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The Vascular Endothelial Growth Factor (VEGF)/VEGF Receptor 2 Pathway Is Critical for Blood Vessel Survival in Corpora Lutea of Pregnancy in the Rodent

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The vascular endothelial growth factor (VEGF)/VEGF receptor 2 (VEGFR-2) pathway regulates proliferation, survival, and permeability of vasculature. This pathway is active during the formation of a corpus luteum, a highly vascularized, endocrine organ with a short life span during the nonpregnant state. In the pregnant state, the life span of corpora lutea is much longer because they play a critical role in supporting pregnancy development. We hypothesized that the VEGF/VEGFR-2 pathway plays a critical role in regulating angiogenic events in the corpora lutea of pregnancy. Injection of the neutralizing anti-VEGFR-2 antibody DC101 (ImClone Systems, Inc., New York, NY) on embryonic d 3.5 (preimplantation) or 6.5 (postimplantation) disrupts function of the corpora lutea of pregnancy in CD1 mice, as evidenced by a decrease in organ size, regression of luteal vessels, and a fall in progesterone secretion within 24 h postinjection. Inhibition of the VEGFR-2 caused removal of endothelial cells, mostly through endothelial cell detachment from the vascular basement membrane. Luteal steroid-producing epithelial cells were eliminated through apoptosis secondary to vasculature becoming dysfunctional. Disruption of luteal function caused arrest of embryonic development. The effect of antibody is specific to the ovary, because pregnancy progresses normally in ovariectomized, progesterone-replaced animals treated with anti-VEGFR-2 antibody. Embryonic blood vessels were not affected directly by the antibody, because it did not reach the embryo. Administration of an antibody against VE-cadherin (E4G10), which specifically blocks endothelial proliferation, did not disrupt luteal function and pregnancy development. Thus, VEGFR-2-mediated endothelial cell signals are critical to maintain functionality of luteal blood vessels during pregnancy. Potential clinical applications of inhibitors of the VEGF/VEGFR-2 pathway include emergency contraception and medical treatment of ectopic and abnormal intrauterine pregnancies. (*Endocrinology* 146: 1301–1311, 2005)

THE CORPUS LUTEUM is a temporary, endocrine structure with a short life span in the nonpregnant state. It is formed from a preovulatory follicle, whose hallmark is a centrally located, fluid-filled cavity. After ovulation this cavity fills rapidly with epithelial and endothelial cells through proliferative events transforming it into a solid structure. Epithelial cells are derived from granulosa and thecal cells, whereas endothelial cells originate from preexisting, thecal layer blood vessels. The corpus luteum is a highly vascularized organ whose vascular density exceeds those of most tumors. Once formed, the vasculature is maintained for a limited period of time in the nonpregnant state. When a new estrous or menstrual cycle is initiated, the corpus luteum regresses as luteal epithelial and endothelial cells are eliminated (1, 2). This is in contrast to the pregnant state, where the presence of blood vessels in corpora lutea is required for a longer period of time to maintain functionality throughout at least part, if not the entire, pregnancy. Because luteal proliferative events are very few in the early stages of pregnancy, at least in rodents, maintenance of function occurs mostly through securing the survival of epithelial and endothelial cells (3).

One important pathway regulating angiogenic events is VEGF acting through two receptors: VEGF receptor 1 (VEGFR-1) and VEGFR-2 (4). Alternative exon splicing results in the formation of four different isoforms with 121, 165, 189, and 206 amino acid forms, respectively (murine VEGF is shorter by one amino acid), with $VEGF_{165}$ being the predominant one. It is thought that VEGF-dependent activation of VEGFR-2 is involved in mediating endothelial cell proliferation, survival, and vascular permeability, whereas VEGFR-1 might play an inhibitory role by sequestering VEGF, and thus preventing its interaction with VEGFR-2 (4). Regulation of angiogenic events in corpora lutea is only partially understood. The VEGF/VEGFR-2 pathway is active in angiogenesis, occurring during the formation of corpora lutea, as evidenced by the fact that substances inactivating VEGF or the VEGFR-2 interfere with luteal blood vessel proliferation (5–7). It is not clear whether this angiogenic pathway is also active in regulating endothelial function in corpora lutea of pregnancy. For example, in rodents, admin-

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Abbreviations: BrdU, 5-Bromo-2′-deoxyuridine; BVD, blood vessel density; ED, embryonic day; H&E, hematoxylin and eosin; n.s., not significant; P4, progesterone; PECAM, platelet endothelial cell adhesion molecule; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

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istration of anti-VEGF antibody seems to interfere with early pregnancy development by affecting predominantly endothelial proliferative events in the uterus, whereas effects on ovarian function seem to be less pronounced (8, 9). In contrast, administration of anti-VEGF antibody to pregnant rats, which were hypophysectomized and hysterectomized, seems to counteract estradiol-dependent rescue of luteal function by decreasing progesterone (P4) levels and slightly diminishing luteal vascular density (10), indicating that luteal function is dependent on VEGF. Observations made in mouse tumor models are relevant in this context, because they suggest that anti-VEGF antibodies not only inhibit angiogenesis, but also induce regression of preexisting blood vessels, suggesting a regulatory role in endothelial survival (11–13). Based on VEGF-related findings in nonpregnant and pregnant corpora lutea as well as in tumors, we hypothesized that the VEGF/VEGFR-2 pathway plays a critical role in the regulation of angiogenic events in corpora lutea of pregnancy.

We report that a single pre- or early postimplantation administration of anti-VEGFR-2 antibody (DC101, ImClone Systems, New York, NY) in pregnant CD1 mice disrupts survival of preexisting luteal blood vessels in the ovary and terminates embryonic development through its effects on ovarian hormone production. Inhibitors of the VEGF/VEGFR-2 pathway, therefore, may be helpful in the development of new types of contraceptives and in the treatment of pathological conditions such as ectopic pregnancy.

Materials and Methods

Behavior of the antibody DC101 in vivo in pregnant animals

Access of maternal substances to embryonic structures is selective due to protective barriers (zona pellucida, trophoblast, and placenta with chorion) surrounding the embryo and fetus. Thus, we first studied the binding pattern of the rat monoclonal anti-VEGFR-2-blocking antibody DC101 (ImClone Systems, Inc.) after administration to pregnant mice to determine whether it can affect maternal and embryonic tissues simultaneously during pregnancy. Animals were injected with saline (control; n = 2) or increasing dosages of DC101 (66, 132, and 264 mg/kg; n = 2/group) on embryonic d (ED) 6.5 or 12.5, and killed on ED 7.5 or 13.5. A dosage of 66 mg/kg animal inhibits ovarian angiogenesis (14). Ovary, uterus containing the embryo, liver, and kidney were removed for characterization of DC101 localization. Frozen tissue sections of these organs from control and anti-VEGFR-2 antibody-treated animals were incubated with mouse absorbed, affinity-purified, biotinylated rabbit antirat IgG secondary antibody (Vector Laboratories, Inc., Burlington, CA). This immunohistochemical approach allowed identification of tissues to which the blocking antibody DC101 was bound. No staining for anti-VEGFR-2 antibody was detected in any of the control group tissue sections. Binding of DC101 to vascular structures in the ovary, uterus, liver, and kidney was consistently detected with the secondary antibody in treatment group animals. In contrast, no DC101 binding to embryonic structures, including vasculature, was detected with the secondary antibody. To test whether DC101 has the ability to bind to embryonic vascular structures, we incubated slides onto which tissue cross-sections of uterus and embryonic tissue from pregnant control animals were mounted, with the blocking antibody DC101 as the primary antibody. Under these conditions, the secondary antibody detected embryonic vascular (ED 13.5) structures through its binding to DC101. We concluded that antibody injected into pregnant animals binds to maternal blood vessels, including ovary and uterus, but does not reach embryonic structures.

