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2-Methoxyestradiol Inhibits Hypoxia-Inducible Factor-1 α and Suppresses Growth of Lesions in a Mouse Model of Endometriosis

Christian M. Becker,^{*††} Nadine Rohwer,[§]
Tae Funakoshi,^{*} Thorsten Cramer,[§]
Wanja Bernhardt,[¶] Amy Birsner,^{*} Judah Folkman,^{*}
and Robert J. D'Amato^{*}

From the Vascular Biology Program,^{*} Children's Hospital Boston, Harvard Medical School, Boston, Massachusetts; the Department of Obstetrics and Gynaecology,[†] Charité—Campus Benjamin Franklin, Berlin, Germany; the Nuffield Department of Obstetrics and Gynaecology,[‡] John Radcliffe Hospital, University of Oxford, United Kingdom; the Department of Gastroenterology,[§] Charité—Campus Rudolf Virchow, Berlin, Germany; and the Department of Nephrology,[¶] Friedrich-Alexander-Universität Erlangen-Nürnberg, Germany

Endometriosis, the presence of ectopic endometrial tissue outside the uterine cavity, is a common disease affecting women during their reproductive years. Current therapeutic success is often unsatisfactory because of limited insight into disease mechanisms. Nevertheless, angiogenesis plays an essential role in the pathogenesis of the disease, making it a potential novel target for therapy. In the current study, we demonstrate in an established mouse model of endometriosis that transient hypoxia in transplanted endometriosis-like lesions results in the up-regulation of hypoxia-inducible factor-1 α (HIF-1 α), leading to the expression of vascular endothelial growth factor (VEGF), a key player in endometriosis-associated angiogenesis. Systemic treatment with the angiogenesis inhibitor 2-methoxyestradiol suppressed HIF-1 α expression *in vivo*, resulting in a decreased downstream expression of HIF-1 α target genes, such as for VEGF, phosphoglycerate kinase, and glucose transporter-1. 2-Methoxyestradiol also suppressed VEGF-induced vascular permeability, as demonstrated in a modified Miles assay. Finally, systemic treatment with 2-methoxyestradiol significantly inhibited the growth of endometriosis-like lesions in a dose-dependent manner. In conclusion, hypoxia appears to play an important role in the pathogenesis of endometriosis and endometriosis-associated angiogenesis, and the angiogenesis

inhibitor 2-methoxyestradiol may be a potential candidate for systemic treatment in the future. (*Am J Pathol* 2008, 172:534–544; DOI: 10.2353/ajpath.2008.061244)

Endometriosis, defined as the presence of ectopic endometrial tissue, is a disease associated with pelvic pain and reduced fertility. Women are affected almost exclusively during their reproductive years, because the establishment and growth of endometriotic lesions is highly estrogen dependent. It is estimated that 10 to 15% of women in the general population suffer from endometriosis, whereas subgroups, such as women undergoing laparoscopy for fertility investigations or hysterectomy, show a higher prevalence.¹ Currently, it is believed that most endometriotic lesions originate from shed endometrium that enters the abdominal cavity through the Fallopian tubes at the time of menstruation.² In women susceptible to the disease, these fragments or cells attach to the peritoneal wall and are then thought to invade the mesothelial cell layer, the underlying basement membrane, and stroma. The endometriotic lesions generate a blood supply by recruiting new blood vessels from the surrounding vasculature.³ This process known as angiogenesis also plays an essential role during many other disease states, such as tumor growth, rheumatoid arthritis, and diabetic retinopathy.⁴ However, blood vessel growth is also vital in physiological processes, such as development, wound healing, and reproduction.⁵

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Address reprint requests to Christian M. Becker, Nuffield Department of Obstetrics and Gynaecology, John Radcliffe Hospital, Womens Centre Headington, University of Oxford, Oxford, OX3 9DU, UK. E-mail: christian.becker@obs-gyn.ox.ac.uk.

