Vascular density and distribution of vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 (Flk-1) are significantly higher in patients with deeply infiltrating endometriosis affecting the rectum

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Objective: To analyze vascular density and immunolocalization of angiogenic vascular endothelial growth factor (VEGF) and its receptor Flk-1 in the proliferative and secretory eutopic human endometrium and in three different sites of endometriosis: the ovary, bladder, and rectum.
Design: Prospective study.
Setting: University hospital.
Patient(s): Thirty women with endometriosis (10 ovarian, 10 bladder, 10 rectal) and 32 control women (10 proliferative endometrium, 10 secretory endometrium, 4 normal ovary, 4 normal bladder, 4 normal rectum).
Intervention(s): Normal endometrial samples were obtained from women during laparoscopic ablation of subserous myoma, and biopsy specimens of endometriosis were obtained from patients undergoing surgery for the diagnosis and treatment of endometriosis. Normal tissues of ovary, bladder, and rectum were obtained from these organs beside the lesions of endometriosis.
Main Outcome Measure(s): Blood vessels were quantified according to the number of von Willebrand factor–positive endothelial cells. The VEGF and Flk-1 distribution were evaluated semiquantitatively by immunohistochemical staining.
Result(s): More blood vessels were found in cases of endometriosis, particularly rectal endometriosis, compared with the respective control samples and with the eutopic endometrium, and they were localized in endometrial stroma around the glands. The VEGF and Flk-1 expression levels were also higher in cases of endometriosis, especially rectal endometriosis.
Conclusion(s): Vascularization and VEGF and Flk-1 expression are significantly higher in deeply infiltrating endometriosis affecting the rectum, reinforcing the hypothesis that antiangiogenesis therapy may constitute a new modality of treatment, especially in cases of deep endometriosis involving the rectum. (Fertil Steril® 2008; 90:148–55. ©2008 by American Society for Reproductive Medicine.)

Key Words: Endometriosis, endometrium, VEGF, angiogenesis, vascularization

Endometriosis, defined as the presence of functional endometrium outside the uterine cavity, is a common disease, causing abdominal pain, dysmenorrhea, dyspareunia, and infertility in about 10% of the female population of reproductive age (1). One of the most widely accepted hypothesis with respect to the development of endometriosis is the retrograde menstruation theory (2). In addition to the retrograde flow of exfoliated endometrium, new blood vessels essential for the survival of the endometrial implant, and therefore the development of endometriosis, must be formed (3).

Although the macroscopic observation of superficial peritoneal endometriosis shows a clear abundance of vasculature, few studies have been carried out on the vascularization of endometriotic lesions. Matsuzaki et al.(4), using the CD34 endothelial marker, found no differences in the vascular density between peritoneal endometriotic lesions but reported a difference in the diameter of vessels, with red lesions

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having more vessels of a small diameter and black lesions significantly more vessels of a larger diameter. Studies carried out to evaluate the vascularization of endometriotic lesions based on the number of von Willebrand factor–positive vessels, another important marker of endothelial cell surface, showed an increase in microvessel density in these lesions (5). More recently, using CD31 endothelial cell marker, it was demonstrated that the number of vessels is greater in superficial and deep endometriotic lesions than in endometrium, and that progestin treatment induced microvascular changes, in particular, a reduction of the number of microvessels (6).

Vascular endothelial growth factor (VEGF) is a heparin-binding glycoprotein with potent angiogenic, endothelial cell–specific mitogenic, and vascular permeability activities, and is considered to play a central role in both physiologic and pathologic angiogenesis. The loss of a single VEGF allele has been reported as lethal in the mouse embryo on days 11 to 12 of gestation, because angiogenesis formation was impaired, resulting in several developmental anomalies (7). It has also been suggested that VEGF has three functions in vascular development: to promote endothelial cell proliferation, to maintain the viability of immature blood vessels, and to facilitate the process of pericyte recruitment (8). VEGF binds to one of two tyrosine kinase receptors: the fms-like tyrosine kinase (flt) or the kinase domain receptor (Flk-1) (9). These receptors are found predominately in endothelial cells (10), and activation leads not only to proliferation and an increase in vascular permeability but also to the expression of a number of the proteolytic enzymes involved in the process of angiogenesis (11).

