

Inhibition of the Vascular Endothelial Cell (VE)-Specific Adhesion Molecule VE-Cadherin Blocks Gonadotropin-Dependent Folliculogenesis and Corpus Luteum Formation and Angiogenesis

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Although it has been previously demonstrated that administration of anti-vascular endothelial growth factor (VEGF) receptor-2 antibodies to hypophysectomized (Hx) mice during gonadotropin-stimulated folliculogenesis and luteogenesis inhibits angiogenesis in the developing follicle and corpus luteum (CL), it is unclear which of the many components of VEGF inhibition are important for the inhibitory effects on ovarian angiogenesis. To examine whether ovarian angiogenesis can be more specifically targeted, we administered an antibody to VE-cadherin (VE-C), an interendothelial adhesion molecule, to Hx mice during gonadotropin stimulation. In tumor models and *in vivo* and *in vitro* assays, the anti-VE-C antibody E4G10 has been shown to specifically inhibit angiogenesis, but VE-C has yet to be inhibited in the context of

ovarian angiogenesis. In addition to studying the effect on neovascularization in the follicular and luteal phases, we also examined the effect of E4G10 on established vessels of the CL of pregnancy. The results demonstrate that E4G10 specifically blocks neovascularization in the follicular and luteal phases, causing an inhibition of preovulatory follicle and CL development, a decrease in the vascular area, and an inhibition of function demonstrated by reduced hormone levels. However, when administered during pregnancy, unlike anti-VEGF receptor-2 antibody, E4G10 is unable to cause disruption of the established vessels of the mature CL. These data demonstrate that E4G10 causes a specific inhibition of neovascularization in the ovary without destabilizing pre-existing vasculature. (*Endocrinology* 146: 1053–1059, 2005)

DEVELOPMENT OF THE preovulatory follicle and the corpus luteum (CL) are processes that are critically dependent on angiogenesis (1). New blood vessels provide substrate for steroidogenesis, allow for delivery of endocrine and paracrine factors to and from the follicle and CL, and supply the preovulatory oocyte with oxygen and other metabolic needs. Manipulation of ovarian angiogenesis may be of therapeutic value by allowing potential augmentation or inhibition of follicular or luteal function, depending on the desired clinical outcome.

The relationship between angiogenesis and ovarian function has been well established, most notably by inhibiting the activity of vascular endothelial growth factor (VEGF) and its receptors. VEGF has multiple roles: an endothelial cell mitogen, a vascular survival factor, and an inducer of microvascular permeability (2). Studies of rodents and primates have shown that immunoneutralization of VEGF by soluble receptors or receptor-blocking antibodies during the ovarian cycle causes inhibition of angiogenesis and impaired devel-

opment of preovulatory follicles and the CL (3–7). These studies implicate VEGF as an important mediator of ovarian angiogenesis but do not address which specific functions are necessary for angiogenesis.

To investigate angiogenic modulation of ovarian vasculature by inhibition of endothelial cell assembly into vascular structures, we chose to target vascular-endothelial cell cadherin (VE-C), an angiogenesis-related adhesion molecule. VE-C, also known as CD144/cadherin-5, is a 125-kDa single-pass transmembrane glycoprotein that associates as *cis*-dimers via the extracellular domains (ECD) on the cell to promote intercellular homophilic adherens junctions (8, 9). The cytoplasmic domain is anchored to the actin cytoskeleton via proteins of the armadillo family, specifically β -catenin, plakoglobin, and p120 (10, 11). The association with actin may be important in modulating paracellular permeability, stabilization of the junctional complex, and intracellular signaling (8, 10). A null mutation in the VE-C gene results in embryonic lethality by embryonic day (E)9.5–10 with the inability of normally differentiated endothelial cells to form vascular structures (12). The involvement of the ectodomain and the distribution of the VE-C complex on the cell surface are thought to be dynamic and variable with the functional state of the cell.