Experiment 1: effect of the anti-VEGFR-2 antibody DC101 on ovarian function and pregnancy development

Seven animals per group were used in all experiments. Implantation occurred on ED 4.5.

Experiment 1a: postimplantation (ED 6.5)

Three-month-old female CD1 mice (Charles River Laboratories, Baltimore, MD) were mated with males of proven fertility overnight. Identification of a vaginal plug the following morning was interpreted as successful mating and counted as d 0.5 of pregnancy. Plugged female mice were randomized into two groups. Control group animals received a single ip injection of saline (0.5 ml) on ED 6.5. Treatment group animals received a single ip injection of anti-VEGFR-2 antibody (66 mg/kg; DC101, ImClone Systems, Inc.) on ED 6.5. Such a dose is sufficient to inhibit angiogenesis in corpora lutea (7). Animals were killed on ED 7.5, and 13.5. A baseline group of animals were also killed on ED 6.5 to serve as a reference for the evaluation of ovaries. Uterus, both ovaries, one kidney, and part of the liver were removed from all animals for histological and/or immunohistochemical/immunofluorescent evaluation.

Experiment 1b: preimplantation (ED 3.5)

Plugged female mice were randomized to two groups. Control group animals received a single ip injection of saline (0.5 ml) on ED 3.5. Treatment group animals received a single ip injection of anti-VEGFR-2 antibody (66 mg/kg; DC101) on ED 3.5. Animals were killed on ED 4.5, 7.5, and 13.5. A baseline group of animals was also killed on ED 3.5 to serve as a reference for the evaluation of ovaries. Uterus, both ovaries, one kidney and part of the liver were removed from all animals for histological and/or immunohistochemical/immunofluorescent evaluation.

Experiment 2: effect of anti-VEGFR-2 antibody DC101 on pregnancy development in ovariectomized, hormone-replaced animals

Substances originating from the products of conception could play an important role in the maintenance of luteal vasculature (15). If so, DC101-induced changes in luteal vasculature could have been indirect, through disruption of pregnancy development by interfering with the function of decidual tissue. We tested for such a possibility by following pregnancy development in ovariectomized, P4-replaced animals exposed to DC101 before or after implantation. Pregnancy in such animals can be maintained by exogenously administered P4 (16). Five animals were used in each group.

Experiment 2a: postimplantation (ED 6.5)

Four 10-mg 21-d release P4 capsules (Innovative Research of America, Sarasota, FL) were placed sc on the left and right sides (two capsules per side) of the neck of pregnant female mice on ED 5.5 using a small trocar provided by the company for this purpose. Ovariectomy was performed in all animals on ED 6.0. Hormone-replaced, ovariectomized animals were then randomized into the control group, which received a single ip injection of saline (0.5 ml) on ED 6.5, and the treatment group, which received an injection of a single dose of anti-VEGFR-2 antibody (66 mg/kg; DC101) on ED 6.5. Animals were killed on ED 7.5 and 13.5. Uteri were removed from all animals for histological and/or immunohistochemical evaluation.

Experiment 2b: preimplantation (ED 3.5)

P4 capsules (Innovative Research of America) were placed sc in plugged female mice on ED 2.5. Ovariectomy was performed in all animals on ED 3.0. Hormone-replaced, ovariectomized animals were then randomized into a control group, which received a single ip injection of saline (0.5 ml) on ED 3.5, and a treatment group, which received an injection of a single dose of anti-VEGFR-2 antibody (66 mg/kg; DC101) on ED 3.5. Animals were killed on ED 13.5. Uteri were removed for histological evaluation.

Experiment 3: effect of anti-VE-cadherin antibody E4G10 administered on ED 6.5 on ovarian function during pregnancy

To determine whether the proliferation of endothelial cells occurs in corpora lutea of pregnancy, we evaluated the effect of the anti-VE cadherin antibody E4G10 (ImClone Systems, Inc.) (17). This antibody specifically blocks angiogenesis, but does not affect blood vessel survival. Plugged female mice were randomized to two groups. Control group animals received a single ip injection of saline (0.5 ml) on ED 6.5. Treatment group animals received a single ip injection of rat anti-VE cadherin antibody (264 μg/kg; E4G10), which specifically blocks angiogenesis, on ED 6.5. Such a dose inhibits angiogenesis during the formation of corpora lutea (18). Animals (n = 3/group and day of death) were killed on ED 7.5 or 13.5. A baseline group of animals was also killed on ED 6.5 to serve as a reference for the evaluation of ovaries. The uterus and both ovaries were removed from all animals for histological and immunohistochemical evaluation.

Identification of implantation sites and evaluation of ovary, uterus, and pregnancy by histology

Each completely dissected uterus containing the embryos was weighed, and the number of implantation sites per animal was counted by visual inspection. Subsequently, one ovary and approximately half of the uterus containing the embryos were immersed in formalin for fixation, then stored in 70% ethanol. Tissues of interest were embedded in paraffin, sectioned at 10 μm, and stained with hematoxylin and eosin (H&E) according to standard histological protocols. To select a corpus luteum suitable for evaluation, 20–30 slides of each ovary were evaluated, the corpus luteum with the largest diameter was identified, and its diameter was measured.

Immunohistochemistry/immunofluorescence

The contralateral ovary, the remaining uterus, one kidney (experiment 1), and part of the liver (experiment 1) were embedded in OCT (Miles, Inc., Elkhart, IN), snap-frozen in dry ice-cooled isopentane, and stored at −80 C degrees. Frozen ovaries were sectioned at 5-μm intervals. Thirty sections were obtained, and every fifth slide was stained with H&E. One to 30 slides were evaluated. The slide containing the largest cross-section through a corpus luteum was identified and given the number 0. The sections preceding (identified by +) and after (identified by −) this H&E section were selected for immunohistochemical/immunofluorescent evaluation.

Endothelial cells (+1). Section +1 was evaluated for the presence of blood vessels in corpora lutea using an antibody against platelet endothelial cell adhesion molecule (PECAM; biotin-conjugated rat antimouse CD 31 monoclonal antibody, 1:200; BD Pharmingen, San Diego, CA) (19, 20) following standard immunohistochemical procedures (7). It is possible that anti-VEGFR-2 antibody influences the expression of PECAM on endothelial cells. We theorized that if the expression pattern of PECAM correlated closely with the expression pattern of a second angiogenic marker, such a possibility would be less likely. Therefore, in a pilot experiment we incubated two consecutive ovarian sections from three control and treatment group pregnant animals (ED 7.5 and 13.5) with antibody against PECAM and antibody against VE-cadherin RDI-MCD144-11D4 (rat antimouse monoclonal antibody, 1:20; Research Diagnostics, Flanders, NJ) following the manufacturer's instructions (18). Because the patterns of staining for PECAM and VE-cadherin nearly completely overlapped in all sections, we conclude that treatment with anti-VEGFR-2 antibody did not significantly affect the expression of PECAM. Only staining for PECAM is shown in this report, because it is more robust.