Immunohistochemical and biochemical studies have shown VEGF to be involved in both the pathogenesis and the maintenance of endometriosis, at least with respect to the peritoneal form of the disease (3, 12, 13). Hull et al. (5) found a large number of pericyte-free vessels in endometriotic lesions compared with the superficial eutopic endometrium. Ectopic endometrial tissue triggers an angiogenic response by attracting endothelial cells to migrate into the graft, and, after tubes are formed, pericytes are recruited to stabilize vessels (14).

Assuming that angiogenesis represents one of the crucial steps in the pathogenesis and persistence of endometriotic foci, the aim of the present study was to quantify blood vessels and determine the immunolocalization of the angiogenic factor VEGF and its receptor Flk-1 in the eutopic human endometrium in both phases of the menstrual cycle and in three different sites of endometriosis: the ovary, bladder, and rectum.

MATERIALS AND METHODS

Patients

This study was conducted following approval by the Internal Review Board of the Clementino Fraga Filho Teaching Hospital of the Federal University of Rio de Janeiro, Brazil (no. 051/01, 2001).

The patients of control and endometriosis groups were similar and all of them were between menarche and menopause. The mean age of the patients evaluated was 31 years (range 23–40 years). Paraffin blocks from 62 patients were used in the study. Thirty-two blocks consisted of tissue from women without endometriosis: 20 normal endometrium, ten of whom were in the proliferative phase (day 10–12 of the cycle) and ten in the secretory phase of the menstrual cycle (day 21–25 of the cycle), and four normal ovary, four normal bladder, and four normal rectum. The remaining 30 blocks consisted of tissue from patients with endometriosis: ten cases in which the disease was affecting the ovary, ten in which the bladder was affected, and ten cases of rectal endometriosis. All patients with endometriosis had stages III or IV of the disease, according to the American Society for Reproductive Medicine classification. Tissue samples of normal endometrium were obtained during laparoscopic ablation of subserous myoma in patients with proven fertility, and endometrial states were established according to the Noyes criteria (15). The biopsy specimens of endometriosis were collected from patients undergoing videolaparoscopy or laparotomy for the diagnosis and treatment of endometriosis, and the lesions were confirmed according to the presence of endometrial glands and/or stroma. All patients had regular (26–30-day) menstrual cycles, and none had received hormone therapy during the 3 months before surgery or GnRH analogues in the preceding 6 months. Paraffin blocks of normal tissues of ovary, bladder, and rectum were obtained from these organs beside the endometriotic lesions.

Immunohistochemistry

Paraffin-embedded tissue sections (5μm thick) were cut, placed on silane-treated slides, and maintained at room temperature. After dewaxing, sections were treated with a solution of 3% H_2O_2 in 0.01 mol/L phosphate-buffere saline (PBS), pH 7.5, to inhibit endogenous peroxidase activity. The slides were then immersed in 10 mmol/L citrate buffer (pH 6.0) and heated in a microwave oven for 5 minutes to retrieve masked antigens; to reduce nonspecific antibody binding, the sections were then incubated with PBS containing a 10% solution of normal goat serum and 5% bovine serum albumin for 30 minutes. Sections were incubated with the following antibodies: polyclonal antibody A-082 (DakoCytomation, Carpinteria, CA) at 1:200 dilution against von Willebrand-related factor, monoclonal antibody SC-7269 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100 dilution against VEGF, and polyclonal anti-VEGFR-2 (Flk-1) antibody (SC-6251; Santa Cruz Biotechnology) at 1:200 dilution. Incubations were carried out overnight and then revealed using StreptAB Complex/HRP Duet Kit (DakoCytomation), with diaminobenzidine (3,3′-diaminobenzidine tablets; Sigma, St. Louis, MO) as the chromogen and counterstained with hematoxylin. For each case, negative control

slides consisted of sections incubated with the antibody vehicle or nonimmune rabbit or mouse serum.