Proliferation of endothelial cells from pre-existing vessels is dependent on an increase in local proangiogenic cytokines and a decrease in inhibitors.⁶ Vascular endothelial growth factor (VEGF) is one of the best studied proangiogenic factors.⁷ It was originally identified as vascular permeability factor because of its enhancing effect on blood vessel permeability, but the mitogenic and survival-promoting effect of the molecule on endothelial cells eventually led to its current name.^{8,9} The VEGF-A gene is alternatively spliced to yield three major isoforms of 189, 165, and 121 amino acids.^{10,11} The splicing variant VEGF₁₆₅ is most abundantly expressed and has been repeatedly described as being overexpressed in the peritoneal fluid of women with endometriosis.^{12,13}

VEGF production is regulated by various stimuli. Hypoxia is one of the most potent factors resulting in VEGF expression.¹⁴ Hypoxia-inducible factor-1 α (HIF-1 α) is believed to play a key role in VEGF-induced angiogenesis.¹⁵ HIF-1 α is a helix-loop-helix transcription factor that is ubiquitously expressed in mammalian cells. Under normoxic conditions, the protein is hydroxylated at prolyl and asparaginyl residues, binds to the von-Hippel-Lindau protein, and undergoes proteasomal degradation.^{16,17} HIF-1 α degradation is prevented in hypoxic environments, and the molecule translocates into the nucleus where it heterodimerizes with HIF-1 β /aryl hydrocarbon nuclear receptor translocator. This heterodimer binds to hypoxia-responsive elements on various genes, such as those encoding glycolytic enzymes, erythropoietin, and VEGF.^{18,19}

In the current study, we hypothesize that endometriosis-like lesions may acquire new blood vessels through hypoxia-induced up-regulation of VEGF. We demonstrate that systemic therapy with the angiogenesis inhibitor 2-methoxyestradiol inhibits the growth of induced endometriosis-like lesions *in vivo*. We propose that this inhibitory effect may be partially due to the effect of the drug on HIF-1 α .

Materials and Methods

Animal Handling

All procedures were performed in the animal facility at Children's Hospital (Boston, MA) in accordance with federal, local, and institutional guidelines. The Institutional Animal Care and Use Committee of Children's Hospital approved all animal handling and procedures. Eight-week-old female C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). They were caged in groups of 5 to 10 mice with free access to chow and water and acclimated for 1 week before any experiment. The animal room was kept constantly at 26°C, 38.5% humidity, with a 12-hour light, 12-hour dark cycle (6:30 AM to 6:30 PM). The laboratories were located just a few meters above sea level.

Surgical Procedure and Treatment

All surgical procedures were performed under inhalative anesthesia with isoflurane (Baxter, Deerfield, IL), and mice were observed until fully recovered. Mice were

ovarectomized through bilateral paravertebral incisions, and the wound was closed with a 5-0 braided silk suture (Ethicon, Somerville, NJ). Two days later, endometriosis was surgically induced as described previously.^{20–23} Briefly, after midline abdominal incision, the uterine horns were removed and opened longitudinally in a Petri dish containing warmed Dulbecco's modified Eagle's medium F-12 (Gibco, Grand Island, NY). Seven biopsies (2 mm in diameter) were taken using a dermal biopsy punch (Mil-tex, Bethpage, NY), and the lesions were autotransplanted to the peritoneal wall (four biopsies) and the mesentery (three biopsies) with a braided silk suture. The wound was closed with a 5-0 suture (all Ethicon). At the end of the procedure, a 17- β estradiol pellet (0.36 mg; 60-day release; Innovative Research of America, Sarasota, FL) was implanted subcutaneously into the interscapular area of all mice.

Treatment was initiated 2 to 3 hours before the procedure. Mice received 200 μ l of 2-methoxyestradiol solution (2-ME₂) (Panzem NCD; generous gift of EntreMed Inc., Rockville, MD) or vehicle per daily oral gavage. For dose-finding experiments ($n = 6$ /group), mice received 10, 30, 60, and 100 mg/kg drug or vehicle for 4 weeks. All experiments were repeated three times. For all other experiments, 100 mg/kg was given. Control mice received vehicle at the identical volume.

At the end of each experiment, mice were euthanized individually under brief isoflurane anesthesia by cervical dislocation. The abdominal cavity was immediately re-opened through the original incision, and the lesions were instantly excised and processed. In the experiments in which tissue was not collected, all seven lesions were measured in two perpendicular diameters (D_1 and D_2) with a caliper, and cross-sectional lesion area (CSA) was calculated using the formula for an ellipse ($D_1 \times D_2 \times \pi/4$) as previously described.^{20,21,24}

Green Fluorescent Protein Imaging

In a different set of experiments, uterine tissue from 8-week-old transgenic mice expressing green fluorescent protein²⁵ was removed and prepared as described above. In these animals, green fluorescent protein is expressed in all tissue because the gene is coupled with the ubiquitin promoter. The lesions were then transplanted to the peritoneal wall of wild-type C57BL/6 mice ($n = 6$). After closing the abdomen, the mice were kept for 4 weeks. Under general anesthesia, the abdominal cavity was then re-opened, and the lesion was visualized using a dissecting microscope with external light source and a green fluorescent protein filter lens. Pictures were captured using a CCD camera.

