenic factors. Among the most potent and extensively studied of these proteins, vascular endothelial growth factor has emerged as a critical vasculogenic regulator in endometriosis. Accordingly, angiogenesis of the nascent endometriotic lesion has become an attractive target for novel medical therapeutics and strategies to inhibit vascular endothelial growth factor action. Vascular endothelial growth factor gene regulation in endometrial and endometriosis cells by nuclear receptors, other transcription factors, and also by infiltrating immune cells is emphasized. New data showing that oxidative and endoplasmic reticulum stress increase vascular endothelial growth factor expression are provided. Finally, we review the clinical implications of angiogenesis in this condition and propose potential antiangiogenic therapies that may become useful in the control or eradication of endometriotic lesions.

Keywords

VEGF; uterus; endothelial

Etiology of Endometriosis

Endometriosis is a common gynecological disorder defined by the proliferation of endometrial glands and stroma outside the confines of the uterine cavity. The disease affects 5% to 10% of all reproductive-aged women and the prevalence rises to 20% to 50% in infertile women. Genetic factors influence susceptibility to endometriosis; however, the mode of hereditary transmission is complex and likely multifactorial.¹ Sib-pair linkage analyses in 1176 families of affected British and Australian women identified a susceptibility locus on chromosome 10q26 locus.² A number of other genetic aberrancies, particularly single nucleotide polymorphisms in relevant nuclear receptors (eg, estrogen receptor- α ³ and estrogen receptor- β ⁴), cytokines,⁵ and even in the vascular endothelial growth factor (VEGF) coding sequence per se in Korean⁶ and South Indian populations⁷ are associated with an increased odds ratio of endometriosis prevalence.

Arguments persist over the histogenic etiology of endometriosis; however, the implantation hypothesis put forward by Sampson more than 80 years ago is the most widely accepted.⁸ Retrograde menstruation,⁹ with subsequent intraperitoneal spillage¹⁰ and mesothelial

attachment and invasion of viable endometrial cells¹¹ is becoming increasingly accepted as the most plausible sequence of events leading to lesion establishment.

Lesion Vascularization

Using the analogy of tumor metastasis,¹² we postulated that angiogenic potential of the derivative endometrium or the intraperitoneal environment would be expected to influence lesion establishment.¹³ Indeed, endometriotic implants often are surrounded by a web of blood vessels (Figure 1) and extrapelvic endometriosis, while rare, typically occurs in well-vascularized organs.¹⁴ Microscopic studies have confirmed neovascularization around and within endometriosis lesions.¹⁵ In human–mouse xenograft models of endometriosis, the VEGF that stimulates angiogenesis is derived from the human endometrial explants, whereas the vasculature supplying the growing human lesions was demonstrated to be of murine origin, based on species-specific antibodies.¹⁶

The process by which angiogenesis occurs within endometriotic implants is not known, but 3 general mechanisms have been proposed: sprouting, elongation, and intussusception. In normal eutopic endometrium, where prominent capillary growth occurs in the late proliferative and early-mid secretory phases of the cycle, vessel elongation is the predominant mechanism.¹⁷ This corresponds to the time in the ovulatory cycle in which human endometrial VEGF messenger RNA (mRNA) reaches its maximum production.¹⁸ The extension of new vessel branches from preexisting capillaries requires proteolytic degradation of extracellular matrix, proliferation and migration of endothelial cells, and ultimately the formation of patent capillary tubules supplying the angiogenic stimulus.¹⁹ Several growth factors and cytokines have been shown to exert chemotactic and proliferative effects on endothelial cells and their surrounding pericytes and many of these have been reviewed extensively.^{13,20} Among the angiogenic proteins synthesized by endometrial and endometriosis cells, VEGF is the prototypical, most potent and most highly regulated endothelial cell mitogen. It also is an important vascular permeability factor.²¹

Localization of Vegf in Endometrium and Endometriosis

Vascular endothelial growth factor immunostaining was observed predominantly in the epithelium of endometriotic implants, although stromal cells also express this protein.¹⁸ Vascular endothelial growth factor concentrations were found to be particularly high in hemorrhagic red implants²² and endometriomas.²³ High concentrations of soluble VEGF accumulates in the pelvic fluid of patients with endometriosis. In addition to its production by endometriotic implants,¹⁸ activated peritoneal macrophages and neutrophils also have the capacity to synthesize and secrete VEGF.^{24,25}