An antibody to the NH₂-terminal portion of the ECD of VE-C (E4G10; ImClone Systems) was developed and characterized that specifically inhibits angiogenesis by preventing new adherens junction formation between endothelial

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Abbreviations: CL, Corpus luteum; DAB, diaminobenzidine; E, embryonic day; ECD, extracellular domain; H&E, hematoxylin and eosin; hCG, human chorionic gonadotropin; Hx, hypophysectomized; PE-CAM, platelet endothelial cell adhesion molecule; PMSG, pregnant mare serum gonadotropin; VE-C, vascular-endothelial cell cadherin; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

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cells without disrupting preexisting junctions (13–15). The efficacy of this agent has been studied using *in vitro* and *in vivo* angiogenesis assays and in tumor models but has yet to be investigated under physiological conditions.

To examine the role of VE-C in ovarian angiogenesis, we administered E4G10 during stimulated ovarian cycles in hypophysectomized (Hx) mice. Follicular maturation and ovulation are normally absent in these animals but can be reliably induced with exogenous gonadotropins (16, 17). Because they lack endogenous ovarian-hypothalamic-pituitary feedback, preovulatory follicle and CL development is not confounded by internal hormonal interactions or the effect of antiangiogenic substances on hypothalamic-pituitary response. We demonstrate that the presence of an anti-VE-C antibody blocks follicular and luteal angiogenesis, consequently inhibiting the ability of gonadotropins to normally stimulate the development of the preovulatory follicle and CL. To examine the ability of anti-VE-C to disrupt mature vessels, we administered E4G10 to pregnant mice with an established CL. With this model, we demonstrate that VE-C inhibition does not cause disruption of mature vasculature; this is in contrast to a VEGF-receptor (VEGFR) antagonist that destabilizes the vasculature of the CL even after it is established in pregnancy. These data suggest a dynamic, specific role of VE-C in ovarian angiogenesis, making it an attractive target for selective inhibition of growing vessels.

Materials and Methods

Angiogenesis and development of the preovulatory follicle and CL

Anti-VE-C antibody. E4G10 monoclonal antibody against an epitope corresponding to a region of the NH₂-terminal domain of murine VE-C was generated as previously described (13).

Ovulation induction and antibody treatment. Female CD-1 mice Hx before 24 d of life (Charles River Laboratories Inc., Wilmington, MA) were used. In Hx mice, follicular development is arrested at the advanced preantral or early antral stage because of lack of endogenous gonadotropins but can reliably develop mature follicles that can be triggered to ovulate if exogenous gonadotropins are administered (18). After arrival, animals are allowed to equilibrate for 1 wk. The lack of significant weight gain (<1.5 g) and vaginal smears consistent with a hypoestrogenic state indicate successful hypophysectomy. Only mice that meet both criteria were included in the experiments. Antibody dosing (250 mg/kg) was empiric, based on previous experiments of dose response (not published). Mice were treated humanely in accord with the policies set forth by the Institutional Animal Care and Use Committee of Columbia University.

For the follicular phase (n = 12 per group), Hx mice were treated with 1 ip injection of 20 IU pregnant mare serum gonadotropin (PMSG) (Sigma Chemical Co., St. Louis, MO) 1 wk after arrival to stimulate gonadotropin-dependent follicular development. Treatment animals were given 1 ml ip injections of E4G10 (4 mg/ml) (ImClone) 12 h before and 48 h after the PMSG injection. Control animals received ip saline injections (1.0 ml) at 12 and 48 h after PMSG. Animals were killed 24 h after the last injection (72 h after PMSG).

For the luteal phase (n = 12 per group), Hx mice were injected ip with 20 IU PMSG as above and 72 h later with 20 IU human chorionic gonadotropin (hCG) (Sigma) to induce ovulation and CL formation. Treatment animals received a single 1-ml injection of E4G10 (4 mg/ml) 2 h before hCG; control group animals received 1.0 ml of saline instead of antibody. Animals were killed 24 h after hCG treatment.

Specimen collection. Immediately after killing, blood was obtained via cardiac puncture for hormonal analysis. Both ovaries and one kidney were carefully dissected. The kidney and one ovary were snap frozen in

OCT and stored at –80 C in preparation for frozen sectioning. The contralateral ovary was weighed after drying on fluid-adsorbing paper, and then preserved in formalin for serial sectioning and hematoxylin and eosin (H&E) staining.