RNA Extraction and Real-Time PCR

Mice were pretreated with 2-methoxyestradiol or vehicle for 4 days ($n = 3$ mice per time point). All experiments were repeated three times. Mice were then ovariectomized, and endometriosis was induced as described above. The endometriosis-like lesions were removed at

different time points. Tissue was snap frozen in liquid nitrogen and then stored at -80°C until further use. Usually two to three lesions were used per time point to yield enough RNA and real-time PCR was performed at least two times per experiment.

Total RNA was harvested with TRIzol Reagent (Invitrogen, Karlsruhe, Germany) and extracted with chloroform/isopropanol. The RNA was reverse transcribed into cDNA using Omniscript RT kit (Qiagen, Valencia, CA) and random primers (Invitrogen) according to the manufacturer's instructions. Quantification of mRNA expression was performed using a DNA Engine Opticon 2 System (Bio-Rad, Hercules, CA). TaqMan Universal PCR Master Mix (Roche, Branchbury, NJ) was used for the real-time PCR reaction according to the manufacturer's protocol. cDNA (50 μg) was used as template to determine the relative amount of mRNA by real-time PCR. Cycle threshold value means the number of PCR cycles required for the detection of fluorescence signal to exceed a fixed threshold. The relative levels of gene expression of target mRNA were normalized against β -actin. The following primers and probes were used: VEGF forward, 5'-AGTCCCATGAAGTGATCAAGTTCA-3'; VEGF reverse, 5'-ATCCGCATGATCTGCATGG-3'; VEGF probe, 5'-(6FAM)TGCCACGTCAGAGAGCAACATCAC(BHQ~6FAM)-3'; phosphoglycerate kinase (PGK) forward, 5'-CTGTGGTACTGAGAGCAGCAAGA-3'; PGK reverse, 5'-CAGGACCATTCCAAACAATCTG-3'; PGK probe, 5'-(6FAM)TAGCTCGACCCACAGCCTCGGCATAT(BHQ~6FAM)-3'; glucose transporter-1 (Glut-1) forward, 5'-GGGCATGTGCTTCCAGTATGT-3'; Glut-1 reverse, ACGAGGAGCACCGTGAA-GAT-3'; Glut-1 probe, 5'-(6FAM)CAACTGTGCGGCCCTACGTCTTC(BHQ~6FAM)-3'.

Hypoxyprobe Detection

For the immunohistochemical assessment of hypoxia in endometriosis-like lesions, in a separate set of experiments, mice ($n = 2/\text{group}$) were sacrificed at different time points (0 hours, 1 hour, 4 hours, 24 hours, 48 hours, 1 week, and 6 months). The experiment was repeated twice. The hypoxyprobe-1 kit (Chemicon International, Temecula, CA) was used according to the manufacturer's protocol. At each time point, 30 minutes before euthanasia, mice received an i.p. injection of 60 mg/kg pimonidazole hydrochloride (hypoxyprobe-1), a 2-nitroimidazole, which forms insoluble protein adducts under hypoxic conditions *in vitro* and *in vivo*. After euthanasia, the collected tissue was immediately fixed in 10% buffered formalin and then paraffin embedded. Five-micrometer-thick sections were subsequently incubated with primary anti-pimonidazole hydrochloride antibody, biotinylated secondary antibody, and streptavidin/peroxidase conjugates (Vector, Burlingame, CA). An isotype-matched irrelevant murine IgG1 monoclonal antibody (Genzyme, Cambridge, MA) directed against Shigella toxin, was used as a control antibody. Counterstaining was performed with hematoxylin.

Immunohistology for HIF-1 α and Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling

Paraffin sections (3 μm) were dewaxed in xylene, rehydrated in a series of ethanol washes, and placed in distilled water before staining procedures. A rabbit polyclonal HIF-1 α antibody (NB100-449; 1:10,000; Novus Biologicals, Littleton, CO) was used to detect HIF-1 α . For signal amplification and visualization of HIF-1 α , a catalyzed amplification system (CSA kit; Dako, Hamburg, Germany) based on a streptavidin-biotin-peroxidase reaction was used according to the manufacturer's instructions. Antigen retrieval was performed for 6 minutes in preheated Dako target retrieval solution, using a pressure cooker. All incubations were performed in a humidified chamber. 3,3'-Diaminobenzidine tetrahydrochloride (Dako) served as chromogen for peroxidase reaction. Substitution of the primary antibody with a nonreactive rabbit IgG control antibody (Invitrogen, Carlsbad, CA) served as a negative control. Between incubations, specimens were washed two to four times in buffer (50 mmol/L Tris-HCl, 300 mmol/L NaCl, and 0.1% Tween 20, pH 7.6). Samples were counterstained with Richardson's staining solution or hematoxylin for 30 seconds and coverslipped using an aqueous mounting media (Aquatex; Merck KGaA, Darmstadt, Germany). Apoptotic cells were visualized in formalin-fixed, paraffin-embedded tissue sections with the DeadEnd fluorometric terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling system (Promega, Madison, WI) according to the manufacturer's instructions.