Nuclear cell number (+2). Section +2 was stained with hematoxylin to identify nuclei of individual cells.

Apoptosis (+3 and +4). Apoptotic cells were detected using rabbit antihuman/mouse caspase 3 antibody (rabbit antimouse antibody, 1:500; R&D Systems, Inc., Minneapolis, MN) following the manufacturer's instructions (21). Because antibody against caspase 3 might detect both active and inactive proteins, we evaluated the presence or absence of apoptosis with a second marker by staining for DNA fragmentation using the ApopTag kit (Intergen, Purchase, NY) following the manufacturer's instructions (14). Quantitative evaluation revealed a close correlation between the two methods (0.82), with the number of cells staining positively for caspase 3 being lower compared with staining with the ApopTag kit. Results with the ApopTag kit are shown because the staining was more robust than caspase staining.

Cell proliferation (+5). Animals were injected 1 h before death with 5-bromo-2′-deoxyuridine (BrdU; 1 ml/100 g animal weight; Zymed Laboratories, Inc., San Francisco, CA). Proliferating cells were identified with a biotinylated mouse anti-BrdU primary antibody (Zymed kit) following the manufacturer's instruction (14).

Pericytes (+6). Pericytes were detected using monoclonal anti-α-smooth muscle actin antibody (antimouse monoclonal antibody, 1:500; Sigma-Aldrich Corp., St. Louis, MO) together with the mouse on mouse kit (Vector Laboratories, Inc., Burlingame, CA) following the manufacturer's instructions.

Sections −1 to −4 were incubated with PECAM in combination with another primary antibody, and immunofluorescence was used for antibody detection. Immunofluorescent staining was performed by incubating sections with biotinylated primary antibodies, then the signals were detected using fluorescent dye conjugate of streptavidin, AlexaFluor 488 (1:200; green) and AlexaFluor 594 (1:200; red; Molecular Probes, Inc., Eugene, OR), applied for double labeling following the manufacturer's instructions (14). Nonbiotinylated primary antibodies were first incubated with a biotinylated secondary antibody, and signal detection was performed as described above.

Basement membrane (−1). Basement membrane was identified with an antibody against collagen IV (rabbit polyclonal antibody, 1:7000; Cosmo Bio Co. Ltd., Tokyo, Japan), a constituent of the vascular basement membrane, following the manufacturer's instructions (22). This allowed for detection of vascular basement membrane surrounding endothelial cells (22).

The non-PECAM antibody used in section −2 was against caspase 3, ApopTag was used in section −3, and an antibody against BrdU was used in section −4. This approach allowed us to detect apoptotic and proliferative endothelial and epithelial cells in corpora luteal of pregnancy.

Controls. Negative controls from control and treatment groups (ovary, uterus, kidney, and liver) were included in all immunohistochemical and immunofluorescent tissue evaluations. To obtain such negative controls, the primary antibody was omitted, but otherwise the procedural steps were the same as those described above. No significant nonspecific staining was observed in any of the tissue samples analyzed in the absence of primary antibody; therefore, data are not shown.

Serum P4

Blood was obtained from all animals by cardiopuncture at the time of death. Serum P4 levels were measured using a ligand-labeled, competitive, chemiluminescent immunoassay (Diagnostic Products Corp., Los Angeles, CA) (14).

Data analysis

Quantitative analysis of endothelial cells (PECAM), proliferation (BrdU), and apoptosis (caspase 3 and DNA fragmentation) was performed using an image analysis system linked to an Eclipse E800 camera (Nikon, Melville, NY). The data were processed for quantitative analysis using ImagePro Plus version 4.01 (Silver Spring, MD). For corpora lutea and uterine sections, this analysis was performed as follows.

Blood vessel density (BVD). The area of interest in a cross-section was outlined, highlighted, and measured in square millimeters. Subsequently, specifically stained tissue (brown staining for PECAM in immunohistochemical sections) in the same area was highlighted and measured in square millimeters. Then the density of specific staining, *e.g.* PECAM staining for vasculature, was calculated by forming the ratio of the area of specific staining divided by the total area of the structure of interest (corpus luteum) multiplied by 100. In uterine cross-sections, the

decidual area was outlined, and BVD was evaluated as described for corpora lutea.

Proliferation and apoptosis. The total area of a cross-section was outlined. The image analysis system was set up to measure the number of dark-stained nuclei or cells (BrdU and apoptosis) using the segmentation part of the computer program. An adjacent section, which was stained with hematoxylin, allowed for counting the total number of luteal cells identified as blue-stained nuclei. A proliferation index was formed (*i.e.* BrdU-positive cells, expressed as a percentage of the total number of luteal cells). For apoptosis, the values obtained in the postimplantation group on ED 6.5 (baseline) and in the preimplantation group on ED 3.5 (baseline) were set at 100%, and the deviations seen on ED 4.5, 7.5, or 13.5 were calculated as the percent change from baseline.

Statistical analysis

Data are presented as the mean ± SE. ANOVA and unpaired t test were used, with a significance level set at $P < 0.05$. Microsoft Excel was used to perform the above statistical tests.

Animals were observed for signs of distress, such as decreased food or water intake, decreased activity, abnormal posture or gait, abnormal behavior or vocalization, and increase in lethargy, for 2 h after the injection of the antibody and for 1 h daily thereafter until death. No systemic side-effects of antibody DC101 were observed in any of the mice.

All experiments were conducted in accordance with the principles and procedures of the NIH Guide for the Care and Use of Laboratory Animals and had been approved by the Columbia University institutional animal care and use committee.

Results

Effect of a single post- or preimplantation injection of anti-VEGFR-2 antibody DC101 on ovarian function and pregnancy development