Histology and immunohistochemistry. Formalin-fixed ovaries were sectioned at 10- μ m intervals, and each section was stained with H&E. Evaluation of H&E sections through entire ovaries were used for differential follicle and CL counting and qualitative comparison of development of follicles and CL. Frozen blocks were sectioned at 10- μ m intervals by standard histological protocols. Antibody to platelet endothelial cell adhesion molecule (PECAM)/CD31 (PharMingen, San Diego, CA) was used to identify blood vessels according to manufacturer's protocols. Rabbit anti-rat IgG was used as secondary antibody (Sigma-Aldrich, Milwaukee, WI). Peroxidase staining with diaminobenzidine tetrachloride (DAB) brown chromogen was used to identify the secondary antibody (DAB substrate kit; Vector Laboratories, Burlingame, CA). Hematoxylin was used for counterstaining.

To verify expression of VE-C in the control ovaries, follicular and luteal phase serial sections were stained with two different primary VE-C antibodies: E4G10 and RDI-MCD144–11D4 (Research Diagnostics, Flanders, NJ). Secondary antibody and peroxidase staining were performed in the usual fashion.

Image analysis. For follicular and CL counting, formalin samples embedded in paraffin were sectioned at 10- μ m intervals through their entirety. Gonadotropin-dependent follicles as defined by Pedersen's classification of mouse follicles as stage 6 or greater (19) were counted in serial sections. CL were identified by morphological properties consistent with luteinized follicles and counted in serial hematoxylin sections. Two independent investigators corroborated follicular and CL counts.

For vascular density, the largest-diameter sections of each follicle and CL were analyzed under a light microscope coupled with a Nikon Eclipse E800 digital camera. Images were analyzed with ImagePro Plus version 4.01 software (Media Cybernetics, Silver Spring, MD). Images were calibrated for magnification, and follicles and CL were measured for diameter and largest cross-sectional area. To assess vascular area, PECAM staining was visually identified as DAB brown staining. These areas were selected for counting. The software identified all other areas with equivalent image properties as the selected area and included them for measurement of vascular area.

In the preovulatory follicles, the thecal area of the largest cross-section was measured, and the endothelial area identified by PECAM-positive cells within this compartment was identified. The area occupied by vasculature was measured and expressed in micrometers (2). In the CL, the vascular density was also calculated as the ratio of the area of PECAM-positive cells to the largest cross-sectional area of the CL.

To examine the effect of E4G10 on permanent vasculature, 10- μ m frozen sections of kidney were stained with PECAM in control and treatment animals.

Hormonal assays. Blood obtained via cardiopuncture was allowed to clot for 1 h and then centrifuged at 900 \times g for 10 min at room temperature. Serum was aspirated, and estradiol and progesterone were measured with ligand-labeled, competitive, chemiluminescent immunoassay (Diagnostic Products Corp., Los Angeles, CA). The interassay coefficients of variation were 11.9 and 11.1% for estradiol and progesterone, respectively.

Statistical analysis. Data are expressed as mean \pm SE. Student's *t* test was used with *P* values <0.05 considered significant, using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA).

Blood vessel maintenance in the CL of pregnancy

CD1 male and female mice were mated overnight, and vaginal plugs were checked in the midmorning to confirm coitus. Postcoital females were weighed and separated into individual cages. On E6.5, all mice were weighed and divided into three groups (n = 5 per group). Group 1 consisted of control animals that received no further treatment. Group 2 mice received 1 ip injection of E4G10, 4 mg. Group 3 animals received 1 ip injection of anti-VEGFR-2/Flk1 antibody (DC101; ImClone), 2.5 mg. DC101 has a known antiangiogenic effect on the CL of pregnancy; ip

administration on E3.5 causes disruption of CL vasculature resulting in prevention of further development of the pregnancy (34). DC101 does not cross the placenta; thus, the antiangiogenic action affects the CL but not the placenta or fetus. On E13.5, mice were weighed and killed. Blood was obtained by cardiac puncture, and serum was assayed for progesterone as above. Uteri were dissected and weighed, and two fetuses per uterus (if present) were dissected, weighed, and preserved in formalin. Ovaries were dissected, weighed, and snap frozen in OCT. Frozen sectioned ovaries were stained for PECAM by standard immunohistochemical methods, and ImagePro software was used to calculate CL vascular density, as above.