Miles Vascular Permeability Assay

Mice were treated for 5 days with 100 mg/kg 2-methoxyestradiol, vehicle, or tap water (control) ($n = 6$ mice/group). We then performed a modified Miles assay to test the effect of the drug on vascular permeability.²⁶⁻²⁹ In brief, anesthetized mice received an injection of Evans Blue into the retro-orbital venous plexus (100 μl of a 1% solution in 0.9% sterile saline). Mice were kept on a warming blanket set at 37°C throughout the procedure. After 10 minutes, 50 μl of 1 ng/ μl human VEGF₁₆₅ or PBS (control) was injected intradermally. Twenty minutes later, the mice were euthanized, the dorsal skin was removed, and lesions were excised using an 8-mm biopsy punch (Miltex, York, PA). Evans Blue was extracted over 5 days at room temperature in formamide (Sigma Aldrich, St. Louis, MO). The absorption (620 nm) was then measured in a 96-well microplate (Sigma Aldrich). The absorption readings from the PBS-injected skin lesions were then subtracted from the lesions injected with VEGF to receive the final result.

Western Blot

Protein lysates (75 μg) were run on a 12% SDS-polyacrylamide gel along an electric current and then transferred to a nitrocellulose membrane. Western blot analysis was

Figure 1. **A:** Hypoxyprobe staining. Representative murine endometriotic tissue removed at different time points after transplantation. **D:** 1 hour. **E:** 4 hours. **F:** 24 hours. **G:** 48 hours. **H:** 1 week. **I:** 6 months. Each mouse was injected with pimonidazole hydrochloride (hypoxyprobe-1) 30 minutes before removal. Eutopic uterine lesions from the same mice were used as negative controls (**A–C**). Staining with hypoxyprobe antibody and counterstaining with hematoxylin. Control endometriotic tissue (4 hours; **J**) stained with an isotype-matched irrelevant murine IgG1 monoclonal antibody directed against Shigella toxin.

performed using a polyclonal rabbit antibody against murine HIF-1 α (1:1000 dilution; NB100-449; Novus Biologicals). To demonstrate equal loading, blots were stripped and reprobed with a specific antibody recognizing β -actin (1:5000 dilution; Sigma Chemicals, Munich, Germany). A mouse anti-rabbit antibody conjugated to horseradish peroxidase (Sigma Chemicals, St. Louis, MO) diluted 1:20,000 was used as the secondary antibody. En-

zyme activity was visualized using the enhanced chemiluminescence method according to the instructions of the manufacturer (NEN, Boston, MA). The experiment was repeated twice, and two to three lesions were used per time point to yield enough tissue. Free Image J software (National Institutes for Health, Bethesda, MD) was used to perform semiquantitative analysis of the Western blot.

Figure 2. **A:** Immunohistochemistry staining of endometriosis-like lesions with anti-HIF-1 α antibody. Counterstaining with hematoxylin. Increasing immunoreactivity (brown color) in glandular epithelial and stromal cells (arrows) over time (0, 1, 3, and 6 hours). Control (6 hours) stained with nonspecific rabbit IgG antibody. **B:** Western blot of endometriotic tissue stained with anti-HIF-1 α antibody from different time points after transplantation. Loading control of same Western blot stained with anti- β -actin antibody. **C:** Real-time RT-PCR of endometriosis-like lesions at different time points using primers for HIF-1 α -dependent genes (VEGF, PGK, and Glut-1). Bars indicate SEM. **D:** Semiquantitative measurement of Western blot for HIF-1 α as seen in **B**. Error bars indicate SEM. **E:** Endometriosis-like lesion on a green fluorescent protein^{+/+} mouse on the peritoneal wall of a wild-type recipient. Typical growth of blood vessels into the endometriosis-like lesion.