Vascular Endothelial Growth Factor Gene Regulation

The regulation of bioavailable VEGF is controlled at the transcriptional and posttranscriptional levels. Long segments of the human VEGF gene promoter have been cloned and several important *cis*-regulatory elements have been mapped (Figure 2). Our group identified a variant estrogen responsive element (ERE) at –1525 bp upstream of the transcription start site²⁶ that is responsible for the 3- to 5-fold induction of VEGF mRNA by estrogens in human endometrial cells *in vitro*.^{18,27} The same genetic element was confirmed as the dominant ERE regulating VEGF in human breast cancer cells.²⁸ In the rat, estrogen induction of VEGF gene expression appears to be mediated by different elements,²⁹ including an hypoxia inducible factor 1 α motif.³⁰ We also identified 3 progesterone receptor half-sites that appear to regulate endometrial VEGF expression as composite elements.³¹ These studies support the obligatory role of ovarian steroids on the development of endometriosis implants in primate models³² and the clinical

observation that estradiol downregulating doses of gonadotropin-releasing hormone (GnRH) analogs decrease the size and vascularity of endometriosis lesions.¹⁴

A high prevalence of endometriosis was observed in primate colonies exposed to the xenobiotic endocrine disruptor dioxin [2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)]³³ and some human case-control studies support a relationship between exposure to these polychlorinated biphenyl (PCB) industrial contaminants and the prevalence of lesions.³⁴ Dioxin binds and activates the nuclear aryl hydrocarbon receptor (AhR), which in turn induces gene transcription. Although direct effects of liganded AhR on the VEGF gene promoter have not been reported, there is indirect evidence to suggest that AhR can activate glycodefin in endometrial epithelial cells³⁵ and glycodefin is reported to stimulate VEGF expression in these cells.³⁶ This phenomenon is discussed in more detail below.

Other transcription factors, including activator protein (AP)-1, stimulatory protein (Sp)-1 and Sp-3, and CCAAT/enhancer-binding protein (C/EBP)- β have been shown to modulate VEGF transcription in various cells and tissues.³⁷ An important zinc finger transcription factor, encoded by the early growth response (Egr)-1 gene, stimulates VEGF expression.³⁸ We discovered that Egr-1 is constitutively upregulated in ectopic and eutopic endometrial tissues from women with endometriosis compared to controls and may be a selective therapeutic target for this condition.³⁹ Other factors known to stimulate VEGF expression are very relevant to the peritoneal environment in endometriosis, and include hypoxia, acidosis, prostaglandin E2, and the inflammatory cytokine interleukin-1 β (IL-1 β).⁴⁰⁻⁴²

At least 5 distinct mRNA species arise via differential splicing of 8 exons in the primary VEGF transcript. In the endometrium, mRNAs encoding the 165 and 121 amino acid (aa) variants appear to be the predominant isoforms^{43,44} generating glycosylated homodimeric proteins of about 45 and 35 kd, respectively. Longer forms of VEGF (189 and 206 aa) are not actively secreted into the extracellular milieu. Instead, their relatively basic carboxyl-termini cause them to reversibly associate with heparan sulfate proteoglycans of the extracellular matrix, where they are believed to exert juxtacrine effects.⁴⁵ Vascular endothelial growth factor binds to a family of tyrosine kinase receptors, particularly VEGF receptors 1 and 2 (Flt-1 and KDR), leading to dimer formation, autophosphorylation and activation of a cascade of mitogen-activated protein kinases.⁴⁵

Oxidative Stress in Endometriosis

Considerable evidence has accumulated supporting a role for macrophage activation and oxidative stress in the peritoneal environment of women with endometriosis, including the detection of oxidatively modified lipoprotein complexes.⁴⁶ In recent studies, incubation of primary endometrial epithelial cells for 24 hours in the presence of minimally oxidized low-density lipoprotein (LDL) increased VEGF secretion by $75 \pm 17\%$ ($P < .05$) whereas no significant effect was noted following treatment with unmodified LDL (an increase of $35 \pm 17\%$).³⁶ We noted a concomitant increase in endometrial cell glycodefin expression under these conditions and had shown that peptides corresponding to the latter protein can stimulate angiogenesis in vitro.⁴⁷ Thus, compounds like progestins⁴⁸ and dioxin³⁵ that modulate glycodefin expression might secondarily affect VEGF production.

