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Single Injections of Vascular Endothelial Growth Factor Trap Block Ovulation in the Macaque and Produce a Prolonged, Dose-Related Suppression of Ovarian Function

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Follicular development is associated with intense angiogenesis and increased permeability of blood vessels under the control of locally produced angiogenic factors such as vascular endothelial growth factor (VEGF). The aim of the present study was to evaluate the effects of transient inhibition of VEGF on pituitary-ovarian function in the macaque. Animals were given a single, iv injection of a potent, receptor-based VEGF antagonist, the VEGF Trap. VEGF Trap was given at a dose of 4, 1, or 0.25 mg/kg in the midfollicular phase or at 1.0 mg/kg in the late follicular phase. Controls were treated with vehicle or a control protein, recombinant human Fc (1 mg/kg). Blood samples were collected once daily for 12 d after injection, and three times per week thereafter until normal ovulatory cycles had resumed. The VEGF Trap produced a rapid suppression of estradiol and inhibin B concentrations at all doses tested, followed by a marked and sustained increase in LH and FSH. Ovulation and formation of a functional corpus luteum, as evidenced by increased serum progesterone levels, failed to occur at the anticipated time. Normal ovarian activity resumed when plasma concentrations of unbound VEGF Trap fell below about 1 mg/liter. When treatment was initiated in the midfollicular phase, control macaques ovulated 7.2 ± 0.4 d later, but ovulation was delayed in a dose-dependent manner by VEGF Trap, occurring 23 ± 0.7, 30 ± 1.4, and 43 ± 0.8 d after injection of 0.25, 1, or 4 mg/kg, respectively. Thus, the VEGF Trap exerts a potent, dose-dependent, but reversible inhibitory effect on ovarian function. (*J Clin Endocrinol Metab* 90: 1114–1122, 2005)

IT IS NOW generally accepted that follicular angiogenesis and vascular permeability are closely regulated by a complex interplay of stimulatory and inhibitory vasoactive factors produced by the theca and granulosa (1). Given the close association between structural and functional vascular remodeling and follicular maturation, it has long been postulated that manipulation of follicular angiogenesis could affect ovarian function (2–4). However, initial pharmacological studies that attempted to establish the link between ovarian angiogenesis and function met with mixed success because they of necessity employed relatively nonspecific compounds whose mechanism of action, and potency, were largely unknown (5–7). Over the last few years, the development of improved reagents targeted to defined angiogenic factors or their receptors has enabled studies that have unequivocally confirmed the importance of angiogenesis in ovarian function, and particularly implicated vascular endothelial growth factor (VEGF)-A and its receptors as key mediators of this process.

In these experiments, the VEGF pathway has been inhibited using antibodies directed against VEGF itself (8, 9), antibodies to the VEGF receptor VEGFR-2/Flk (10, 11), a VEGF receptor tyrosine kinase inhibitor (12), or by decoy VEGF receptors (13, 14). We employed highly potent receptor-based VEGF antagonists. Initial studies in marmosets used a prototypical receptor-based antagonist, VEGF $Trap_{A40}$, which comprised the immunoglobulin domains 1–3 of VEGFR-1 fused to the Fc portion of human IgG. Surprisingly, acute systemic administration during the early luteal phase inhibited not only luteal angiogenesis (15) but also follicular angiogenesis (16). In subsequent studies a successor molecule, VEGF $Trap_{R1R2}$, was employed to evaluate the effects of inhibition of VEGF throughout the follicular phase. These studies confirmed that selective inhibition of VEGF markedly attenuated thecal angiogenesis and restricted follicular growth in the marmoset (17).

