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ENDOMETRIOSIS

Urine vascular endothelial growth factor-A is not a useful marker for endometriosis

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Objective: To determine whether urine VEGF is elevated in women with endometriosis.

Design: Prospective observational study.

Setting: Tertiary care government and private hospitals.

Patient(s): During laparoscopy for pelvic pain or infertility, urine was collected and possible endometriosis lesions were excised. Of 62 women, 40 had histology-proven endometriosis and 22 had no histological proof of the disease.

Intervention: None.

Main Outcome Measure(s): Urine VEGF-$A_{121,\ 165}$ was measured and compared in women with and without biopsy-proven endometriosis.

Result(s): Urine VEGF levels corrected for creatinine excretion were similar in women with (83.6 ± 11.3 pg/mg Cr) and without (88.5 ± 10.4 pg/mg Cr) endometriosis ($P = .77$). The frequency distribution of urine VEGF measurements for women with and without endometriosis was similar. No significant difference was noted in urine VEGF levels when comparing endometriosis stages or in those with endometriomas compared to controls. Urine VEGF did not vary significantly over the menstrual cycle or between groups by cycle phase. No cutoff point discriminated individuals with and without the condition.

Conclusion(s): It is unlikely that urine VEGF-$A_{121,\ 165}$, as measured in this study, will be a useful non-invasive marker for endometriosis. (Fertil Steril® 2004;81:1507–12. ©2004 by American Society for Reproductive Medicine.)

Key Words: Endometriosis, VEGF, diagnosis

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Angiogenesis—the formation of new blood vessels—appears to be critical for the peritoneal attachment and development of endometriosis (1). Although the factors essential for neovascularization of endometriotic tissue have not been defined, vascular endothelial growth factors (VEGFs) may mediate this activity. The VEGFs, a family of dimeric glycoproteins denoted as VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor, have potent angiogenic, mitogenic, and vascular permeability activities (2). The VEGF-A is expressed in humans as five splice variants of a single gene, with 121, 145, 165, 189, and 206 amino acid residues (3). The most frequently expressed variants, VEGF-A_{121} and VEGF-A_{165}, are synthesized by human endometrium (4) and are freely secreted (3) and excreted in urine (5).

In women with endometriosis, VEGF mRNA and/or protein have been detected in endometrium and endometriosis lesions (1, 4), peritoneal fluid macrophages, and peritoneal fluid (1). These data, and the finding of elevated serum levels in women with endometriomas (6), suggest that VEGF might be a marker of endometriosis. To our knowledge, urinary VEGF has not been measured in women with endometriosis. Urinary VEGF increases in women after hCG stimulation, presumably because of ovarian VEGF production (7) and is a marker for certain urinary diseases including renal cell carcinoma, bladder cancer

(2), minimal change nephrotic syndrome (8), and chronic renal failure (9).

We hypothesized that urinary VEGF may be increased in women with endometriosis. We also investigated whether elevated urinary VEGF levels are correlated with the stage of the disease. Such correlation would suggest a new non-invasive and inexpensive way to both diagnose endometriosis and evaluate its treatment, and might allow for earlier diagnosis of the disease without need for surgical confirmation.

MATERIALS AND METHODS

Patients were recruited for the study at Georgetown University Medical Center and at the Clinical Center of the National Institutes of Health. At Georgetown, women had laparoscopy (by CW or EW) as part of clinical care for infertility or as part of a research protocol for pelvic pain. At the National Institutes of Health, women underwent laparoscopy for pelvic pain as part of a research protocol. Forty-seven women had surgery at the Warren G. Magnuson Clinical Center of the National Institutes of Health, Bethesda, MD, from 1999 to 2001, and 15 at Georgetown University Hospital, Washington, D.C. in 1998. The procedures occurred on various days of the menstrual cycle.

Possible endometriotic lesions were resected using an neodymium:yttrium aluminum garnet (Nd:YAG) contact laser (Surgical Laser Technologies, The Oaks, PA) and sent to pathology for confirmation. Women were considered to have endometriosis if at least one biopsy was positive. At surgery, the extent of endometriosis was determined using the American Society of Reproductive Medicine (ASRM) revised classification (10).