Results

VE-C is expressed in the ovary

Expression of VE-C in the ovary was verified with immunohistochemistry using two different primary antibodies. E4G10 displayed only weak affinity for vasculature, even at high concentrations. However, MCD144-11D4 showed marked localization to thecal and luteal vasculature (Fig. 1). This expression of VE-C colocalized with anti-PECAM staining (not shown), verifying specificity to endothelial cells.

E4G10 inhibits follicular angiogenesis and development

Table 1 summarizes follicular phase results. Follicular development in control animals was uniform with distinct localization of PECAM to the thecal layer, whereas treatment-group follicular development appeared disorganized with scant PECAM staining in the thecal layer (Fig. 2). The weight of treatment group ovaries was significantly lower than the weight of control group animals. The number of gonadotropin-induced preovulatory follicles per ovary in treatment animals was significantly less than controls. In the treatment group, the advanced follicles were mostly stage 6, with few if any stage 7 or 8 follicles per ovary. The maximum cross-

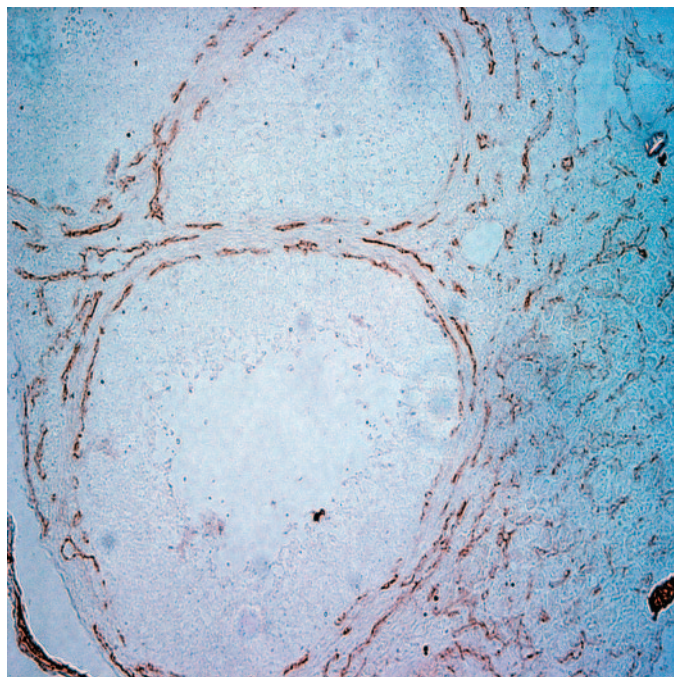


FIG. 1. VE-cadherin expression confirmed in ovary as identified by anti-VE-C antibody, MCD144-11D4. Expression overlaps with expression of PECAM, localizing to vascular endothelial cells.

TABLE 1. Follicular phase

	Control	Treatment
Ovarian weight (mg)	5.70 ± 0.54	3.50 ± 0.55
Advanced follicular area (μm^2)	$1.6 \times 10^5 \pm 1.34 \times 10^4$	$1.09 \times 10^5 \pm 1.71 \times 10^4$
Thecal vascular area (μm^2)	3440 ± 266.4	987.2 ± 243.8
Vascular density (%)	12.7 ± 2.5	1.2 ± 0.98
Estradiol (pg/ml)	63.59 ± 5.14	38.51 ± 2.91

Values are mean ± SE; $P < 0.05$.

sectional area of the most advanced treatment group follicles was significantly smaller when compared with preovulatory control group follicles. The vascular area detected in the theca layer of the most advanced treatment group follicles was significantly less than that of similar control group follicles. Also, the blood vessel density, calculated by dividing the vascular area by the total thecal area multiplied by 100, was significantly less in the treatment group compared with controls. Estradiol levels in the treatment group were significantly lower than the control group. The weight of treatment mice did not significantly differ from controls before or after treatment. No mouse in either control or treatment group exhibited signs of toxicity.