Histomorphometry

All tissue sections were examined by two blinded observers using a ×40 objective lens of a light microscope (Nikon, Tokyo, Japan) connected to a digital camera (Coolpix 990; Nikon). Ten fields of immunostained section (von Willebrand factor, VEGF, and Flk-1) were chosen at random and captured from each specimen. Quantification was assessed on captured high-quality images (2048 × 1536 pixels buffer) using the Image Pro Plus 4.5.1 (Media Cybernetics, Silver Spring, MD). Data were stored in Adobe Photoshop, version 3.0, to enable uneven illumination and background color to be corrected. The number of transversal sections of von Willebrand factor–stained vessels in the lesions was counted, and the number of vessels per square millimeter of the lesion was calculated, as described by Nap et al. (16).

A semiquantitative evaluation of immunohistochemical staining for VEGF and Flk-1 was performed according to the method described by Donnez et al. (3). This method involves the analysis of the distribution and the intensity of staining within the glandular epithelium or stroma. The histologic scores (H) for VEGF and Flk-1 were calculated using the formula $H = \Sigma Pi$, where i is the intensity ranging from 0 (negative cells) to 3 (high staining intensity) and P is the percentage of stained cells for each given i, with P values of 1, 2, 3, 4, and 5 indicating <15%, 15%–50%, 50%–85%, >85%, and 100% positive-staining cells, respectively. The staining result was expressed as mean ± standard deviations.

Statistical Analyses

All statistical calculations were carried out using the StatXact-5 software program (CYTEL Software Corporation, Cambridge, MA). The differences between groups were calculated using nonparametric analyses (Mann-Whitney U test). A P value of $<.05$ was established as statistically significant.

RESULTS

The distribution of the von Willebrand factor–positive endothelial cells was observed in the vessels localized throughout the stroma, mainly around the glands (Fig. 1). Compared between groups, there were more positive microvessels in the stroma around the glands in samples of rectal endometriosis than in samples of ovarian or bladder endometriosis, in the eutopic endometrium in both menstrual phases, or in samples of normal ovary, bladder, and rectum (Fig. 1). These observations were confirmed by the increase in microvessel density in endometriotic lesions, as shown by the histomorphometry evaluation (Table 1).

The immunoreactivity of VEGF was detected focally in the cytoplasm of glandular epithelial cells and diffusely in stromal cells in both eutopic and ectopic endometrial tissues (Fig. 2). In rectal and bladder endometriosis, immunoreactivity was found predominantly in the glandular epithelium, whereas in ovarian endometriosis immunoreactivity was detected principally in the endometrial stroma. VEGF was more abundant in cases of endometriosis than in the proliferative or secretory eutopic endometrium. In the ectopic endometrium, histologic scores of VEGF were statistically higher in rectal endometriosis (Table 1).

The Flk-1 immunodistribution was similar to that of VEGF. Immunoreactivity was observed in the same cells and was more intense in the cases of endometriosis than in the eutopic endometrium (Fig. 3). Comparing the different sites of endometriosis and the proliferative and secretory endometrium, Flk-1 histologic scores were higher in endometriosis tissue, particularly in cases of rectal endometriosis (Table 1).