Postimplantation (injection of anti-VEGFR-2 antibody DC101 on ED 6.5). The diameter of control group corpora lutea remained unchanged in postimplantation pregnancy. In contrast, luteal size decreased significantly by 24 h after antibody administration in treatment group animals (30% decline compared with controls) and reached its nadir on ED 13.5 (Fig. 1A and Table 1A), indicating that due to the action of anti-VEGFR-2 antibody DC101, treatment group corpora lutea function must have been compromised. Such dysfunction of treatment group corpora lutea was reflected by the fact that a significant decline in ovarian P4 secretion occurred, starting 24 h after antibody administration, compared with controls (Fig. 2A). Evaluation at the cellular level revealed that in the treatment group luteal BVD had decreased significantly 1 d after antibody administration (ED 7.5; Fig. 1B), and was maintained at this level as pregnancy progressed (Table 1A). In contrast, BVD in control group corpora lutea remained unchanged as pregnancy advanced to ED 13.5 (Fig. 1B and Table 1A). Qualitative evaluation of luteal blood vessels demonstrated that they stained positively for PECAM, but were mostly negative for α-smooth muscle actin, a marker for pericytes (Fig. 1C). The absence of pericytes is considered a hallmark of immature microvasculature, indicating increased vulnerability when deprivation of VEGF action occurs (20). Subsequently, we examined possible mechanisms of cell elimination in corpora lutea. Although apoptosis was very low throughout pregnancy in control group corpora lutea (Fig. 1D), it increased significantly 24 h after antibody administration (Fig. 1D) and remained above the control value with the continuation of pregnancy (Table 1A). Immunofluorescent double staining for apoptosis and PECAM demonstrated that apoptotic epithelial cells dominated over apoptotic endothelial cells in treatment group corpora lutea (Fig. 3B). The apoptotic ratio of epithelial to endothelial cells was approximately 15:1 throughout pregnancy. Apoptosis and endothelial cell regression were rarely seen in treatment group corpora lutea before 24 h after antibody administration (our unpublished observation), indicating that a time period of approximately 24 h was necessary to obtain significant treatment effects. Based on these observations, it can be concluded that one mechanism of epithelial cell elimination is apoptosis, whereas nonapoptotic mechanisms seem to be more important for luteal endothelial cell elimination. One possibility is that endothelial cells could have become detached from basement membranes, leaving vascular basement membrane ghosts behind as dysfunctional blood vessel residua (2, 20). Such ghosts would be a historical record of previously existing blood vessels, which have become dysfunctional (20). Double staining with antibodies against collagen IV, a vascular basement membrane component (20), and PECAM, an

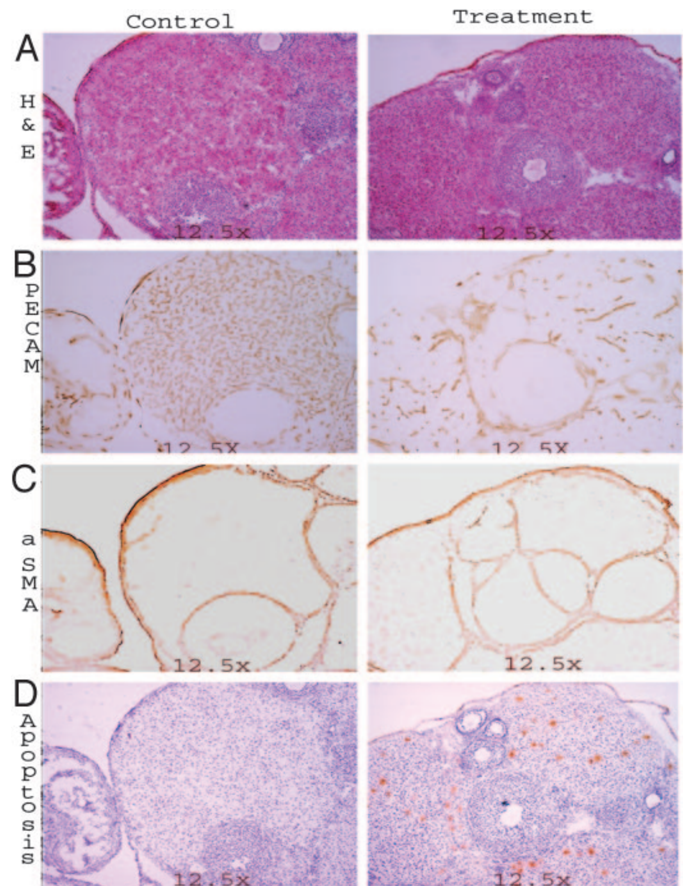

FIG. 1. Cross-section through the corpora lutea of pregnancy evaluated on ED 7.5 from animals injected with saline (control; *left column*) or DC101 (treatment; *right column*) on ED 6.5 using serial sections. A, H&E staining of corpora lutea; note the decreased size of treatment corpus luteum. B, PECAM staining shown in *brown*; note the decrease in vascular density in the treatment group. C, α-Smooth muscle actin staining; note the absence of staining inside control and treatment group corpora lutea. D, Apoptosis (ApopTag) staining shown in *brown*; note an increase in staining in the treatment group.

TABLE 1. Effects of postimplantation administration of DC101 (ED 6.5) on ovary, uterus, and pregnancy

A. Diameter, vascular density, and apoptosis in control and treatment group corpora lutea

ED	Diameter (mm)			Vascular density (%)			Apoptosis (%)		
	Control	Treatment	*P*	Control	Treatment	*P*	Control	Treatment	*P*
6.5	0.63 ± 0.05			100 (baseline)			100 (baseline)		
7.5	0.61 ± 0.08	0.45 ± 0.05	0.02	101 ± 1.9	32 ± 3.9	0.01	102 ± 4.1	568 ± 12.4	<0.01
13.5	0.65 ± 0.04	0.39 ± 0.04	0.01	112 ± 4.5	39 ± 2.4	0.01	99 ± 5.4	184 ± 6.5	0.05

B. Uterine weight and implantation sites in control and treatment group corpora lutea

ED	Uterine weight			Implantation sites		
	Control (g)	Treatment (g)	*P*	Control	Treatment	*P*
6.5	0.38 ± 0.05			13.1 ± 1.3		
7.5	0.43 ± 0.06	0.41 ± 0.08	n.s.	13.5 ± 0.9	13.1 ± 0.7	n.s.
13.5	6.89 ± 0.73	0.25 ± 0.11	<0.01	13.0 ± 1.1	0	<0.01

endothelial cell marker (19), revealed that in treatment group corpora lutea, many basement membranes were devoid of endothelial cells (collagen IV-only structures or basement membrane ghosts; Fig. 4, treatment groups A–C). In contrast, most of the basement membranes in controls colocalized with endothelial cells (collagen IV staining associated with PECAM staining; Fig. 4, control groups A–C). These findings indicate that the endothelial cells must have regressed through the action of anti-VEGFR-2 antibody DC101, possibly through detachment from the basement membrane, making these remnant vessels dysfunctional. Cell proliferation was noncontributory to the observed phenomena, because it was very low in control and treatment group corpora lutea throughout pregnancy [0.2–0.3% of all cells per corpus luteum; not significant (n.s.); Fig. 3A]. The above data also seem to indicate that no recovery of luteal function through new epithelial and endothelial cell division occurred, because no increase in luteal size or vascular density was seen on ED 13.5 in the treatment group, even though the effects of antibody injection are short term (23).