E4G10 inhibits luteal angiogenesis and development

Table 2 summarizes luteal phase results. Control animals appeared to have uniform development of CL with distinct staining of vasculature within the luteinized granulosa layer, whereas the treatment animals appeared to have disorganized luteal development with a paucity of PECAM staining and unovulated preovulatory follicles (Fig. 3). The weight of treatment group ovaries was significantly lower than the weight of control group ovaries. The number of CL in treatment group ovaries was significantly less than that in the control group. The maximum cross-sectional area of treatment group CL was also significantly reduced compared with controls. The amount of vasculature formed in treatment group CL was significantly reduced relative to the control group CL. The percentage of vascular area of the entire CL area was significantly less in treatment animals compared with controls. Serum progesterone levels were significantly lower in treatment mice compared with control mice. The weight of treatment mice did not significantly differ from controls before or after treatment. No mouse in either control or treatment group appeared to exhibit signs of toxicity.

E4G10 does not disrupt the CL of pregnancy

All animals had a similar weight gain between E1 and E6.5. The control and E4G10 mice continued to have a similar degree of weight gain between E6.5 and E13.5, consistent with normal pregnancy. Anti-VEGFR-2 (DC101) treated mice had a net weight loss between E6.5 and E13.5. Comparison of the results from the three experimental groups are summarized in Table 3. Uterine and fetal weights were not statistically significant between the control and the E4G10 groups, and the number of implantation sites between these

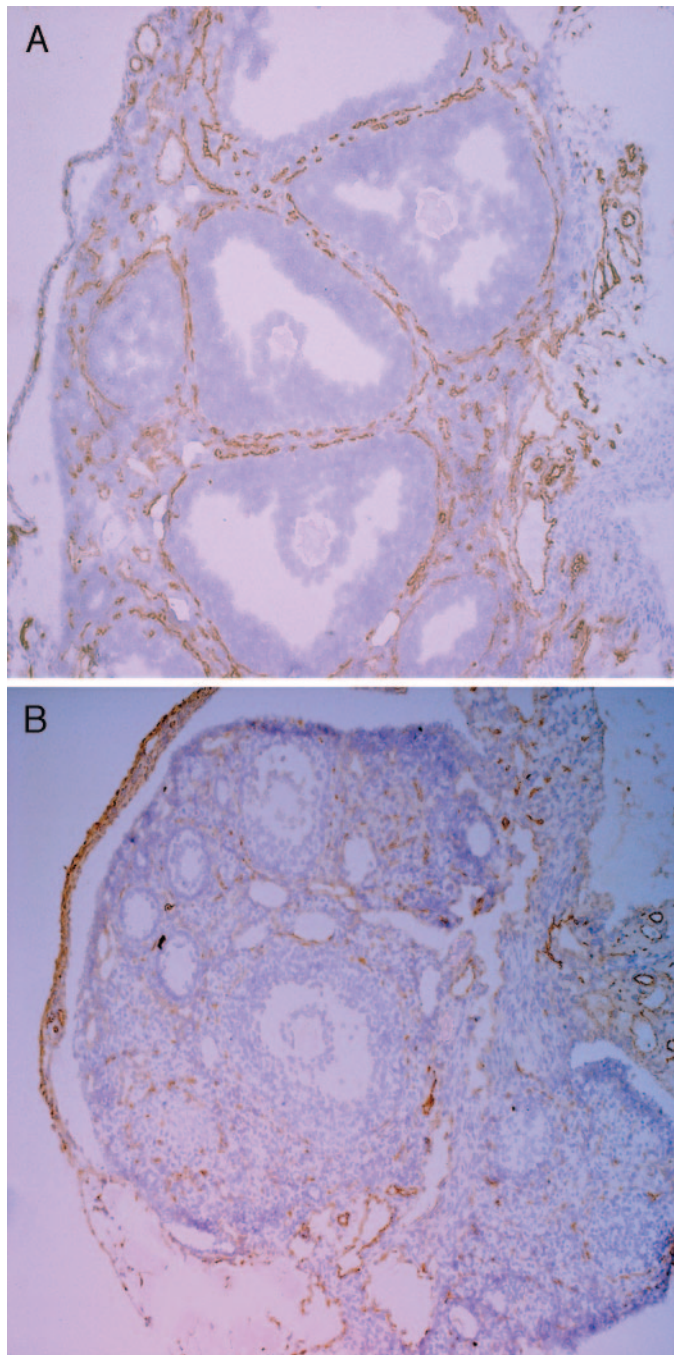


FIG. 2. Follicular phase. A, Control; PECAM staining (*brown*) identifies discrete localization to thecal layer around well-developed pre-ovulatory follicles; B, E4G10-treated, showing sparse PECAM staining surrounding few advanced follicles.