DISCUSSION

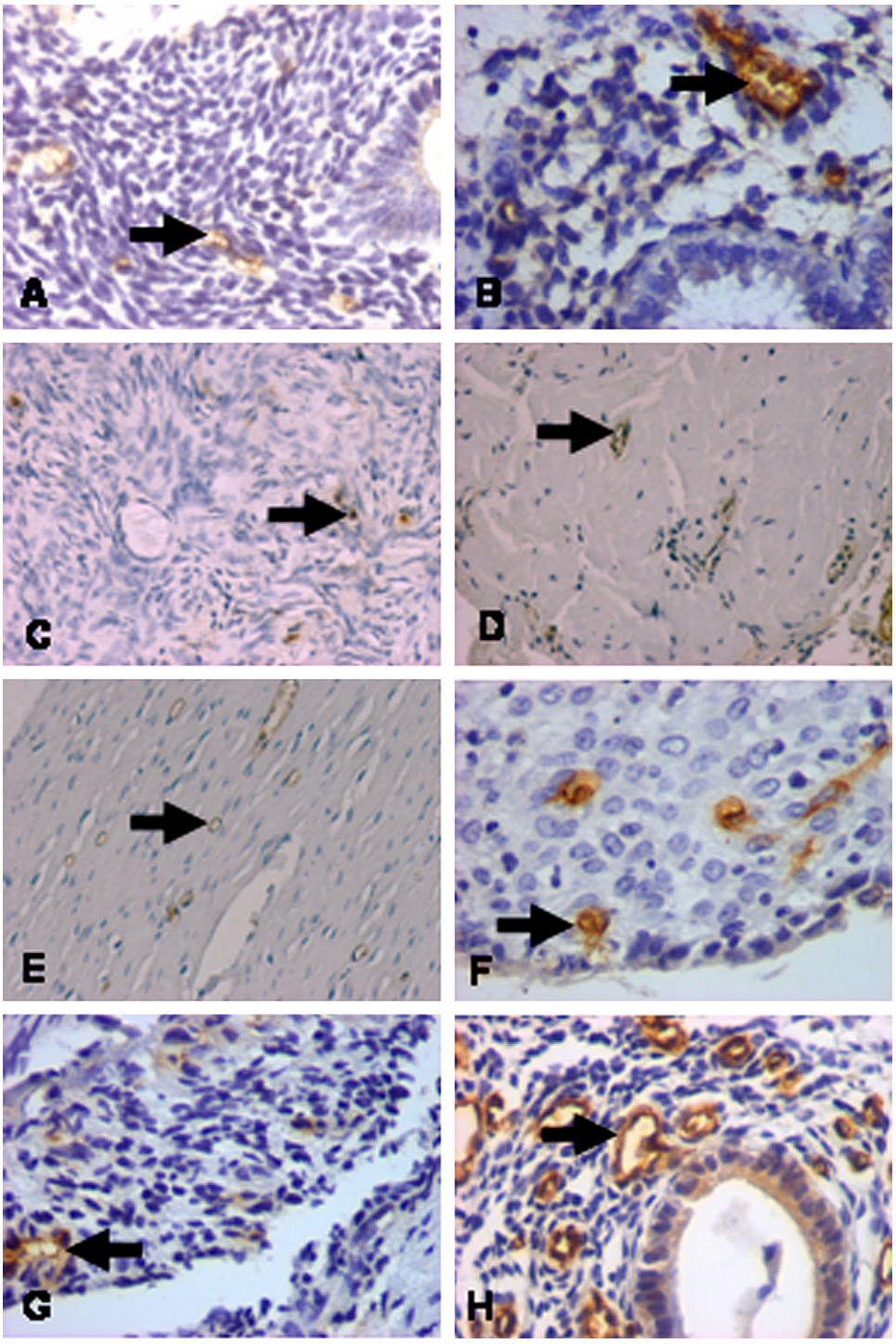
The present study show that angiogenesis and VEGF and Flk-1 expression are significantly higher in deeply infiltrating endometriosis affecting especially the bladder and the rectum. Deeply infiltrating endometriosis is a particular form of the disease that is strongly associated with pelvic pain–related symptoms such as dysmenorrhea, deep dyspareunia, chronic pelvic pain, and painful defecation (17). The intensity of these symptoms seems to be correlated with the depth of infiltration, the location, and the tendency of some of these lesions to invade neuronal structures (18). Koninckx et al. (19) described the characteristics of this form of infiltrative endometriosis and defined the degree of infiltration. Deep endometriosis was defined as a lesion that penetrates more than 5 mm into the subperitoneal tissues. The aggressive behavior of deep endometriosis seems to be associated with failure of the defense mechanisms of peritoneal fluid. It was recently demonstrated that endometriosis is an inflammatory disease involving a relative increase in cytokines characteristic of the pattern of T_H2 immune response (20).