The biological significance of these ovarian antibody effects was assessed by examining pregnancy development. Uterine weight increased significantly in the control group as pregnancy progressed (0.43 ± 0.06 g on ED 7.5 to 6.89 ± 0.73 g on ED 13.5), reflecting appropriate growth of the uteri and progression of embryonic development (Table 1B). The number of implantation sites (on the average, 13) did not change significantly over time (Table 1B). Uterine weights in treatment group animals were similar to those in control group

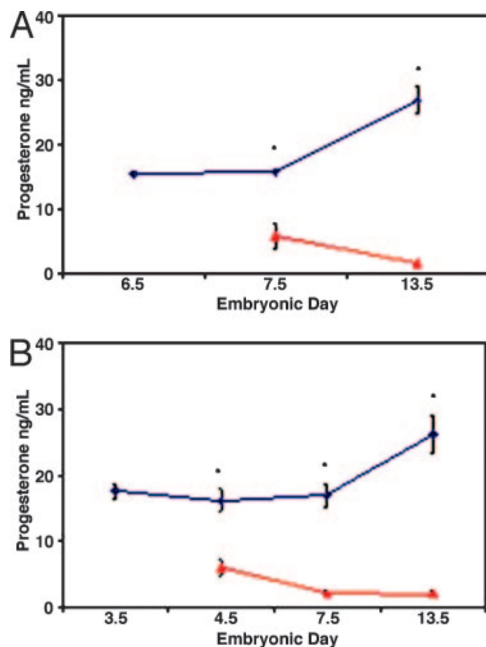

FIG. 2. Maternal P4 levels. A, Postimplantation experiment. The control group (*blue line*) was injected with saline on ED 6.5; the treatment group (*red line*) was injected with DC10 on ED 6.5. B, Preimplantation experiment. The control group (*blue line*) was injected with saline on ED 3.5; the treatment group (*red line*) was injected with DC10 on ED 3.5. *Asterisks* indicate a statistically significant difference.

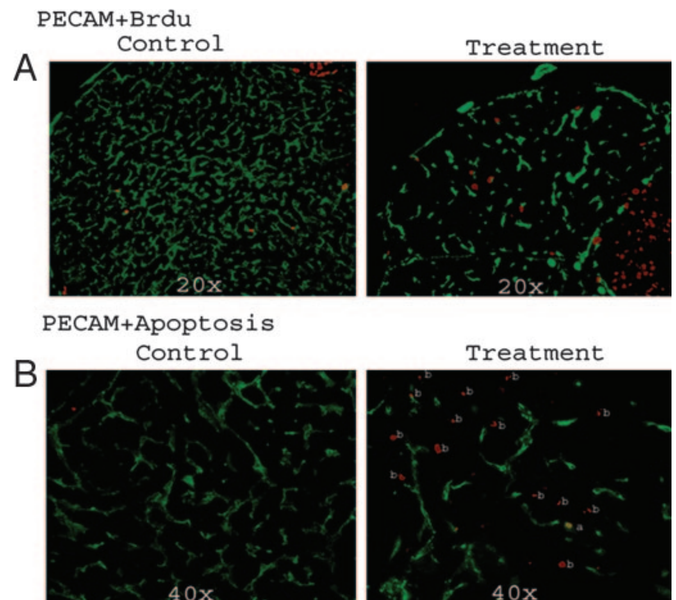

FIG. 3. Immunofluorescence staining for proliferation and PECAM (A) and apoptosis and PECAM (B) of control and treatment group corpora lutea from an animal injected with saline (control; *left column*) or DC101 (treatment; *right column*) on ED 6.5 and evaluated on ED 7.5. Proliferation (A): *green*, blood vessels stained for PECAM; *red*, proliferative cells. Please note low proliferation in control and treatment. On *lower right* in treatment proliferative cells of a follicle. Apoptosis (B): *green*, blood vessels stained for PECAM; *red*, apoptotic cells. Please note nearly complete absence of apoptosis in control; note presence of apoptotic cells without simultaneous staining for PECAM (epithelial cells; b) in treatment; also note few cells stained *yellow* representing apoptotic endothelial cells (a).

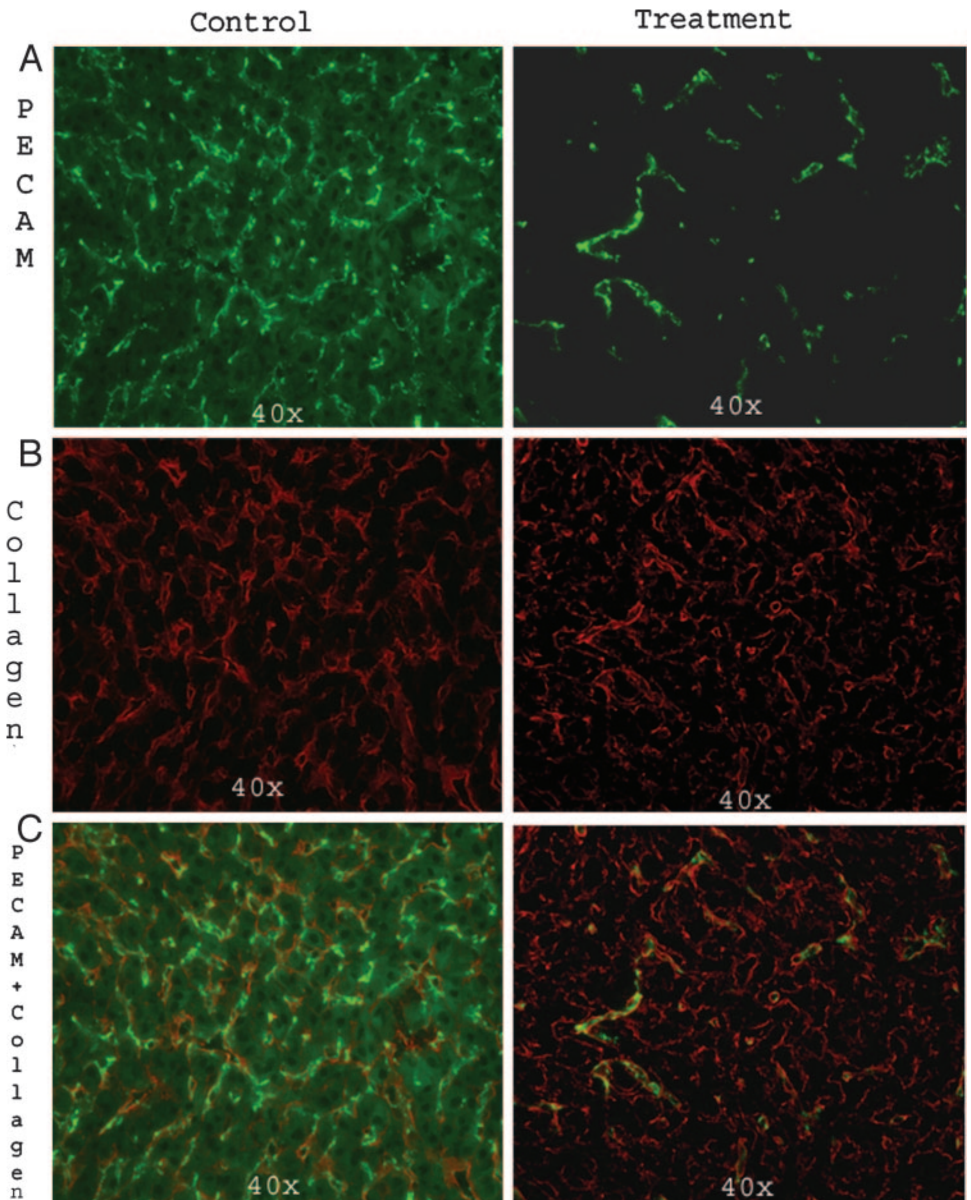

FIG. 4. Immunofluorescent double staining for vascular basement membrane protein collagen IV and endothelial cell marker PECAM from animals injected with saline (control; *left column*) or anti-VEGFR-2 antibody DC101 (treatment; *right column*) on ED 6.5 and killed on ED 7.5. A, Staining of endothelial cells in *green* for PECAM. Note the decrease in staining in the treatment group. B, Staining for collagen IV in *red*. Note that the density of staining is similar in treatment and control groups. C, Staining for PECAM and collagen VI. Note in controls significant colocalization of PECAM (*green*) with collagen IV (*red*), indicating that most endothelial cells are surrounded by vascular basement membrane. In the treatment group, many vascular basement membranes are devoid of endothelial cells.