two groups was statistically similar. Fetuses in the E4G10 group appeared normal compared with controls in size and morphology. The anti-VEGFR-2 group had a significantly lower average uterine weight compared with the control and E4G10 groups, and no discernable fetuses were visible on E13.5. Immunohistochemical staining to PECAM of ovarian sections showed similar total vascular area in the control and E4G10 groups, whereas the DC101 group had significantly

TABLE 2. Luteal phase

	Control	Treatment
Ovarian weight (mg)	7.283 ± 0.58	4.050 ± 0.64
CL area (μm^2)	$1.74 \times 10^4 \pm 5.06 \times 10^3$	$6.38 \times 10^4 \pm 8.5 \times 10^3$
CL vascular area (μm^2)	$1.31 \times 10^3 \pm 2.47 \times 10^3$	$1.50 \times 10^3 \pm 2.99 \times 10^2$
Vascular density (%)	8.41 ± 1.45	2.1 ± 0.37
Progesterone (ng/ml)	8.409 ± 1.162	3.244 ± 0.5783

Values are mean ± SE; $P < 0.05$.

decreased vascular area. Serum progesterone levels were similar between the control and E4G10 groups but significantly lower in the DC101 group

E4G10 does not affect permanent vasculature of the kidney

H&E and immunohistochemistry staining of renal glomeruli against PECAM was comparable between controls and treatment animals in follicular and luteal phases. The overall number of glomeruli and morphology were similar between treatment and control groups in both follicular and luteal phases.

Discussion

VE-C is shown here for the first time to be expressed in ovarian vasculature and essential for proper ovarian angiogenesis. Inhibition of VE-C with an immunoneutralizing antibody, E4G10, during gonadotropin-induced ovarian stimulation results in inhibition of follicular and CL angiogenesis. Preventing junction formation between endothelial cells by blocking VE-C with E4G10 inhibits neovascularization but does not disrupt preexisting junctions. Established vasculature of the CL of pregnancy was unaffected by E4G10 administration. Thus, we conclude that anti-VE-C specifically inhibits neovascularization in the ovary but, unlike anti-VEGFR-2, does not cause vascular disruption.

Inhibition of new blood vessel development has significant effects on ovarian function. In the follicular phase, the thecal vascular area of the treatment group was decreased by 72% compared with controls. There were fewer advanced follicles in the treatment group, corresponding to the smaller average antral follicle size in the treatment group compared with controls. The morphological findings correlated with functional findings, because estradiol levels in treatment mice were reduced by 40% compared with controls. The greater increase in ovarian weight of controls compared with treatment also corresponds with the lesser degree of hypertrophic gonadotropin effect on E4G10-treated ovaries.

Findings in the luteal phase were similar. The vascular area of the CL was reduced by 90% in the treatment group compared with controls. The number of CL and the size of the largest CL were also significantly reduced in the treatment group. The serum progesterone levels in treatment mice were reduced by approximately 60% compared with controls. As in the follicular phase, ovarian weight was less in the treatment animals, reflecting a lesser degree of stimulation-induced hypertrophy. Because the E4G10 was adminis-