When the exfoliated endometrium enters the peritoneal cavity and becomes attached to the mesothelial layer through attachment proteins such as the cadherins, a process of angiogenesis is essential for further implantation and the development of peritoneal endometriosis (3). The present study is the first to demonstrate the immunohistochemical distribution of von Willebrand factor, VEGF, and Flk-1 at three different sites of endometriosis and in the proliferative and secretory endometrium.

Analysis of the microvessels density assessed by the counting of capillary density demonstrated that angiogenesis is higher in endometriotic lesions compared with respective control ssamples and with the eutopic endometrium, principally in cases of rectal endometriosis. Inan et al. (21) assessed the immunohistochemical localization of von Willebrand factor and CD-34 in ovarian endometriosis and also reported that vascularization was more intense in the endometriotic

FIGURE 1

Von Willebrand Factor staining of vessels in proliferative endometrium (**A**), secretory endometrium (**B**), normal ovary (**C**), normal bladder (**D**), normal rectum (**E**), ovarian endometriosis (**F**), bladder endometriosis (**G**), and rectal endometriosis (**H**). The distribution of the von Willebrand Factor–positive vessels was localized throughout the stroma and concentrated around the glands (*arrows*). Note a significant augmentation of the number of blood vessels in rectal endometriosis. Magnification ×400.



Machado. Vascular density in rectal endometriosis. Fertil Steril 2008.

TABLE 1

Histologic scores of von Willebrand factor (vWF), vascular endothelial growth factor (VEGF), and kinase domain receptor Flk-1 in proliferative and secretory endometrium, in normal ovary, bladder, and rectum, and in cases of ovarian, bladder, and rectal endometriosis. Values are mean ± standard error.

Tissue	vWF (number of vessels/mm^2)	VEGF (% of positive-staining cells)	Flk-1 (% of positive-staining cells)
Proliferative endometrium	5.0 ± 0.74[b]	8.6 ± 1.17[b]	13.5 ± 0.53
Secretory endometrium	3.5 ± 0.70[b]	3.0 ± 1.05[b]	11.0 ± 0.88
Normal ovary	4.8 ± 0.75		
Normal bladder	6.3 ± 0.67		
Normal rectum	10.4 ± 0.89		
Ovarian endometriosis	6.5 ± 0.70[a]	13.3 ± 0.48	14.0 ± 1.43
Bladder endometriosis	8.0 ± 0.63[a]	12.9 ± 0.57	15.2 ± 0.92
Rectal endometriosis	16.5 ± 0.53[a,c]	14.5 ± 0.53[c]	17.9 ± 1.10[c]

[a] $P<.05$ (the scores for vWF are significantly higher in endometriotic specimens compared with respectively control samples).
[b] $P<.05$ (the scores for vWF and VEGF are significantly lower in both phases of the eutopic endometrium compared with endometriotic specimens).
[c] $P<.05$ (the scores are significantly higher in rectal endometriosis for vWF, VEGF, and Flk-1 compared with cases of ovarian and bladder endometriosis).

Machado. Vascular density in rectal endometriosis. Fertil Steril 2008.

lesions compared with the control samples. The present results are also in agreement with those of Groothuis et al. (14), who observed deeply invasive endometriosis in the sigmoid and rectovaginal septum and reported these lesions to be well vascularized. In addition, those authors described most of the blood vessels as being mature, as illustrated by the fact that they were associated with α smooth muscle actin-positive pericytes.