animals on ED 7.5 (~0.4 g; Table 1B), but were significantly decreased on ED 13.5 (0.25 ± 0.11 g; Table 1B). In treatment group animals, the number of implantation sites was similar to that in controls on ED 7.5; however, on ED 13.5, no implantation sites were seen (Fig. 5A and Table 1B). Histologically, serial sections of control group uteri on ED 7.5 and ED 13.5 (Fig. 5B) revealed normal development corresponding to gestational age. Treatment group embryos appeared similar to controls on ED 7.5. However, there was a complete absence of embryonic structures and placentas by pregnancy d 13.5 (Fig. 5B and Table 1B).

Preimplantation (injection of anti-VEGFR-2 antibody DC101 on ED 3.5). Similar to the postimplantation experiment, the size of treatment group corpora lutea decreased 24 h after antibody administration and reached its nadir on ED 7.5 (Table 2A). Due to effects of the antibody injection on ED 3.5, luteal BVD had decreased significantly on ED 4.5 and was not able to recover (Table 2A). In the treatment group, apoptosis (mostly epithelial cells) increased significantly 24 h after antibody administration compared with controls, and no additional change was seen with continuation of pregnancy (Table 2A). Cell proliferation was rarely observed in corpora lutea in the treatment and control groups (0.2–0.3%; n.s.). Abnormal function of treatment group corpora lutea was also reflected by a significant decline in P4 secretion starting 24 h after antibody administration and reaching a nadir on ED 7.5 (Fig. 2B).

Similar to the postimplantation experiment, the biological significance of the ovarian effects of antibody were evaluated by observing pregnancy development. Uterine weights in treatment group animals increased up to ED 7.5 similar to control values (Table 2B), but decreased to nonpregnant levels on ED 13.5 (0.20 ± 0.04 g; Table 2B). In treatment group animals, the number of implantation sites was similar to that

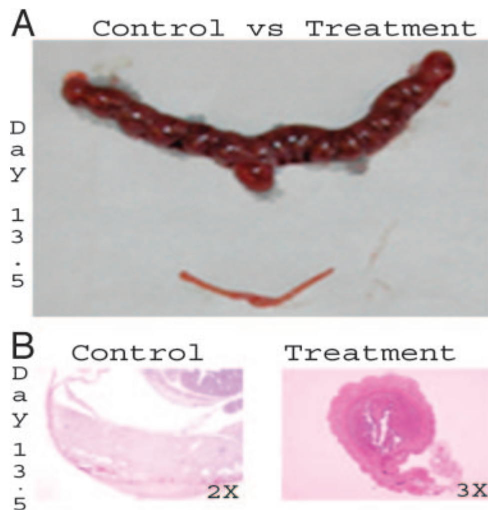

FIG. 5. Comparison of control and treatment group uteri in intact, pregnant animals injected on ED 6.5 with either saline (control) or DC101 (treatment) and killed on ED 13.5. A, Gross specimens; note the small size of the treatment group uterus. B, Cross-section; the control shows part of the placenta and liver in the developing embryo. Note the absence of products of conception in the treatment group uterus on the *right*.

in controls on ED 4.5; however, on ED 7.5 the sites were less pronounced compared with controls, and on ED 13.5 no implantation sites were seen (Table 2B). Histologically, serial sections through control group uteri on ED 4.5, 7.5, and 13.5 revealed normal development corresponding to gestational age. Treatment group animals looked similar to controls on ED 4.5; however, no clear-cut embryonic structures were seen on ED 7.5, and complete absence of embryonic structures and placenta was observed on pregnancy d 13.5.

Effect of a single post- or preimplantation injection of DC101 on pregnancy development in ovariectomized, P4-replaced animals

Postimplantation (injection of anti-VEGFR-2 antibody DC101 on ED 6.5). Maternal serum P4 concentrations in ovariectomized, hormone-replaced mice on ED 13.5 were similar to those in intact pregnant animals with normal ovarian function (30.97 ± 10.45 *vs.* 27.00 ± 4.15 ng/ml; n.s.) (15). Uterine weights (control, 6.89 ± 1.90 g; treatment, 6.72 ± 1.92 g; n.s.) and the number of implantation sites on ED 13.5 (~13 implantation sites/uterus; n.s.; Fig. 6A) were similar in ovariectomized, hormone-replaced control and treatment group animals. Histologically, serial sections through areas containing placenta and embryos on ED 13.5 in both groups revealed normal development structures corresponding to gestational age (Fig. 6B). Therefore, postimplantation decidual function must not have been affected significantly by DC101, because normal progression of pregnancy in hormone-substituted, ovariectomized animals was observed in the treatment group. Evaluation of vasculature (PECAM staining) in decidua surrounding the embryo in ED 7.5 animals revealed no difference in BVD between control and treatment groups (34.5 ± 1.2% *vs.* 35 ± 1.9%; n.s.; Fig. 7B). To verify that the anti-VEGFR-2 antibody does not affect uterine function, we repeated the experiment using the same experimental design (n = 3), but a higher dosage of DC101 (132 mg/kg). Results were very similar to those seen on ED 13.5 using the lower dosage (data not shown).

Preimplantation (injection of anti-VEGFR-2 antibody DC101 on ED 3.5). Similar to the postimplantation experiments, maternal serum P4 concentrations in the ovariectomized, hormone-replaced mice did not differ significantly from those in pregnant mice with intact ovaries on ED 13.5 (31.70 ± 8.40 *vs.* 26.1 ± 5.76 ng/ml; n.s.). Uterine weights (control, 6.71 ± 0.92 g; treatment, 6.47 ± 0.21 g; n.s.) and number of implantation sites on ED 13.5 (~13 implantation sites/uterus; n.s.) showed no significant difference. Histologically, serial sections through areas containing placenta and embryos in both groups revealed normal development structures corresponding to gestational age. Because pregnancy progressed normally in hormone-substituted, ovariectomized animals, the functionality of the decidua must not have been affected significantly by the administration of anti-VEGFR-2 antibody DC101 before implantation.

Effect of VE-cadherin antibody

Administration of a single dose of VE-cadherin antibody on ED 6.5 had no effect on pregnancy development. There was no difference in uterine weight (control, 6.8 ± 0.87 g; treatment, 7.1 ± 0.95 g; n.s.), number of implantation sites (~13/uterus in control and treatment groups; n.s.), histological appearance of embryos, or P4 levels (control, 25.6 ± 1.7 ng/ml; treatment, 25.1 ± 4.1 ng/ml). Also, luteal BVD was similar (baseline control ED 6.5, 100%; control ED 7.5, 102 ± 3.6%; treatment, 99.2 ± 4.1%; n.s.).