Collection of urine to test for markers for endometriosis was approved by the Institutional Review Boards (IRBs) of Georgetown University and the National Institute of Child Health and Human Development. All patients gave informed oral and written consent before surgery.

Urine Collection and Storage

Urine was collected into sterile plastic tubes when the bladder was catheterized after induction of anesthesia. Samples from subjects obtained after normal voiding and catheterization (n = 3) gave similar results (94.2 ±17.6 pgVEGF/mg Cr vs. 84.8 ±14.1pgVEGF/mg Cr, respectively), suggesting that catheterization does not effect VEGF level. As there is no diurnal variation in the urine VEGF-to-creatinine ratio (7), no effort was made to obtain the samples at a specific time of day, although most were collected in the morning.

Previous experiments from the Folkman laboratory demonstrated that VEGF-A is stable at 4°C for at least 1 week, and other investgators have demonstrated stability for 24 hours at room temperature (9). Based on this information, samples were initially placed in an ice water bath, refrigerated, frozen at −20°C within 1 day of surgery and shipped on dry ice to Dr. J. Folkman's laboratory. Subsequently, samples were kept at −70°C until the week they were assayed, when they were thawed for 1 hour at room temperature and then stored at 4°C. Prior to assay, samples were centrifuged at 3000 rpm for 10 minutes at 4°C to remove cellular particles and insoluble material.

VEGF and Creatinine Measurements

We measured human VEGF-A by ELISA immunoassay (R&D Systems Inc., Minneapolis, MN). The kit detects isoforms 121 and 165. Urine creatinine was measured using commercial assays at Covance Laboratories (n = 47) or Brigham and Women's Hospital (n=15).

Statistical Analysis

Urinary VEGF concentrations were evaluated using both absolute values and those corrected for urinary concentration by dividing VEGF values by creatinine values. Values in each patient group were analyzed according to the time of the menstrual cycle: proliferative, the beginning of menses until 16 days before the next menses (n = 12 in control group, n = 17 in endometriosis group), periovulatory, 13–15 days before the next menses (n = 4 in each group), and luteal, 1–12 days before the next menses (n = 6 in control group, n = 16 in endometriosis group). There were no menstrual cycle data from three women in the endometriosis group.

Data were analyzed statistically using StatView software (StatView 5, SAS Institute, Cary, NC). Nominal variables, such as cycle phase and endometriosis history, as well as demographics were compared among cases and controls using a series of χ^2 tests. Unpaired *t*-tests were used to compare VEGF values as well as demographic data including age, body mass index (BMI), pelvic pain duration, and cycle day among cases and controls. The VEGF value for stage of disease, presence or absence of endometriomas, and cycle phase were compared by ANOVA. Jonckheere's nonparametric test for trend in ANOVA also was used for comparing VEGF values and stage of disease or menstrual cycle phase. Correlation analyses were used to assess the relationship between VEGF values and cycle day, as well as between VEGF values and subject age. All comparisons were analyzed by pathology as well as clinical diagnosis. Statistical significance was accepted at $P<.05$.