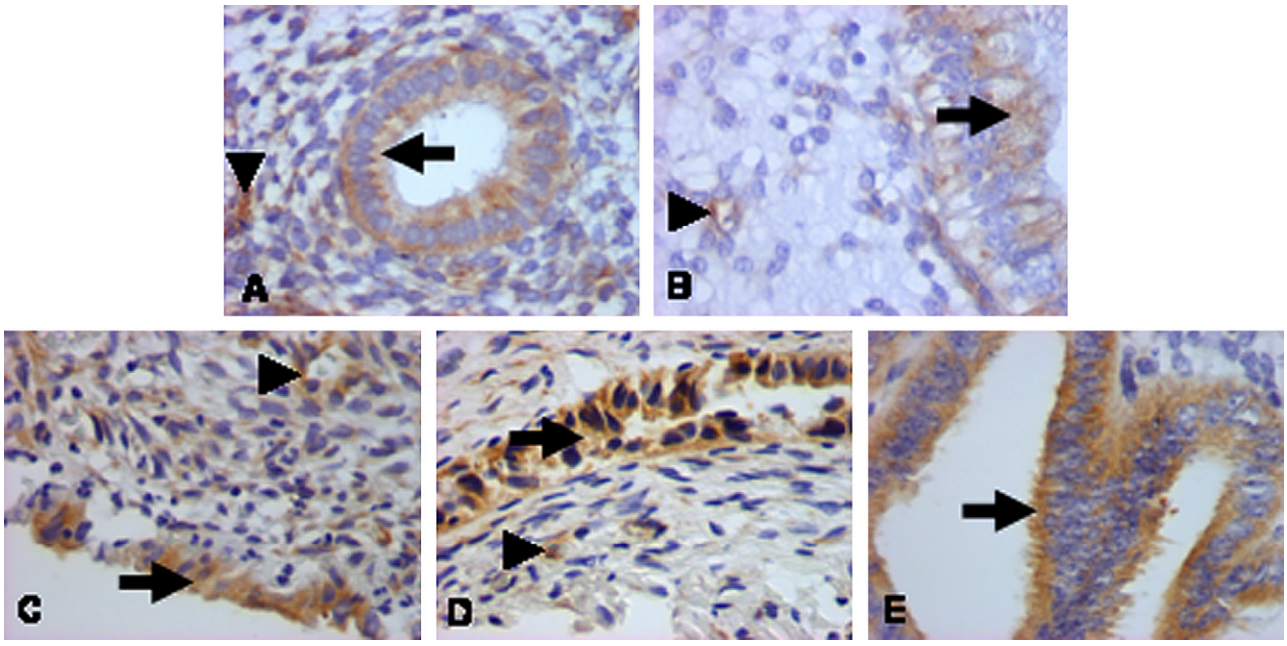
Several authors have described angiogenesis as being an important step in the implantation and development processes through which menstrual endometrial fragments enter the peritoneal cavity, with VEGF as one of the most important mediators of angiogenesis (3, 22, 23). We were able to demonstrate that the immunoexpression of VEGF was more intense in endometriotic lesions compared with control samples and localized predominantly in the glandular epithelium of cases of rectal and bladder endometriosis, with a distribution pattern similar to that seen in the eutopic endometrium. In ovarian endometriosis, the present results are similar to those of Goteri et al. (24), who reported higher immunoreactivity of VEGF being localized in the stroma. This finding probably occurred owing to the particular characteristics of ovarian endometriosis, because a predominance of stroma occurs in these cases. Donnez et al. (3) reported that immunohistochemical expression of VEGF was higher in red peritoneal lesions than in black lesions. The red lesions were characterized by high VEGF levels, which may provoke an increase in the subperitoneal vascular network, thereby facilitating implantation and viability within the retroperitoneal space.

The VEGF receptors flt and Flk-1 are tyrosine kinase receptors expressed predominately in endothelial cells (25). The binding of VEGF to these receptors results in a number of responses, including an increase in mitogenesis, changes in cell morphology, enhanced migration, and the release of various proteolytic enzymes (26). In the present study, the Flk-1 immunodistribution pattern was very similar to that seen with VEGF. The localization of Flk-1 was predominantly in the glandular epithelium of rectal and bladder endometriosis samples and of the eutopic endometrium, whereas in ovarian endometriosis immunoreactivity was detected principally in the endometrial stroma. Histologic scores of Flk-1 were once again significantly higher in cases of rectal endometriosis. These observations are in agreement with the findings of Wang et al. (27), who reported a higher VEGFR (Flt-1 and Flk-1) expression in endometriosis lesions of the peritoneal and abdominal wall which may have been associated with neovascularization.