The small size of the marmoset restricts the number of blood samples that may be obtained to monitor the concomitant effects of VEGF inhibition on pituitary and ovarian hormones. In contrast, the stump-tailed macaque is an old world primate with a body weight of 12–15 kg and menstrual cycles similar to the human female, whose hormone profiles can be determined by well-established assays from blood samples collected at close intervals. We used this species previously to evaluate the effects of GnRH analogs (18) before initiating clinical investigations in women (*e.g.* Ref. 19). The first objective of the current study was to assess the acute and longer-term effects of a single, iv injection of the VEGF $Trap_{R1R2}$ at the midfollicular phase in the macaque. This phase was selected for detailed study because the follicle that will eventually ovulate is being selected at this time. Our earlier studies in the marmoset suggested that VEGF-mediated angiogenesis is of crucial importance in the selection

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Abbreviations: OHSS, Ovarian hyperstimulation syndrome; PCOS, polycystic ovarian syndrome; VEGF, vascular endothelial growth factor.

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and growth of the dominant follicle and that if VEGF action were effectively abrogated, the follicular cycle could not continue and a new phase of follicular recruitment would have to be initiated. A second objective was to determine the minimal dose of VEGF $Trap_{R1R2}$ that would be required to interrupt follicular development and whether the duration of the subsequent suppression of ovarian function would also be dose related. A third objective was to assess the effects of acute VEGF inhibition during the late follicular phase when the thecal vasculature of the ovulatory follicle is already fully developed.

Materials and Methods

Animals

Thirteen adult female stump-tailed macaques aged 7–22 yr and weighing 10–16 kg used in the study were captive bred in the United Kingdom or Holland and housed in a unit opened in 1996 and designed with an emphasis on environmental enrichment (20). The animals moved freely from their living rooms via a tunnel to cages for their water supply and sleeping area and collection of blood samples.

Vaginal swabs were taken with a cotton-tipped applicator each morning and the pattern of menstrual bleeding recorded. The animals were trained to enable blood sample collection by femoral venipuncture without anesthesia and with minimal or no restraint. All the animals in the study had regular ovulatory menstrual cycles as determined from menstrual pattern and serum concentrations of estradiol-17β and progesterone in blood samples obtained three times per week before treatment. The study was approved by the local Primate Ethical Committee and carried out under a project license granted by the United Kingdom Home Office.

Treatments

Endogenous VEGF was inhibited by administration of VEGF $Trap_{R1R2}$, a recombinant, chimeric protein comprising Ig domain 2 of human VEGF-R1 and Ig domain 3 of human VEGF-R2, expressed in sequence with the human Fc. Compared with earlier versions of receptor-based fusion proteins, the VEGF $Trap_{R1R2}$ exhibits greater affinity for VEGF-A (affinity constant ~1 pM) as well as improved bioavailability and pharmacokinetic properties (21). VEGF $Trap_{R1R2}$ (Regeneron Pharmaceuticals, Inc., Tarrytown, NY) was provided at a concentration of 24.3 mg/ml in 2-ml aliquots in buffer composed of 5 mM phosphate, 5 mM citrate, 100 mM NaCl (pH 6.0), and 0.1% wt/vol Tween 20, with either 20% glycerol or 20% sucrose. Human Fc, for control treatments, was provided at a concentration of 19.7 mg/ml in buffer composed of 40 mM phosphate and 20 mM NaCl (pH 7.4). VEGF Trap vehicle alone also was employed during some control cycles. The compounds were stored at −20 C until required, at which time they were thawed. Any compound remaining was stored at 4 C and used within 2 wk.

In a pilot study in two macaques, a single iv injection of 12.5 mg/kg, VEGF $Trap_{R1R2}$ was found to effectively inhibit ovarian function for more than 40 d. Therefore, in the main study, we elected to investigate the response to 4.0, 1.0, and 0.25 mg/kg (n = 4 per group) administered as a single dose (iv) during the midfollicular phase, d 6–8 of the cycle. Late-follicular-phase administration was studied at the intermediate dose of 1 mg/kg (n = 4). In comparable control cycles, macaques were treated with either 1 mg/kg human Fc (iv) during the mid- (n = 3) or late follicular phase (n = 2) or vehicle administered during the mid- (n = 3) or late follicular phase (n = 3).