Effects of DC101 on permanent vasculature

Liver and kidney tissue sections from all control and treatment animals were evaluated histologically (H&E) and immunohistochemically (PECAM) in experiment 1. No difference between control and treatment groups was detected (data not shown).

Discussion

Pre- and postimplantation administration of a single dose of anti-VEGFR-2 antibody DC101 disrupted maternal ovarian function through elimination of preexisting luteal blood vessels. As a secondary effect, pregnancy development was abnormal. In contrast, pregnancy proceeded normally in ovariectomized, anti-VEGFR-2-treated animals substituted with P4. Embryonic blood vessel development was not affected directly by the antibody, because it did not reach the embryo. Therefore, during pregnancy, the activated VEGF/VEGFR-2 pathway is of critical importance for the survival and maintenance of luteal vasculature in the ovary.

Corpora lutea of pregnancy are highly vascularized and have a much longer life span than nonpregnant corpora lutea. Angiogenesis is very active in the early luteal phase immediately after ovulation when corpora lutea are being formed (5). During the early stages of pregnancy, luteal angiogenesis is minimal (3) (our data), and preexisting vessels are maintained in these corpora lutea for at least part of, if

TABLE 2. Effects of preimplantation administration of DC101 (ED 3.5) on ovary, uterus, and pregnancy

A. Diameter, vascular density, and apoptosis in control and treatment group corpora lutea

ED	Diameter (mm)			Vascular density (%)			Apoptosis (%)		
	Control	Treatment	*P*	Control	Treatment	*P*	Control	Treatment	*P*
3.5	0.53 ± 0.05			100 (baseline)			100 (baseline)		
4.5	0.61 ± 0.07	0.48 ± 0.07	0.03	104 ± 4.9	37 ± 4.5	0.01	103 ± 5.7	578 ± 10.1	<0.01
7.5	0.63 ± 0.08	0.39 ± 0.05	0.03	103 ± 2.3	31 ± 1.1	0.01	108 ± 6.1	353 ± 9.1	0.01
13.5	0.65 ± 0.05	0.38 ± 0.04	0.01	111 ± 6.9	34 ± 3.2	0.01	110 ± 6.3	191 ± 8.3	0.05

B. Uterine weight and implantation sites in control and treatment group corpora lutea

ED	Uterine weight			Implantation sites		
	Control (g)	Treatment (g)	*P*	Control	Treatment	*P*
3.5	0.25 ± 0.06					
4.5	0.29 ± 0.08	0.25 ± 0.08	n.s.	13.2 ± 1.0	12.8 ± 0.8	n.s.
7.5	0.41 ± 0.06	0.37 ± 0.11	n.s.	13.1 ± 0.9	13.1 ± 1.0	n.s.
13.5	6.85 ± 0.95	0.20 ± 0.04	<0.01	12.9 ± 0.9	0	<0.01

not the entire, pregnancy. Corpora lutea function as endocrine organs to maintain pregnancy. After systemic administration of DC101, a blocking antibody directed against VEGFR-2, in the pre- or early postimplantation period, specific antibody binding is detected in many of the endothelial cells of the microvasculature present in pregnant corpora lutea. As a consequence of the blockage of the VEGF/VEGFR-2 pathway through binding of DC101 to luteal vasculature, endothelial cells are eliminated rapidly, as demonstrated by a decrease in BVD in all corpora lutea of pregnancy 24 h after antibody administration. Apoptotic endothelial cells are rarely seen before 24 h after antibody administration, indicating that other mechanisms besides apoptosis (24) must be involved in the elimination of luteal blood vessels. Modlich *et al.* (2) described an alternative mechanism of endothelial cell regression, which occurs during physiological luteolysis. They suggest that endothelial cells can spontaneously detach from the basement membrane, which makes the vessel dysfunctional and leaves behind a denuded membrane. Our observations of the existence of multiple denuded membranes without endothelial cells in treatment corpora lutea suggests that endothelial detachment might be the principal mechanism through which the anti-VEGFR-2 antibody removed endothelial cells. Such a mechanism of action is also seen in tumors treated with angiogenic inhibitors (22). The vascular basement membrane ghosts can be considered a historical record of previously existing blood vessels in treatment group corpora lutea (20). In contrast to endothelial cells, a major mechanism of elimination of steroid-producing epithelial cells is apoptosis. Because no binding of DC101 to luteal epithelial cells is detected, it is likely that elimination of epithelial cells by apoptosis occurs indirectly due to insufficient oxygen supply secondary to the reduction in vasculature. It becomes clear from these findings that elimination of cells in the corpora

FIG. 6. Comparison of control and treatment group uteri in ovariectomized, hormone-replaced, pregnant animals injected with saline or DC101 on ED 6.5 and killed on ED 13.5. A, Gross specimens; note that uterine size and number of embryos are similar in control and treatment groups. B, Cross-section; note that the dimensions of organs such as placenta and developing liver of the embryo are very similar in control and treatment groups.

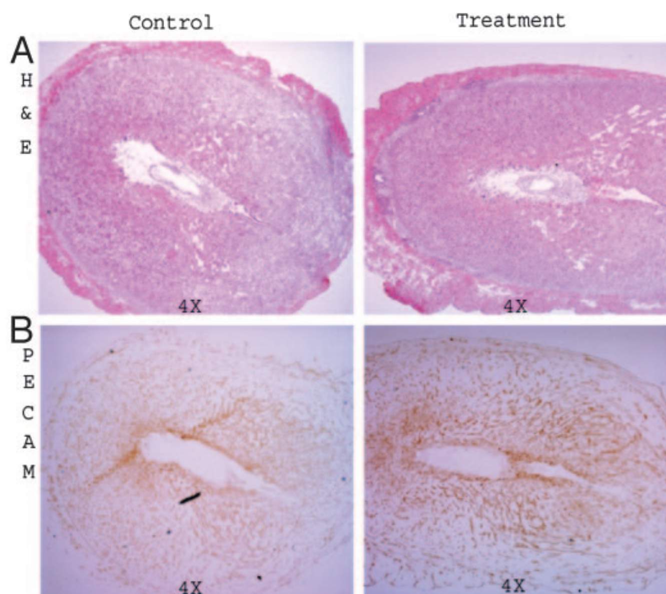

FIG. 7. Cross-sections through pregnant uteri from ovariectomized, hormone-replaced animals injected with saline (C, control) or DC101 (T, treatment) on ED 6.5 and killed on ED 7.5. A, H&E staining. A centrally located embryo surrounded by decidua can be seen in C and T. B, The vascular density of blood vessels surrounding the embryo is similar for C and T groups.

lutea of pregnancy is a very complex phenomenon, and we cannot rule out that it might also involve forms of cell death different from conventional apoptosis. As a consequence of the decreases in blood supply and steroid-producing cells, pregnant corpora lutea function is severely compromised. As P4 production and secretion decline, decidual tissue becomes dysfunctional, and ultimately the uterus, whose function is dependent on luteal support, can no longer maintain normal pregnancy development. In agreement with Kashida *et al.* (10), who treated pregnant rats with anti-VEGF antibody, we found that luteal endothelial cell elimination through the anti-VEGFR-2 antibody was not complete, whereas P4 returned to nonpregnant values. These researchers suggested that such a constellation indicates that VEGF acting through VEGFR-2 might also be involved in regulating vascular permeability, which influences the release of P4 into the bloodstream. Ultimately, due to abnormal P4 levels, the products of conception arrests and embryos are absorbed.