Statistical Analysis

For statistical analysis, GraphPad InStat and the Sigma Stat statistics software package were used. The mean, SD, and SEM were calculated for the mice in each group as described previously.^{20,21,24} Repeated-measures analysis did not show any significant difference in lesion size within animals.

The least square means of the CSA for established lesions (lesions >0 mm²) were compared among groups using one-way analysis of variance. When the overall analysis of variance indicated a significant F-test, pairwise comparisons among treatments with control mice were conducted using the post hoc Dunnett test. Two-tailed values of $P < 0.05$ were considered statistically significant.

Results

Hypoxia Occurs Early in Endometriosis-Like Lesions

Endometriosis-like lesions were removed at different time points after transplantation. The lesions stained positively for a monoclonal antibody against hypoxyprobe-1, thereby indicating that these specific tissues were hypoxic (Figure 1, D–I). No immunoreactivity was seen in

the uterine endometrial tissue (Figure 1, A–C) or in the control endometriotic tissue (Figure 1J). Hypoxia emerged as soon as 1 hour after transplantation, and immunohistochemical staining remained strong during the first 48 hours. However, no staining was present in the lesions 1 week and 6 months after transplantation.

HIF-1 α Is Up-Regulated in Hypoxic Endometriotic Tissue

Staining of endometriosis-like lesions with a monoclonal antibody against murine HIF-1 α showed distinctive, time-dependent patterns (Figure 2A). The glandular epithelial cells stained positive for HIF-1 α independent of the time point. However, in stromal tissue, no immunoreactivity for HIF-1 α was found at baseline, but it increased dramatically in tissues removed 1, 3, and 6 hours after transplantation. Western blot analysis of endometriosis-like lesions demonstrated a similar pattern (Figure 2B). Staining with a monoclonal antibody against HIF-1 α demonstrated that the protein showed increased expression up to 48 hours after transplantation ($P < 0.01$). At 72 hours, expression decreased, and none was detected in lesions 3 weeks after surgery (Figure 2D).

Figure 3. Systemic 2-methoxyestradiol inhibits growth of endometriosis-like lesions. Endometriosis-like lesions were measured in two perpendicular diameters (D_1 and D_2) with a caliper, and CSA was calculated using the formula of an ellipse ($D_1 \times D_2 \times \pi/4$) as previously described.^{20,21,24} **A:** Mean CSA of endometriosis-like lesions ($n = 7$ lesions) of mice treated with vehicle (■) or 2-methoxyestradiol (□) ($n = 6$ /group). **B:** Mean CSA after 4 weeks of oral treatment with different doses of 2-methoxyestradiol. Bars indicate the SEM.

Next, we determined HIF-1 α activity by measuring mRNA levels of target genes (Figure 2C). Real-time RT-PCR revealed that mRNA levels of VEGF and the two glycolytic enzymes PGK and Glut-1 were strongly elevated 24 hours after transplantation ($P < 0.001$). These levels decreased subsequently over time and were back to baseline levels after 3 weeks.

2-Methoxyestradiol Inhibits the Growth of Endometriosis-Like Lesions

Mice with surgically induced endometriosis were treated for various periods with 2-ME₂ or vehicle. Endometriosis-like lesions from vehicle-treated mice decreased in size within 1 week after surgery compared with baseline but increased in size thereafter (Figure 3A). Treatment with 100 mg/kg 2-ME₂ suppressed the growth of endometriosis-like lesions after 1, 3, and 5 weeks compared with matched controls, and the inhibitory effect increased with the duration of treatment [37% ($P < 0.01$), 44% ($P < 0.001$), and 63% ($P < 0.001$), respectively].

A dose-dependent effect was established when mice were treated for 4 weeks with different doses of 2-ME₂. Doses of 10, 30, and 100 mg/kg suppressed the growth of the endometriosis-like lesions by 21% ($P < 0.02$), 41% ($P < 0.001$), and 64% ($P < 0.001$), respectively, compared with vehicle controls (Figure 3B).

Figure 4. Systemic 2-methoxyestradiol treatment reduces expression of HIF-1 α target genes. Results of a representative experiment using real-time RT-PCR of endometriosis-like lesions removed after different time intervals after autotransplantation. Mice had been treated systemically with 2-methoxyestradiol before the induction of endometriosis. Lesions were removed at different time points (baseline, 1 hour, 3 hours, 24 hours, 72 hours, and 1 week) and tested for mRNA expression of VEGF (**A**), PGK (**B**), and Glut-1 (**C**). Bars indicate SEM.