After treatment with VEGF $Trap_{R1R2}$, vehicle, or Fc, (d 0), blood samples were collected at 0 and 15 min and 4, 6, and 8 h and then daily for the next 12–14 d. Thereafter, blood samples were collected three times per week until normal ovulatory cycles were reestablished, as evidenced by elevation of progesterone levels consistent with luteal values measured in pretreatment cycles for that macaque. Because of limits in numbers of animals available, five animals received two treatments with VEGF $Trap_{R1R2}$. In addition, one animal had been treated in the pilot study. In this case, antibodies to the VEGF $Trap_{R1R2}$ were detected during the second treatment cycle, and the affected cycle was excluded from further analysis (see *Results*).

Because no differences were noted in the effects of vehicle and Fc administration (neither treatment produced an appreciable affect on ovarian or pituitary hormones), control cycles from both control treatment conditions were combined for statistical analyses.

Assays

Estradiol-17β and progesterone were measured by RIAs as described previously (22), detection limits being 30 pM and 0.7 nM, respectively. LH and FSH were measured by RIAs based on recombinant cynomolgus monkey LH and FSH supplied by the National Hormone and Pituitary Program (Dr. A. F. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases). FSH was measured using anticynomolgus FSH with a detection limit of 2 μg/liter (National Institute of Child Health and Human Development, rec-mo-FSH-RP-1, AFP-6940A), and interassay coefficient of variance of 12%. LH was measured using a rabbit antiserum to cynomolgus LH (AFP342994) used at a final dilution of 1:750,000. Rec-mo LH-RP-1 (AFP-6936H) was used for radioiodination as instructed and results expressed as micrograms per liter of the same preparation. Assay sensitivity was 0.3 μg/liter and interassay coefficient of variance 11%. Inhibin B was measured throughout the study period in animals in the VEGF Trap treatment groups only. The assay was as described previously (20) and had a detection limit of 10 ng/liter.

VEGF $Trap_{R1R2}$ was measured by an ELISA, using human VEGF 165 to capture and an antibody to the human Fc region as the reporter (21). Capture of the VEGF $Trap_{R1R2}$ by VEGF coated on the microplate requires a vacant VEGF binding site. Consequently, this ELISA specifically detects only VEGF Trap that is not already bound to endogenous VEGF. Serum samples were diluted in assay buffer and run against standards also prepared in assay buffer. Each dilution level was assayed, and those that read on the linear part of the standard curve, in which the samples ran parallel to that of the reference standard, were selected for analysis. If in the initial assay, values were below the limit of detection, samples were reassayed neat and the standards spiked with an equivalent volume of mouse serum. Assay sensitivity was 0.14 μg/liter, and interassay variation based on low-, medium-, and high-quality controls were less than 10%. Concentration *vs.* time curves were constructed from ELISA-generated VEGF Trap values obtained from individual animals. The pharmacokinetic parameter estimates were determined by fitting the serum concentration *vs.* time profile to a noncompartmental model (WinNonLin, version 2.0, Pharsight Corp., Mountain View, CA).

Data analysis and statistics

The day of ovulation was defined as the day of the LH peak. Peak levels of estradiol were typically noted on the previous day or in some cycles on the same day as the LH peak. In normal cycles, the LH peak was followed within 1 d by a rise in progesterone levels, which were sustained for 2 wk. In pre- and posttreatment cycles in which blood samples were obtained three times per week, gonadotropin and ovarian steroid levels were evaluated to provide a best estimate of the day of ovulation.

Data for ovarian and pituitary hormones as well as VEGF $Trap_{R1R2}$ concentrations for each individual animal were plotted with reference to the day of treatment (d 0). Data around d 0 were also plotted as mean ± SEM values for each treatment group: beyond the point at which daily blood samples were available (after 12–14 d), mean values were obtained by averaging samples taken from all animals at the nearest equivalent times (*e.g.* in a group of four, collections of n = 1 at d 20, n = 2 at d 21, and n = 1 at d 22 were averaged and plotted as n = 4 at d 21).

Data for time to ovulation after treatment and effects of treatment on hormone concentrations were subjected to ANOVA using the Prism program 4 for Macintosh (GraphPad Prism, San Diego, CA) followed by Bonferroni's multiple comparison tests. To determine effects of treatment within an animal, the mean of the three pretreatment values for each hormone was used. The posttreatment period subjected to statistical analysis for each group was based on the observed duration of response to treatment defined as the average period of suppression of estradiol below pretreatment value. The final day of response was the one that preceded three consecutive values above pretreatment value.