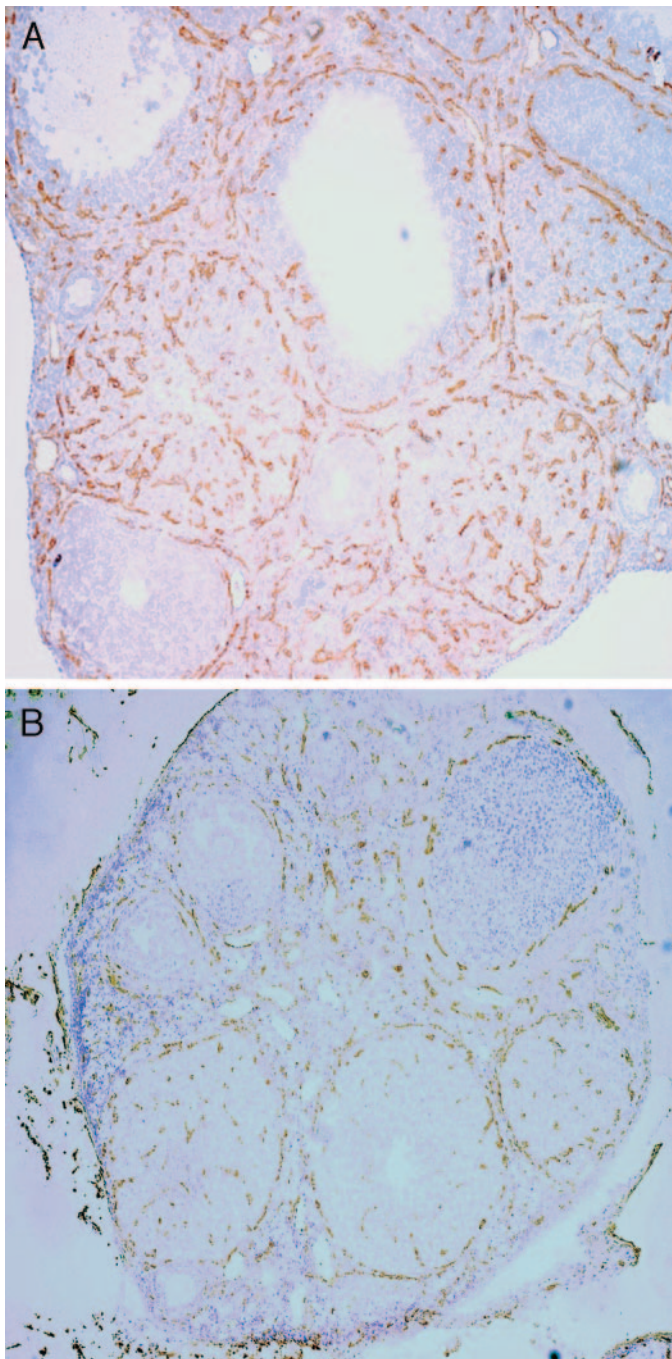


FIG. 3. Luteal phase. A, Control; dense PECAM staining (*brown*) identifies vessels within several well developed CL; B, E4G10-treated, showing few CL with paucity of PECAM staining.

tered before hCG, it is theoretically possible that ovulation was partially inhibited or may have occurred abnormally as a result of impaired preovulatory angiogenesis. In the follicles that ovulated, luteinization occurred, but the degree of angiogenesis and hormonal secretion was dramatically reduced. These effects may have been a result of abnormal ovulation or the inhibition of angiogenesis in the early CL. By administering E4G10 shortly before triggering ovulation, we intended to ensure that antibody would be in circulation at the time of early CL develop-

TABLE 3. Pregnancy

	Control	Anti-VE-C	Anti-Flk
Uterus weight (g)	8.27 ± 1.30 ^a	8.1 ± 0.63 ^a	0.27 ± 0.13
Implant sites	12.5 ± 2.5 ^a	11 ± 3.0 ^a	0
Fetal weight (mg)	155 ± 19.7 ^a	167.3 ± 19.59 ^a	0
Progesterone (ng/ml)	24.23 ± 1.86 ^a	27.44 ± 4.97 ^a	1.95 ± 0.89
Vascular density (μm ²)	11935 ± 3054 ^a	10997 ± 4687 ^a	2209 ± 741

Values are mean ± SE.

^a *P* < 0.05 compared with anti-Flk.

ment, but we cannot rule out the possibility that some of the effects may have been secondary to inhibition of ovulation. The experiments during pregnancy demonstrate that once established, the CL vasculature and function is unaffected by E4G10 treatment.