Systemic Treatment with 2-Methoxyestradiol Inhibits HIF-1 α Expression in Endometriotic Tissue

Endometriosis-like lesions were removed from mice that had been treated systemically with 2-ME₂ or vehicle. When the endometriotic tissue was analyzed from the control mice, HIF-1 α protein was not detectable at baseline, but strong expression was seen 6 hours after transplantation. At that time point, HIF-1 α expression was strongly suppressed in mice treated with 2-ME₂ (Figure 5A).

We then determined mRNA levels of HIF-1 α target genes. Real-time PCR analysis of RNA isolated from endometriosis-like lesions showed reduced transcription of HIF-1 α target genes. Systemic 2-methoxyestradiol treatment significantly reduced RNA levels for VEGF ($P < 0.03$ at 24 hours), PGK ($P < 0.001$ at 3, 24, and 72 hours), and Glut-1 ($P < 0.001$ at 3 hours; $P < 0.01$ at 24 hours) (Figure 4 A–C).

Vascular Permeability Is Reduced by 2-Methoxyestradiol

Mice treated with 2-ME₂ showed reduced vascular permeability in a modified Miles assay (Figure 5B). Leakage of Evans Blue into the perivascular space after intradermal VEGF injection was suppressed in mice that had been pretreated with 2-ME₂ compared with controls. This was quantified by measuring absorbance, which was significantly lower in the excised tissue areas of mice that received 2-ME₂ when compared with mice treated with placebo or tap water [0.020 (SEM 0.005); 0.054 (SEM 0.005); 0.052 (SEM 0.013), respectively; $P = 0.0002$] (Figure 5C).

Discussion

Endometriosis, despite its relatively high prevalence in the female population, still remains a somewhat enigmatic disorder. Little is known about the underlying mechanisms responsible for this often debilitating disease. Sampson's theory of retrograde menstruation and transplantation of endometrial tissue in the abdominal cavity is the most widely accepted hypothesis to explain the pathogenesis of endometriosis. However, the fact that retrograde menstruation occurs commonly in women shows that other contributory factors for this disease must exist. It has been suggested that women with endometriosis may have impaired immunity or an altered expression of surface ligands on the endometrium or the mesothelial cell layer facilitating adhesion and survival of endometrial tissue.^{30–33} Recently, Donnez et al proposed that endometriotic lesions at various sites may be different,^{34,35} a concept that has been supported by data demonstrating the differential expression of aromatase.³⁶ Although it has not directly been demonstrated in humans, data from various animal models suggests that single endometrial cells or tissue fragments invade the mesothelial cell layer and basement membrane after attachment to the peritoneal wall.³⁷ Maas et al³⁸ reported in

the chick embryo chorioallantoic membrane CAM assay that glandular and stromal cells from endometrial fragments begin to invade the mesothelial cell layer within 48 to 72 hours of transplantation. Similar results were shown in another *in vitro* study using human mesothelial cell cultures.³⁹ The Dutch group also demonstrated that the presence of intact glandular structures and stromal components are required for the invasive potential of endometrial cells and the formation of endometriosis-like lesions. However, other data suggest that endometriotic lesion evolve from single cells, and it is currently unclear whether endometriosis is a monoclonal disease.^{40–44}

Regardless of the underlying theory, a basic requirement for the establishment and growth of all endometriotic lesions is the acquisition of new blood vessels to ensure sufficient supply with oxygen and nutrients (Figure 2E). Angiogenesis is dependent on the local balance between proangiogenic cytokines and endogenous angiogenesis inhibitors. In tumors, it has been proposed that an equilibrium of these factors leads to a dormant state, in which the number of proliferating cells equals the number of cells undergoing apoptosis.^{6,45,46} A shift in the balance toward the proangiogenic phenotype leads to tumor growth and metastases.⁴⁷ Cells undergo apoptosis as soon as the distance between the supplying blood vessel and the growing cells exceeds the maximal distance of oxygen diffusion. Many elegant tumor studies demonstrate that large amounts of VEGF are expressed in these hypoxic areas, which provokes a mitotic stimulus for the endothelial cells to proliferate and to form new blood vessels.^{48–50} The VEGF gene contains a hypoxia-responsive element to which, at the time of oxygen deprivation, the transcription factor HIF-1 α binds, resulting in expression of VEGF.^{51–55}

In our mouse model, we have shown that hypoxia is present in surgically induced endometriosis-like lesions during the early stages of transplantation and invasion. We demonstrated that hypoxyprom-1 (pimonidazole hydrochloride, a 2-nitroimidazole that forms a protein adduct to hypoxic cells *in vitro* and *in vivo*^{56–58}) accumulated

in the lesions immediately after transplantation, and this immunoreactivity increased during the first 24 to 48 hours. During the same period, uterine endometrial tissue was negative for hypoxyprobe-1 staining. Although it is not surprising that transplanted tissue is hypoxic, we also found that the lesions were hypoxyprobe-1-negative 1 week after transplantation, suggesting supply of oxygen to the transplanted tissue.