According to this definition, average duration of response was 30, 17, and 10 d for the 4, 1, and 0.25 mg/kg doses, respectively. Similar estimates of the duration of response were obtained using suppression of inhibin B as an end point. Area under the curve for progesterone and estradiol peaks was compared for pre- and posttreatment cycles. Differences were considered significant at a level of $P < 0.05$.

Results

Midfollicular phase treatment

A representative example of the hormone profile for vehicle-treated or Fc-treated control cycles is shown in Fig. 1. After control injections, plasma estradiol levels continued to rise, increasing sharply 4–6 d later, before ovulation. The ovulatory surge in LH/FSH took place 7.2 ± 0.4 d (mean ± SEM) after treatment and was followed by a sustained elevation in plasma progesterone, which reached a plateau 8–12 d post ovulation before falling to follicular phase values around luteal d 14–16. Of the six midfollicular control cycles studied, ovulation was not observed at the anticipated time in one vehicle-treated animal, and this cycle was excluded from further evaluation. This animal had an extended follicular phase of 20 d, but this was distinguished from the response to VEGF Trap treatment in that the delay in ovulation was not accompanied by a suppression of estradiol levels or a prolonged rise in LH and FSH (see below).

The VEGF $Trap_{R1R2}$ was well tolerated at all doses tested. Typical hormonal responses observed after administration of