It is interesting to note that in both the follicular and luteal phases, the degree of vascular inhibition does not linearly correlate with the degree of functional inhibition. That is, the hormonal secretion from the follicles and CL is suppressed to a lesser degree than the vascular area within these structures. This suggests that under physiological conditions, blood vessel development occurs to a greater degree than is necessary for normal endocrine function, such that normal hormonal secretion can be supported by less than maximal vascular proliferation. Furthermore, the degree of inhibition of follicular angiogenesis and growth to the preovulatory stage by antagonizing VE-C was of a similar magnitude as the inhibition caused by VEGFR-2 inhibition with DC-101 (7). Because DC101 is known to have significant effects on inhibiting vascular permeability, whereas E4G10 does not affect permeability (13), changes in permeability are perhaps less important to ovarian angiogenesis than previously proposed (20). In contrast, inhibition of VEGFR-2 function had more significant consequences for CL function (5) than did inhibition of VE-C. Because VEGFR-2 is required for both proliferative and permeability responses to VEGF, our data may suggest that both processes are required for full CL function.

To examine the effects of VE-C inhibition on mature ovarian vasculature, we treated pregnant mice with E4G10. Although neovascularization is highly active in the CL during the early luteal phase, the pregnant state represents a time when the CL vessels have already maximally proliferated such that vascular stabilization and maintenance appear to be more significant processes than neovascularization in the pregnant CL (21, 22). When injected into pregnant mice on E6.5, E4G10 appeared to have no effect on either the CL or the developing fetuses. CL stained with equal vascular density in both control and E4G10 groups. In contrast, VEGFR-2 inhibition appears to not only inhibit neovascularization but also cause destabilization of mature vessels in the pregnancy CL. Vessels in other organ systems, such as the renal glomeruli, do not appear affected by anti-VEGFR-2 treatment. Thus, it appears that the recently established vasculature of the CL may be dependent on VEGF for maintenance and stabilization, whereas quiescent vasculature may be stabi-

lized by non-VEGFR-2 mechanisms. Administration of DC101, an anti-VEGFR-2 antibody, causes a dramatic disruption of the vessels of the pregnancy CL, resulting in an interruption of the ongoing pregnancy. E4G10, however, causes no discernable effect on the pregnancy CL compared with controls. In part, the difference in effect between anti-VEGFR-2 and anti-VE-C inhibition may involve permeability effects; anti-VEGFR-2 has demonstrated significant effects on permeability during early pregnancy (23, 24), whereas VE-C inhibition may not affect vascular permeability (13, 15). It appears that E4G10 specifically targets formation of new blood vessels by preventing interendothelial adherens junctions, without disrupting those that are preexisting. Thus, we concur with other investigators that E4G10 binds an epitope of the VE-C ECD that is involved in angiogenesis but is either insignificant to junctional maintenance or inaccessible once the junction is formed, secondary to conformational change (13, 15). In addition, it appears that the expression of VE-C and VEGF are related, although the details of their relationship have yet to be fully elucidated (25, 26).

In conclusion, these data support previous studies that have demonstrated that angiogenesis in the ovary is important for follicular and luteal function. Angiogenesis is a process that occurs as a result of multiple events that include degradation of the extracellular matrix; budding, proliferation, and migration of endothelial cells; formation of capillary tubes; remodeling of capillary networks; and maturation and stabilization of vessels involving the recruitment of stromal cells (27). Because multiple steps are involved in the process, many targets are available for modulation of angiogenesis for various potential clinical purposes (28). The mechanism of angiogenic inhibition that we propose here is the prevention of adherens junction formation, such that new vessels cannot form, whereas the integrity of established vasculature is preserved. E4G10 provides a potent mechanism for specific inhibition of ovarian neovascularization without disrupting mature vessels. The lack of effect on mature vessels would presumably reduce undesirable side effects of permeability changes possibly associated with other angiogenic therapies (29). The clinical applications of specific ovarian neovascular inhibition potentially include contraception (30), prevention of recurrent ovarian cysts (31), management of polycystic ovary syndrome (32), and adjuvant treatment for malignant ovarian disease (33).

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