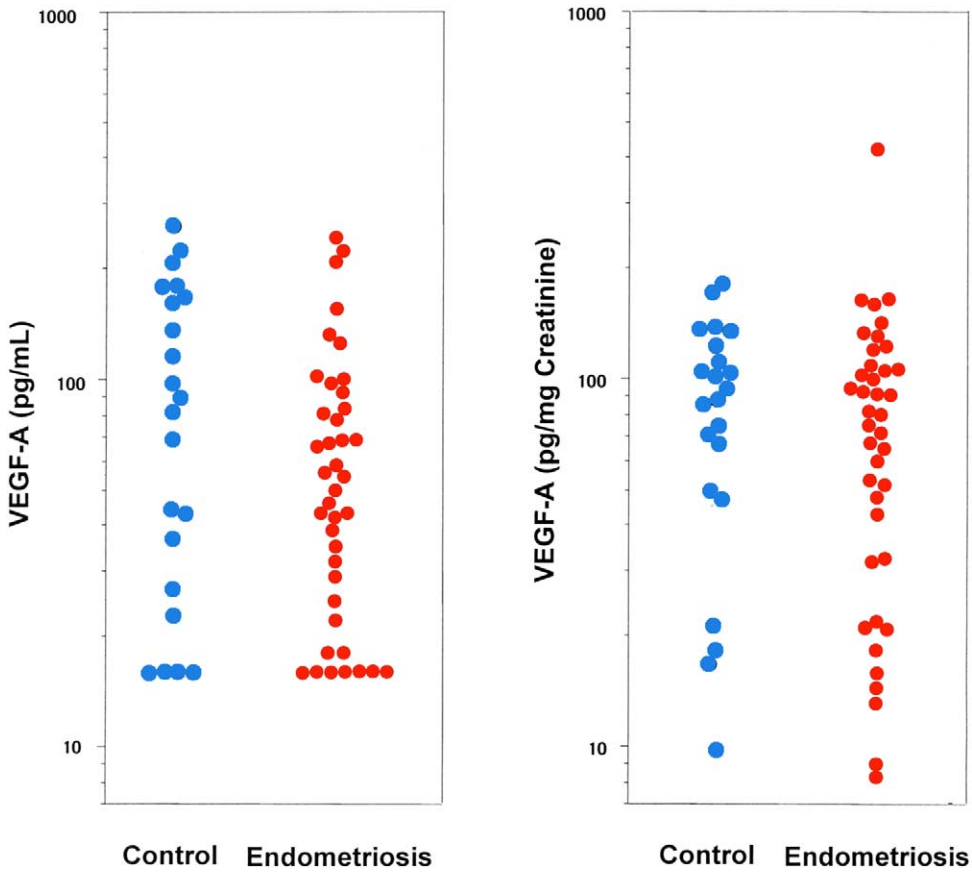
RESULTS

Subjects

The women in the study (n = 62) were 19–46 years old (mean age 32.5 ± 6.8 years) and had a BMI of 17.8–44.5 (mean BMI 25.7 ± 5.9). Ethnic and racial classification included Asian (n = 2), Hispanic (n = 3), African American (n = 11), Indian (n = 3), Caucasian (n = 42), and unknown (n = 1). Fifty-three women had surgery for pelvic pain. The

FIGURE 1

Left: Urine VEGF-A values in individual women with and without endometriosis. *Right:* Urine VEGF-A per milligram creatinine values in individual women with and without endometriosis.



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findings at surgery for the other nine women were a paratubal cyst (n = 1), secondary infertility (n = 2) with and without tubal adhesions, ovarian mass (n = 1), uterine leiomyomata (n = 1), and primary infertility (n = 4).

Of 40 women with histologically confirmed endometriosis, 11 were classified as stage I, 17 as stage II, 7 as stage III, and 5 as stage IV. Twenty-eight of the 40 had a self-reported history of endometriosis diagnosed by laparoscopy, laparotomy, or both.

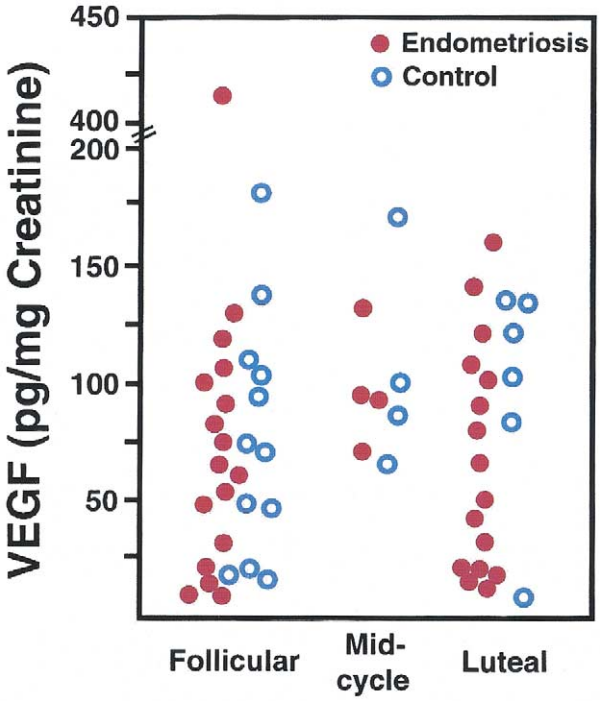
Twenty-two women with no histological evidence of endometriosis were considered to be the control group. Of these, six were believed to have endometriosis at surgery (5 with ASRM stage I and 1 with stage II), and of these six, three had a previous surgical diagnosis of endometriosis. The remaining 16 had no clinical evidence of endometriosis, but 7 of these reported a history of endometriosis. The control and endometriosis patients were similar in age ($P = .47$), BMI ($P = .57$), ethnicity ($P = .60$), gravidy ($P = .79$), parity ($P = .46$), cycle phase ($P = .26$), cycle day ($P = .66$), and duration of pelvic pain ($P = .93$). As expected, women with endometriosis were different from controls with respect to previous history of surgical diagnosis of endometriosis ($P = .04$).