These results correlate well with our findings from a recent study.²⁴ Using *in vivo* bioluminescence imaging of transgenic luciferase-expressing endometriosis-like lesions in wild-type mice, we demonstrated that transplanted lesions emit light after systemic i.v. injection of the substrate luciferin 3 to 4 days after surgery. No signal was seen beforehand suggesting that connection to the circulation was not established yet. Others have also reported, using immunohistochemistry, that new blood vessels could first be detected in endometriosis-like lesions 3 days after transplantation.^{59–62} These results and the findings of the current study support the concept that hypoxia is an early phenomenon in the development of transplanted lesions, which diminishes once new blood vessels start to invade into the tissue. Assuming that Sampson's theory of retrograde menstruation and transplantation/invasion is correct, one can propose that the small tissue fragments are similarly hypoxic until they achieve a connection to the systemic circulation. Hypoxia is a strong stimulus for the growth of new blood vessels under the regulation of the HIF-1 α -VEGF pathway.^{14,51,53} Generally, under low oxygen levels, the transcription factor HIF-1 α is not hydroxylated, avoiding binding to the von-Hippel-Lindau protein and thereby inhibiting its ubiquitination and degradation. HIF-1 α then translocates into the nucleus, forms a complex with HIF-1 β /aryl hydrocarbon nuclear receptor translocator, and binds to hypoxia-responsive elements on various genes.^{63,64} The VEGF gene contains a hypoxia-responsive element, and the HIF-1 α /HIF-1 β /aryl hydrocarbon nuclear receptor translocator complex is known to induce the expression of VEGF strongly.¹⁴

In this study, we demonstrate for the first time that HIF-1 α is expressed in surgically induced endometriosis-like lesions. Western blot analysis of endometriotic tissue from this mouse model shows that the protein is expressed early and that levels decline after approximately 72 hours. Interestingly, immunohistochemical studies reveal that HIF-1 α is expressed mainly in the epithelial layer 1 hour after transplantation, whereas expression is increased in the stromal cell compartment at later time points. Because HIF-1 α undergoes posttranscriptional modifications, we decided to test for mRNA levels of downstream genes, such as VEGF, Glut-1, and PGK. Using real-time PCR, we confirmed our findings by showing strong up-regulation of the hypoxia-inducible genes during the 1st day after transplantation and a subsequent decrease in mRNA levels over time.

It has recently been proposed that the suppression of angiogenesis may be an attractive approach for the treatment of endometriosis.³ Our group and others have been able to show that the use of angiogenesis inhibitors is effective in suppressing endometriotic growth in various

murine models. Systemic therapy with either the endogenous angiogenesis inhibitor endostatin, a proteolytic fragment of collagen XVIII, or 27-amino acid endostatin peptides significantly inhibited the growth of endometriosis-like lesions.^{20,21} Similar results were seen with the TNP-470 derivative caplostatin.²⁴ Because VEGF levels are increased in the peritoneal fluid of women with endometriosis compared with controls,^{12,13} the inhibition of VEGF has been suggested as a means of influencing angiogenesis in endometriosis.⁶⁵ Soluble VEGF receptors and anti-VEGF antibodies showed efficacy in animal models.^{62,66}