Endoplasmic Reticulum Stress in Endometriosis

Less than 20 years ago, a new type of cellular injury or stress was described that may be relevant to the condition of endometriosis. Endoplasmic reticulum (ER) stress occurs predominantly in specialized epithelia with dynamic secretory function. Unlike cytoplasmic proteins, proteins secreted to the cell surface typically require proper glycosylation, folding and association with chaperone partners for exocytotic delivery. Protein misfolding can occur under conditions of

ER stress, which include nuclear factor-kappa B (NF- κ B) activation due to active cytokine stimulation and lipid peroxidation. Both of these phenomena are prominent in endometriosis. The unfolded protein response results in the activation of amino acid transport and new chaperone protein synthesis, but also in the induction of proapoptotic genes.⁴⁹ In our laboratory, we used tunicamycin to induce ER stress by interfering with protein glycosylation within the ER lumen (Figure 3). Endometrial stromal cells were incubated without (control) or with 2.5 μ g/mL tunicamycin for 16 hours. Vascular endothelial growth factor secreted into the culture supernatant was measured using a standardized enzyme-linked immunosorbent assay (ELISA) and VEGF mRNA was quantified in cell lysates by quantitative real-time reverse transcription polymerase chain reaction (PCR; qRT-PCR) standardized relative to glyceraldehyde 3 phosphate dehydrogenase (GAPDH) mRNA and normalized to control cultures (1-fold). Tunicamycin-induced ER stress led to a 48-fold increase in VEGF protein and a 9-fold increase in VEGF mRNA accumulation. The immunoglobulin-binding chaperone protein GRP78 was used as a positive control in these experiments, and its mRNA increased 45-fold in response to the tunicamycin treatment, as reported by others in breast carcinoma cells.⁵⁰

Clinical Correlations

Oosterlynck et al⁵¹ first reported a relationship between peritoneal angiogenic activity and endometriosis using the chick chorioallantoic membrane as a bioassay. We confirmed a similar correlation between endometriosis diagnosis and pelvic fluid angiogenic activity, analyzed using an autologous human endothelial cell [³H]thymidine incorporation model.¹³ However, a slightly different pattern was noted when we specifically quantified VEGF with a newly developed ELISA. Advanced endometriosis (American Society for Reproductive Medicine; ASRM stages III-IV) was associated with elevated peritoneal VEGF concentrations, but mild cases (ASRM I-II) were indistinguishable from controls without laparoscopic evidence of disease.¹⁸ Pelvic fluid VEGF levels also were noted by Kpker et al⁵² to correlate with advanced disease stage. Despite higher levels of VEGF in the peritoneal fluid, serum VEGF concentrations are not increased in patients with endometriosis^{53,54} nor are menstrual effluent concentrations elevated in those women relative to controls.⁵⁵

A recent report of women with ovarian endometriomas showed high correlations between histological microvascular density within the ovarian cysts and power-Doppler blood flow measurements. When the patients were stratified according to their degree of dysmenorrhea and pelvic pain symptoms, a highly significant and direct association with blood flow was noted.⁵⁶ Previous studies also have documented elevated concentrations of VEGF in ovarian endometriomas.²³

Vascular endothelial growth factor expression appears to be augmented in another highly symptomatic presentation of endometriosis, women with deeply invasive rectovaginal disease. Machado et al noted a strong correlation between VEGF expression and microvascular density in this special subset of patients.⁵⁷ No obvious explanations exist to explain the apparently emerging correlation between angiogenesis and pain, but new findings of enervation of endometriotic lesions by sensory fibers⁵⁸ raises the hypothesis that neurovascular growth factor signals may simultaneously stimulate the recruitment of nerves as well as new capillaries to the nascent lesion.