The distribution of VEGF levels was similar between the two groups (Fig. 1), except for one stage I endometriosis patient with an increased normalized VEGF of 423 pg/mg Cr. Urine VEGF-A, expressed either as picograms per milliliter or as picograms per milligram of creatinine, was similar in women with and without histological evidence of endometriosis (67.8 ± 9.1 pg/mL vs. 100.1 ± 16.5 pg/mL, $P = .07$, and 83.6 ± 11.3 pg/mg Cr vs. 88.5 ± 10.4 pg/mg Cr, $P = .77$).

There was no significant difference in urine VEGF-A when the 6 women with a clinical—but not histological—diagnosis of endometriosis were included in the endometriosis group (46 endometriosis patients, 16 patients in the control group; $P = .43$ for pg/mg Cr). However, this comparison was statistically significant when creatinine was not

FIGURE 2

Urine VEGF-A per milligram creatinine values in individual women with and without endometriosis, displayed according to the phase of the menstrual cycle when they were collected.



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considered (P = .03 for pg/mL). When only women without a current diagnosis or previous history of endometriosis were compared to those with a current diagnosis, there was no significant difference in urine VEGF-A (9 patients in the control group, 53 endometriosis patients (P = .56 for pg/mg Cr and P = .65 for pg/mL). Thus, no diagnostic cutoff point could be set to discriminate between patients with and without endometriosis.

Urinary VEGF as picograms per milligrams of Cr did not correlate with age (P = .45), ethnicity (P = .69), reproductive history, viz. gravidity (P=0.89) and parity (P = .90), cycle day (P = .86) or phase (P = .34). No statistical significance was found in urinary VEGF-A excretion among the different menstrual phases when we analyzed them within the control and endometriosis groups separately (P values ranged from .31 to .51). When analyzing cycle phase variation in VEGF levels for a trend to higher levels in the luteal phase, the Jonckheere test was not significant (P = .268); thus, there was no trend for a difference in VEGF levels between endometriosis patients and controls at each cycle phase. Examination of individual results also shows nearly complete overlap between healthy women and those with endometriosis at all phases of the menstrual cycle (Fig. 2).

Jonckheere's nonparametric test found no trend for correlation of VEGF values with the ASRM stage of endometriosis (I, II, III, IV), either when only patients with histologically proven endometriosis were considered (P = .16) or when women with clinical endometriosis were considered (P = .08). Additional comparisons were made for seven women with ovarian endometrioma considered as a separate group, compared to women with pathology-proven endometriosis and controls, without finding a significant difference (P = .72).

DISCUSSION

Endometriosis is a common gynecological disorder that affects 8%–10% of all reproductive age women, and is found in up to 50% of asymptomatic women undergoing laparoscopic tubal ligation (11). Pelvic pain or infertility caused by endometriosis often cannot be distinguished on clinical grounds. The positive predictive value for suspected lesions at surgery vs. histological diagnosis of endometriosis lesions is only 45% (12). In addition, neither magnetic resonance imaging (MRI) nor ultrasound imaging reliably identifies endometriosis lesions (13). As a result, there is a need for development of noninvasive diagnostic tests for this condition.

Because endometriosis is characterized by pelvic inflammation, various groups have investigated the diagnostic utility of serum markers that are increased in inflammatory states. In one large study of 130 women, serum interleukin-6 (IL-6) values of > 2.0 pg/mL had a sensitivity of 90% and specificity of 67% for the diagnosis of endometriosis, but serum concentrations of IL-1β, IL-8, IL-12, IL-13, and tumor necrosis factor-α did not differ between patients with and without endometriosis (14). One potential drawback of the use of IL-6 as a diagnostic test is that it may be elevated in conditions other than endometriosis, such as spontaneous bacterial peritonitis (15), ovarian cancer (16), and ovarian torsion (17), therefore potentially reducing the specificity of the test in women with pelvic pain. Lipoprotein(a) is another possible marker of inflammation, which is slightly increased in women with endometriosis (18).