The use of angiostatic agents in the prevention of the growth of endometriotic lesions has been demonstrated by many authors. Hull et al. (5) showed that antiangiogenesis agents inhibit the growth of explants in an in vivo model of endometriosis by disrupting the vascular supply, and this effect is likely to apply to human disease. Nap et al. (16), using the nude mouse model of endometriosis, demonstrated that inhibitors of angiogenesis effectively interfere with the maintenance and growth of endometriosis. In another study, Nap et al. (28) evaluated the vascularization and endometriosis-like lesion formation after transplantation of human endometrium, together with angiostatic agents, to the chicken chorioallantoic membrane. These agents significantly inhibited this angiogenic response to the presence of human endometrium, and the administration of the anti–human VEGF

FIGURE 2

Proliferative endometrium (**A**), secretory endometrium (**B**), ovarian endometriosis (**C**), bladder endometriosis (**D**), and rectal endometriosis (**E**) immunostained with an antibody against vascular endothelial growth factor (VEGF). The glandular epithelium of the eutopic endometrium in both phases and of the bladder and rectal endometriosis exhibits positive VEGF immunostaining (*arrows*); in ovarian endometriosis the distribution of the immunoperoxidase product is observed in endometrial stroma (*arrow*). The immunoreaction is higher in cases of endometriosis compared with proliferative and secretory endometrium, especially in rectal endometriosis. Note also some blood vessel immumunoreactivity in the stromal tissues (*arrowheads*). Magnification ×400.



Machado. Vascular density in rectal endometriosis. Fertil Steril 2008.

antibody also significantly antagonized the endometrium-induced angiogenic response. Park et al. (29) reported the inhibition of endometriosis development in Rhesus monkeys by blocking the VEGF receptor. The presence of endometriosis was visually and histologically confirmed in 5 of 6 (83%) monkeys in the control group compared with 1 of 6 (17%) monkeys in the study group.

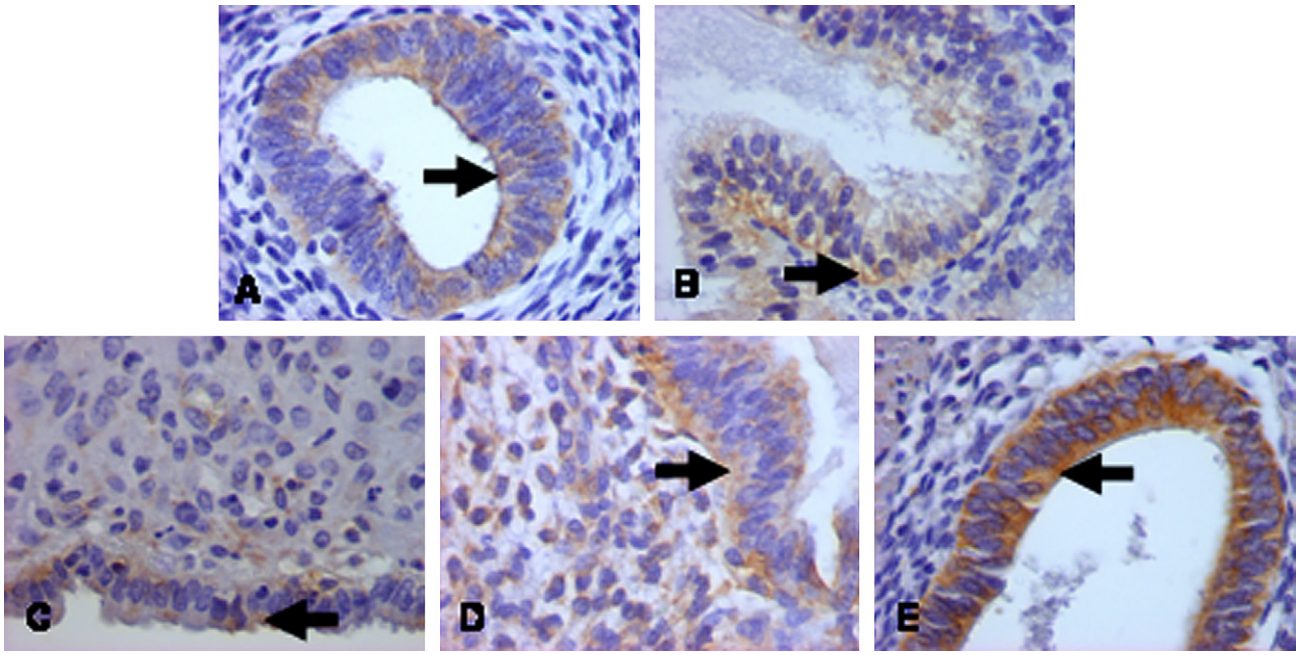
The present results show that angiogenesis is predominantly found in rectal endometriosis compared with bladder and ovarian endometriosis and normal tissues. Although rectal and bladder endometriosis are examples of deeply infiltrating endometriosis, the vascularization and the expression of VEGF and its receptor is significantly higher in cases affecting the rectum. These data indicate that endometriotic lesions are heterogeneous, as suggested by different studies. Heilier et al. (30) showed a significantly different expression of aromatase in the peritoneal and ovarian endometriotic tissues and deep endometriotic nodules of rectovaginal septum, which strengthens the theory of three distinct clinical entities. In addition, it was demonstrated that deep endometriotic lesions do not respond to a progestin treatment as much as endometrium and superficial endometriotic lesions, suggesting a difference in susceptibility of deep lesions (6). Taking into account these different observations, the present results reinforce the idea that deep endometriosis represents a specific pathologic entity and are in agreement with Brosens and Brosens (31), who proposed that the specific microenvironment of deep lesions determines the phenotype of the tissue.