In agreement with previous studies (23), permanent vasculature, including microvasculature present in organs such as kidney and liver, was not affected by the injection of antibody. Such microvasculature is mature, that is endothelial cells are associated with pericytes, which may make them more resistant to the effects of antiangiogenic substances. In contrast, we cannot rule out that high dose, repeated administration of antibody might interfere with function of these organs, because administration of adenovirus-expressing and -producing soluble Flt to pregnant rats can cause damage in these organs 8 d after the injection (25). In contrast to kidney or liver vasculature, luteal blood vessels found during pregnancy are immature; that is, endothelial cells are devoid of pericytes. This anatomical observation might explain the greater vulnerability of such blood vessels to the effects of antiangiogenic agents. The survival and maintenance of immature vasculature depend on VEGF, possibly acting through VEGFR-2 (11, 12, 20). Tumor microvasculature contains a significant number of immature vessels devoid of pericytes. Survival of such tumor vasculature is severely compromised when the VEGF stimulus is blocked and tumors start to regress (11, 12, 26). We showed that luteal function in treatment group animals did not recover after discontinuation of antibody treatment. This was evidenced by the fact that no new blood vessels were formed, and corpora did not increase in size even though the effects of the antibody are temporary (~3 d) (23). Therefore, the persistent vascular basement membranes devoid of endothelial cells seen after antibody treatment do not provide a scaffold for vascular regrowth after cessation of treatment with DC101 (20). Our observation in the rodent is in contrast to findings in the marmoset. Pregnancies continued to develop normally after discontinuation of treatment with a VEGF-inactivating agent, which had been initiated shortly after ovulation, probably due to renewed luteal angiogenic activity (27). A possible explanation of this difference is that a short-term decrease in P4 can be better tolerated in nonhuman primates, whose pregnancy lasts much longer than that in rodents. Our findings might also provide some clues for strategies used to facilitate rapid tissue regression during luteolysis in rodents (1). It may be that throughout the luteal phase, blood vessels are maintained in a vulnerable state dependent on VEGF for survival. At a particular time point during the life span of a corpus luteum, when its endocrine function is no longer required, the local endothelial survival stimulus in the form of VEGF is withdrawn, and blood vessels become dysfunctional through endothelial cell removal. As a secondary effect, P4-producing epithelial cells are eliminated, and gradually corpora lutea cease to exist.

The VEGF/VEGFR-2 pathway regulates two very distinct, biological events pertaining to the vasculature of corpora lutea: endothelial cell proliferation during the formation of corpora lutea, and endothelial cell stabilization and survival during pregnancy to secure its endocrine function. Therefore, the same VEGF stimulus, through its binding to VEGFR-2 on endothelial cells, is a proliferative and/or a survival signal for endothelial cells. Different VEGFR2-dependent intracellular signaling pathways may be activated in this situation. Akt is involved in endothelial cell survival, whereas phospholipase Cγ and MAPK p44/42 stimulate endothelial cell proliferation (28, 29). It is likely that other angiogenic regulators, such as angiopoietins acting through Tie 2, also participate in the regulation of these events (30).

It cannot be ruled out that substances originating from the products of conception could play a supportive role in the maintenance of luteal vasculature and function. Administration of anti-VEGFR-2 antibody DC101 might have disrupted ovarian function indirectly by interfering with pregnancy development through its effects on decidual tissue. Decidua is a proliferative, highly vascularized tissue derived from stromal uterine cells during pregnancy that is absolutely required for normal pregnancy development (31). The possibility that blockage of the VEGF/VEGFR-2 pathway might disrupt pregnancy development through interference with uterine function was suggested by two recent studies (8, 9). We tested for such a possibility by following pregnancy development in ovariectomized, P4-replaced animals exposed to DC101 before and after implantation. P4 replacement was able to completely reverse the effects of the VEGFR-2-blocking antibody administered before or after implantation of the embryo, and pregnancy continued to develop normally. We conclude that the effects of the antibody given to intact pregnant animals must have occurred specifically at the level of the ovary. Because pregnancy was able to progress normally, potential effects of DC101 on decidual tissue must have been insignificant. This conclusion is also supported by the finding that the BVD of decidua was not different between control and treatment groups. Therefore, deciduas of mice seem to be more resistant to the antivascular effects of anti-VEGFR-2 antibody DC101 compared with the corpora lutea of pregnancy. Based on our findings, it is likely that the disruption of pregnancy development through the administration of anti-VEGF antibody, as reported by Rabbani and Rodgers (8) and Rockwell *et al.* (9), can be explained by the effects of antibody on luteal function. Support for an ovarian action of our antibody comes from studies in the monkey, in which pregnancy continued to develop normally in the presence of substances inactivating VEGF function, which caused a temporary decrease in luteal P4 secretion (27). A possible explanation might be that decidual endothelial cells behave differently when exposed to the antibody

compared with luteal endothelial cells, through a difference in either the endowment of VEGFR-2 or associated downstream signaling molecules. In contrast, we cannot rule out that administration of antibody at a higher dosage, initiation of treatment before d 3.5, and/or administration over a longer period of time could have affected decidual vasculature and function significantly and, as a secondary effect, could disrupt pregnancy development.

It is informative to compare the effects of DC101, an antiangiogenic and antisurvival agent, with those of a substance that only blocks angiogenesis. An antibody developed against VE-cadherin, E4G10, has such a profile (17). Both antibodies inhibit angiogenesis during the formation of corpora lutea. In contrast to DC101, luteal function during pregnancy was not affected by E4G10. When administered to pregnant animals, the histological appearance of corpora lutea, BVD, and serum P4 measurements were similar to those in controls. These observations indicate that E4G10 did not interfere with luteal endothelial cell survival and confirm that blood vessel proliferation is not important for the function of corpora lutea during pregnancy (31).

Antibody DC101 shows strong antiangiogenic and antisurvival properties in dynamic structures with high vascular activity, such as corpora lutea, whereas it appears to be innocuous to mature vasculature in permanent organs, such as kidney, at least when administered short term. Therefore, blockage of the VEGF/VEGFR-2 pathway with DC101 has the potential for several applications in obstetrics and gynecology. New contraceptive strategies could focus on the development of agents that disrupt ovarian blood vessel function through blocking the VEGF/VEGFR-2 pathway. The ability of the antibody to inhibit pregnancy development in the preimplantation period suggests that this knowledge could be used to control fertility by developing another effective emergency morning-after alternative that might have fewer side-effects. Furthermore, the antibody's ability to disrupt pregnancy development in the postimplantation period indicates that it also has the potential to be used alone or in combination with drugs such as prostaglandins or antiprogestins to terminate abnormal intrauterine pregnancies as well as for the treatment of ectopic pregnancies. These treatment possibilities have to be tested in a nonhuman primate model, because the effects of interference with the VEGF/VEGFR-2 pathway on pregnancy might vary in different species (27).

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