In the present study, we used 2-ME₂ to treat mice with induced endometriosis. 2-ME₂ is an angiogenesis inhibitor currently being tested in phase II trials for cancer. We showed that 5 weeks of systemic 2-ME₂ treatment suppressed endometriotic growth in a dose-dependent manner. When 2-ME₂ was given at a dose of 30 mg/kg, a 39% reduction in growth was seen. By increasing the dose to 100 mg/kg, the lesion area was significantly reduced by 64%. In addition, the inhibitory effect was apparent by the end of the 1st week after transplantation. Interestingly, we found that lesions from the control mice became smaller during the 1st week, which was probably due to apoptosis and tissue necrosis. However, the effect of 2-ME₂ was significantly greater at that time point and increased to 58% during that experiment. The experiments were performed on nonestablished lesions, therefore making it a preventative therapy, ie, before lesion establishment. Other groups have shown that antiangiogenic therapy in different rodent models can inhibit growth of established lesions.^{62,66} We previously demonstrated that the use of endostatin does not significantly inhibit growth of established lesions in our mouse model of surgically induced endometriosis.²⁰ In our model, we believe that angiogenesis inhibitors work best during early stages of vessel growth (migration and sprouting of endothelial cells), and vascular growth has been demonstrated during the 1st week after transplantation. However, because other models have demonstrated efficacy in established lesions, it is possible that angiogenesis inhibitors, such as 2-ME₂, may work both in "prevention" and "treatment" settings. In a clinical scenario, patients may undergo surgical removal of lesions or conventional hormonal treatment, which is then supplemented by adjuvant antiangiogenic therapy.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling staining of endometriotic lesions removed at 1, 6, and 96 hours showed increase immunoreactivity in tissue from animals systemically treated with 2-ME₂ (Supplemental Figure S1, see <http://ajp.amjpathol.org>). The effect appears to be strongest during the early time points. This finding could be explained by the fact that the 2-ME₂ concentration in the transplanted tissue is likely to remain high immediately after transplantation. The longer no connection to the host vascular system exists, the less drug is available locally, which makes the difference of apoptotic cells between the treated and nontreated mice less apparent. In addition, our Western blot and RNA data suggest that HIF-1 α and target gene expression levels fall after 2 to 3 days in endometriotic

lesions. It is conceivable that, in addition to decreased 2-ME₂ levels, less "substrate" for 2-ME₂ may result in less obvious differences in apoptosis. This data and the inhibitory response on lesion growth are well in line with our experience with other angiogenesis inhibitors in this model. Endostatin and endostatin peptides inhibited the growth of endometriosis-like lesions by up to 47%, and the *N*-(2-hydroxypropyl)methacrylamide copolymer-linked TNP-470 compound caplostatin was 59% effective.^{20,21,24} However, 2-ME₂ has the advantage of being orally bioavailable.

2-ME₂ has been shown to suppress endothelial cell function by inhibiting microtubule dynamics.⁶⁷ Recently, an additional pathway to explain some of the action of the drug has been described. Mabeesh et al⁶⁸ showed that 2-ME₂ down-regulates HIF-1 α at a posttranscriptional level *in vitro*. It has also been reported that 2-ME₂ decreases nuclear binding activity of HIF-1 α in head and neck squamous cell carcinoma.⁶⁹ In the present study, we showed for the first time that 2-ME₂ inhibits the *in vivo* expression of HIF-1 α -inducible genes VEGF, Glut-1, and PGK as measured by real-time PCR during the first few hours after transplantation. These results are supported by our findings that HIF-1 α was also reduced in endometriotic tissues at the protein level.

These data suggest that 2-ME₂ may act through a dual mechanism in endometriosis. The indirect effect is through the inhibition of HIF-1 α . The inhibition of HIF-1 α leads to suppressed VEGF levels, as shown by the finding of reduced VEGF mRNA expression, which removes the stimulus for angiogenesis. The direct effect is via inhibition of endothelial cell function. The observation that 2-ME₂ produces a marked reduction in vascular permeability in response to a fixed amount of injected VEGF in the Miles assay demonstrates the direct inhibitory effect. VEGF is known to have approximately 50,000 times higher permeabilizing activity than histamine.¹⁰ Therefore, the diminished extravasation of Evans Blue in 2-ME₂-treated mice implies an inhibitory effect of the drug on VEGF stimulation of the endothelium. This is also illustrated by previous experiments that showed that 2-ME₂ inhibits angiogenesis induced by a pellet of VEGF placed in the cornea.⁷⁰ In addition, it is conceivable that the herein described suppressive effects on endometriotic growth are supported by the known inhibition of 2-ME₂ on tubulin polymerization by interacting at the colchicine site as previously demonstrated in tumors.^{67,70} However, although VEGF is a major player in endometriosis, other cytokines have been demonstrated to induce angiogenesis in endometriosis both directly and indirectly through an up-regulation of VEGF expression (for review, see Ref. 71).

In summary, we have shown that hypoxia plays an important role in induced endometriosis-like lesions in this established mouse model. New blood vessel growth that is essential for the survival and development of this tissue is induced by the expression of angiogenic factors, such as VEGF, which is up-regulated by oxygen deprivation and subsequent production of HIF-1 α . The antiangiogenic compound 2-methoxyestradiol inhibits HIF-1 α expression *in vivo* and angiogenesis, which is likely to contribute to suppression of the growth of the lesions. It

may therefore be a valuable novel therapeutic approach to the treatment of endometriosis.

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