Finally, bowel endometriosis is one of the greatest concerns of specialists treating infiltrative endometriosis, because of the severity of its symptoms, concomitant infiltration of pelvic organs, the possibility of bowel obstruction resulting from the progression of the disease, and the technical difficulties of its surgical removal (32). Abrao et al. (33), in 2006, observed a high frequency of lymph node involvement when endometriosis involved significant areas of the thickness and circumference of the bowel. These studies raise doubts about whether deeply infiltrating endometriosis can still be considered a clinically benign disease, because it shares some of the characteristics of malignancy, such as abnormal morphology, deregulated cell growth, cellular invasion, and neoangiogenesis.

In conclusion, our results confirm that VEGF-induced angiogenesis is a critical aspect of the pathophysiology of this

FIGURE 3

Immunohistochemical staining with vascular endothelial growth factor (VEGF) receptor Flk-1 antibody in proliferative endometrium (**A**), secretory endometrium (**B**), ovarian endometriosis (**C**), bladder endometriosis (**D**), and rectal endometriosis (**E**). The pattern of distribution of the Flk-1 staining is very similar as with the VEGF study, with anti–Flk-1 antibody immunoreactivity in glandular epithelium of the proliferative and secretory endometrium and of the bladder and rectal endometriosis (*arrows*); the endometrial stroma in the ovarian endometriosis also exhibits positive Flk-1 immunostaining (*arrow*). The concentration of the immunostaining with Flk-1 is more abundant in cases of endometriosis compared with the eutopic endometrium in both phases and again higher in rectal endometriosis, as in the VEGF study. Magnification ×400.



Machado. Vascular density in rectal endometriosis. Fertil Steril 2008.

disease. Although VEGF is fundamental to endometrial angiogenesis, details of how and when different cell types produce VEGF, and of how production and activity is controlled by estrogen and progesterone, remain to be clarified. Moreover, new pharmaceutic agents affecting inflammation, matrix metalloproteinase activity, and the immune system make it clear that intervening on angiogenesis may prevent or inhibit the development of endometriosis, and this finding reinforces the hypothesis that antiangiogenesis therapy may be used as a new modality of treatment for endometriosis, especially in cases of deeply infiltrating and particularly rectal endometriosis.

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