Antiangiogenic Therapies for Endometriosis

At the present time, the authors are aware of only 1 clinical trial of an antiangiogenic agent for the treatment of pain associated with ovarian endometrioma. In an abstract presented at the 2002 meeting of the ASRM (Seattle), Scarpellini et al⁵⁹ reported that 8 of 10 women with stage IV endometriosis achieved remission of pain and resolution of ovarian cysts following a 6-

month course of combined goserelin (GnRH analog) and thalidomide (300 mg/d). In some women, the symptom relief persisted following discontinuation of the GnRH analog while the patients received only thalidomide therapy. These promising pilot findings are well supported by a series of studies examining antiangiogenesis in preclinical models of endometriosis. Thalidomide was shown to inhibit IL-8, an angiogenic cytokine, in endometriotic stromal cells via interference with the transcription factor NF- κ B.⁶⁰ Neutralizing VEGF antibodies and soluble VEGF receptors prevented endometrial xenograft growth and angiogenesis in athymic nude mice¹⁶ and similar findings were observed when immunocompetent mice given intraperitoneal uterine autografts were cotreated with endostatin, a cleavage product of collagen XVIII.⁶¹ At the most recent meeting of the ASRM (Washington, D.C.), the dopamine agonist cabergoline was reported to inhibit neoangiogenesis in the nude mouse xenograft model.⁶² Human endometrial fragments grafted onto chick chorioallantoic membranes also have been used to evaluate potential angiostatic therapeutics, including endostatin, anti-VEGF antibodies and the antibiotic TNP470.⁶³ In an abstract published in 2004, anti-VEGF antibodies prevented endometriosis in a Rhesus model of endometriosis.⁶⁴ Thiazolidenediones, peroxisome proliferator-activated receptor- γ (PPAR- γ) agonists that we have shown can inhibit endometrial VEGF gene expression in vitro,⁶⁵ reduced the surface area of endometriotic lesions in baboons with surgically induced endometriosis.⁶⁶

Summary

Endometriosis is a common disease resulting from a dysregulated interaction between exfoliated menstrual endometrium and host tissue responses that allow ectopic lesions to implant and survive. We propose that a complex network of locally produced steroids, cytokines, oxygen free radicals, and possibly environmental toxins induce lesion growth via neovascularization. Vascular endothelial growth factor is a key mediator of this effect, promoting the recruitment of capillaries toward the growing lesions. Microvascular density and functional blood flow appear to correlate directly with pain symptoms, particularly in ovarian endometriomas and deeply invasive rectovaginal disease. Future therapeutic strategies to target VEGF have the potential to block the establishment or progressive growth and invasion of endometriotic lesions. However, we must not ignore the likely teratogenic actions of these same antiangiogenic drugs and cotreatment with effective contraceptives is prudent in reproductive-age women.

Acknowledgments

This work was supported by NIH grants HD33238, HD37321, and HD44008.

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Figure 1.
Laparoscopic photograph of endometriotic implants on the peritoneum overlying the uterosacral ligament.

Figure 2.

The *cis*-acting sequences in the human VEGF gene promoter. The genomic region extending from -2274 to +745 bp, relative to the transcriptional start site was cloned by Mueller et al²⁶ and several key transcriptional elements of the promoter are represented. The positions of the *cis*-regulatory elements are relative but are not spaced precisely. AP-1 = activator protein 1; C/EBP = CCAAT/enhancer binding protein; DR1 = direct repeat 1 (peroxisome proliferator response element and retinoic acid X receptor response element); Egr-1 = early growth response-1; ERE = estrogen response element; PRE/2 = progesterone response element half-site; Smad = this TGF-beta signaling protein derives its name from a contraction of the *C. elegans* “Sma” and the *drosophila* “mothers against decapentaplegic” proteins; Sp1 = stimulatory protein 1; TGF- β = transforming growth factor; VEGF = vascular endothelial growth factor.

Figure 3.

Endoplasmic reticulum stress results in VEGF upregulation. Endometrial stromal cells were incubated without (control) or with 2.5 µg/mL tunicamycin (Tun) for 16 hours to mimic conditions of ER stress. Vascular endothelial growth factor secreted into the culture supernatant was measured using a standardized ELISA (left ordinate) and VEGF mRNA was quantified in cell lysates by quantitative real-time reverse transcription PCR (qRT-PCR) standardized relative to GAPDH mRNA and normalized to control cultures (right ordinate). A large increase in GRP78 mRNA expression was noted as a positive control for ER stress (described in the text). ER = endoplasmic reticulum; ELISA = enzyme-linked immunosorbent assay; GAPDH = glyceraldehydes-3 phosphate dehydrogenase; mRNA = messenger RNA; PCR = polymerase chain reaction; VEGF = vascular endothelial growth factor.