Other investigators have postulated that endometrial products, such as cancer antigen (CA)-125, would be elevated in peritoneal fluid, and possibly serum, in women with endometriosis (19). Some investigators found CA-125 to be elevated, but only in women with more extensive endometriosis (20, 21). Other reseachers reported that as a group, women with minimal endometriosis do not have higher circulating levels of CA-125 than women without endometriosis, but by using a cutoff point of >16 U/mL, minimal endometriosis was diagnosed with 71% sensitivity and 71% specificity (21). However, like IL-6, CA-125 is elevated in

other conditions, including uterine leiomyomata, pelvic inflammatory disease, and ovarian cancer, potentially reducing its specificity. In addition, some, but not all, investigators have found that CA-125 levels fluctuate during the menstrual cycle (22).

In this study, urine VEGF-A levels did not predict the presence or extent of endometriosis, as values were similar between the endometriosis and control groups, and among all endometriosis stages. The urine VEGF levels found in this study in women with and without endometriosis (83.6 ± 11.3 pg/mg Cr and 88.5 ± 10.4 pg/mg Cr, respectively) fall within the normal range reported by another study of men and women (80 – 109 ng/g Cr) (23). This is surprising because VEGF is increased in the peritoneal fluid of some women with endometriosis (24) and in the serum of some with endometriomas (6).

One possible explanation for our findings is that urine VEGF levels may be increased only in a particular phase of the cycle (e.g., the proliferative phase) when peritoneal fluid levels are highest. There are few data regarding variability of serum VEGF during the normal menstrual cycle, and none regarding urine values. In healthy women, endometrial levels of VEGF are greatest in the late luteal phase and during menses, suggesting that serum and urine levels may be greatest at that time (25). One study documented a relative increase in early proliferative compared to periovulatory serum VEGF concentrations, but luteal phase values were not different (26). However, other investigators have not found a phase-specific difference in serum VEGF concentrations (7, 27, 28). Our analysis of urine VEGF supports the concept that there is no phase-specific difference either in control women or those with endometriosis. However, it is possible that the relatively small sample size might obscure a difference. It also is possible that elevated VEGF would be more likely in women with endometriomas, but again the number with endometriomas in this study was small and a group analysis of these women did not reveal a significant difference.

Another explanation for the normal urine VEGF-A values in women with endometriosis is that VEGF from the peritoneum either does not circulate in the blood or is cleared quickly from the bloodstream by a nonrenal mechanism. For example, endothelial cells clear recombinant human VEGF very efficiently, supporting this concept (29). Although circulating levels of VEGF-A are increased in cancers of the breast, ovary, uterus, and gastrointestinal tract (2), it is likely that the number of angiogenic cells in metastatic disease is greater than the number of endometriosis cells. Although VEGF-A also is increased in the blood and urine of diabetics with renal disease (30), its urinary excretion has not been examined in other nonrenal disorders apart from post-hCG ovarian hyperstimulation (7) and little is known about the renal clearance of VEGF isoforms.

It is also possible that the similar urine VEGF-A values in the control and endometriosis groups in our study reflect occult inflammation, angiogenic conditions, or occult endometriosis in the control women. If this were the case, VEGF would not be specific enough to be a useful diagnostic tool. Another possible limitation to our study was the selection of the control group. Healthy women without pelvic pain would be an ideal comparison group to distinguish between endometriosis and "normal" patients. However, by evaluating both women not suspected of having endometriosis and those with pelvic pain, this study approximated better the diagnostic dilemma faced in ordinary practice.

In conclusion, the present study shows that the levels of VEGF-A in random urine samples do not predict the presence of endometriosis. However, given the small number of women studied at various time of the menstrual cycle, levels of VEGF-A in early follicular phase urine or blood samples should be examined before completely eliminating the possibility of using VEGF-A for the diagnosis of endometriosis.

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