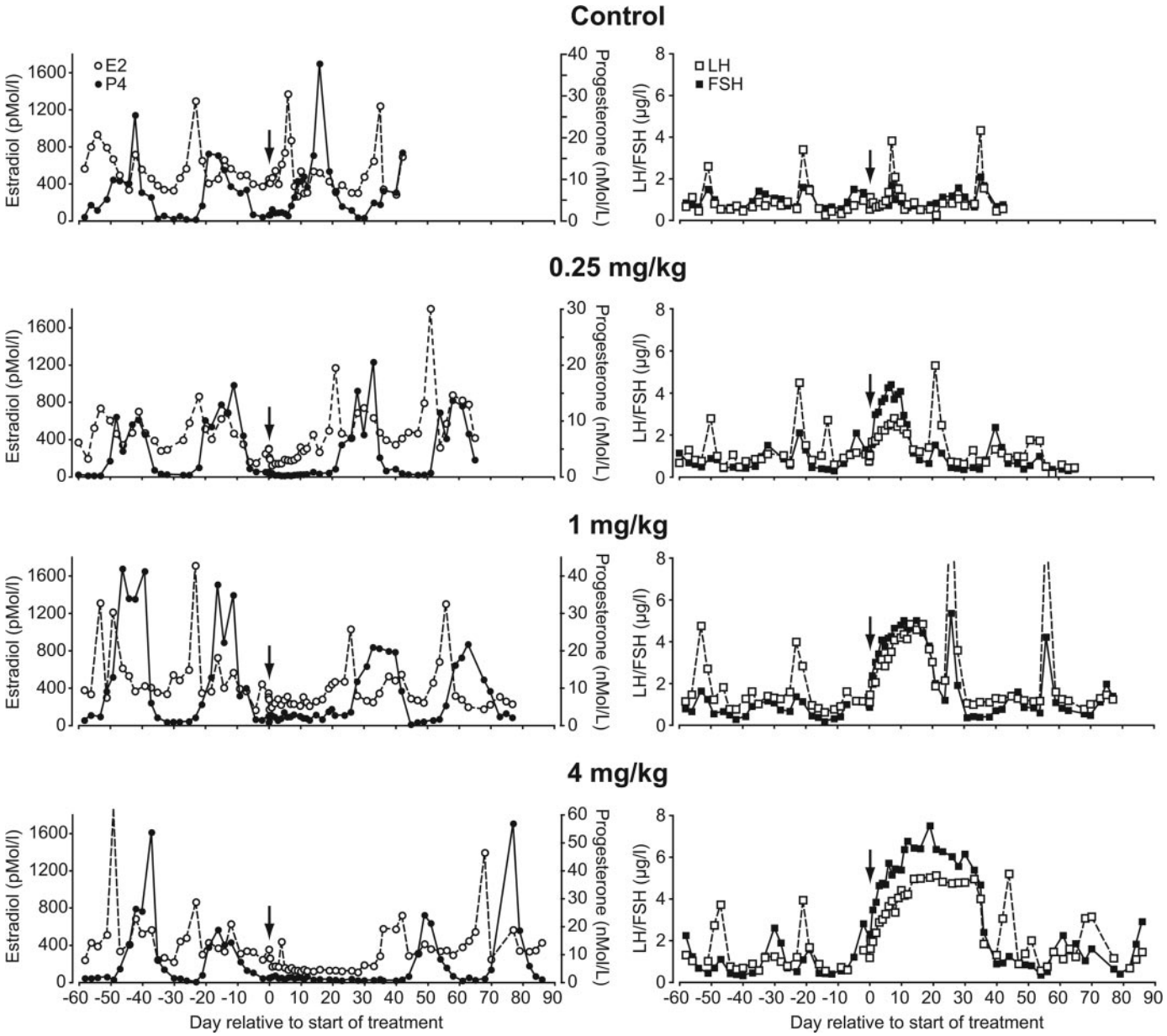

FIG. 1. Serum concentrations of estradiol (*open circles*), progesterone (P4, *closed circles*) (*left panel*), FSH (*closed squares*), and LH (*open squares*) (*right panel*) in individual macaques injected in the midfollicular phase with either Fc (1 mg/kg, iv) (control) or 0.25, 1, or 4 mg/kg VEGF $Trap_{R1R2}$ iv (*arrow*). Note the suppression of estradiol, failure of ovulatory progesterone rises, and increases in LH and FSH after treatment, followed by dose-related recovery of normal pituitary-ovarian function.

0.25, 1.0, and 4.0 mg/kg of the VEGF $Trap_{R1R2}$ are shown in Fig. 1. Pituitary-ovarian function was clearly altered in every case at each dose tested, and the observed changes in hormonal profiles followed a consistent and distinctive pattern. Treatment with VEGF $Trap_{R1R2}$ not only inhibited the late follicular phase rise in plasma estradiol but also resulted in a rapid decline in estradiol concentrations to early follicular phase levels. Within 2–3 d of injection, a progressive increase in plasma LH and FSH was observed, and the FSH to LH ratio was increased in all animals relative to those normally observed during the preovulatory surge of LH and FSH (Fig. 1).

Comparison of mean data for control and treated cycles is shown in Fig. 2. In the posttreatment period, serum estradiol levels in VEGF Trap-treated cycles were significantly lower than in control cycles for all doses tested ($P < 0.0001$). A sustained reduction of estradiol levels below pretreatment follicular values was observed in the 4 mg/kg group ($P < 0.0001$), in which estradiol levels remained significantly below normal follicular levels between d 6 and 28 post treatment. Conversely, VEGF Trap treatment resulted in a significant stimulatory effect ($P < 0.01$) on LH concentrations at all three dose levels. Serum FSH levels also were significantly higher ($P < 0.0001$) than control cycle values in all three treatment groups. FSH appeared to rise and reach a plateau slightly more rapidly than LH. The rate of the rise in serum gonadotropins appeared unaffected by dose of VEGF Trap, although peak values seen between 15–20 in the higher dose groups may not have been obtained in the 0.25 mg group as a result of the more rapid recovery.

Evaluation of percentage change from pretreatment values during the first 48 h after injection showed that inhibin B concentrations exhibited a more rapid and marked decline than estradiol, being significantly suppressed as early as 8 h (0.25 and 1 mg/kg doses), whereas estradiol was not significantly different from pretreatment value until 24 or 48 h (Fig. 3).

Pharmacokinetics and pharmacodynamics

Mean peak concentrations of free VEGF $Trap_{R1R2}$ measured in the first postinjection blood sample at 15 min were

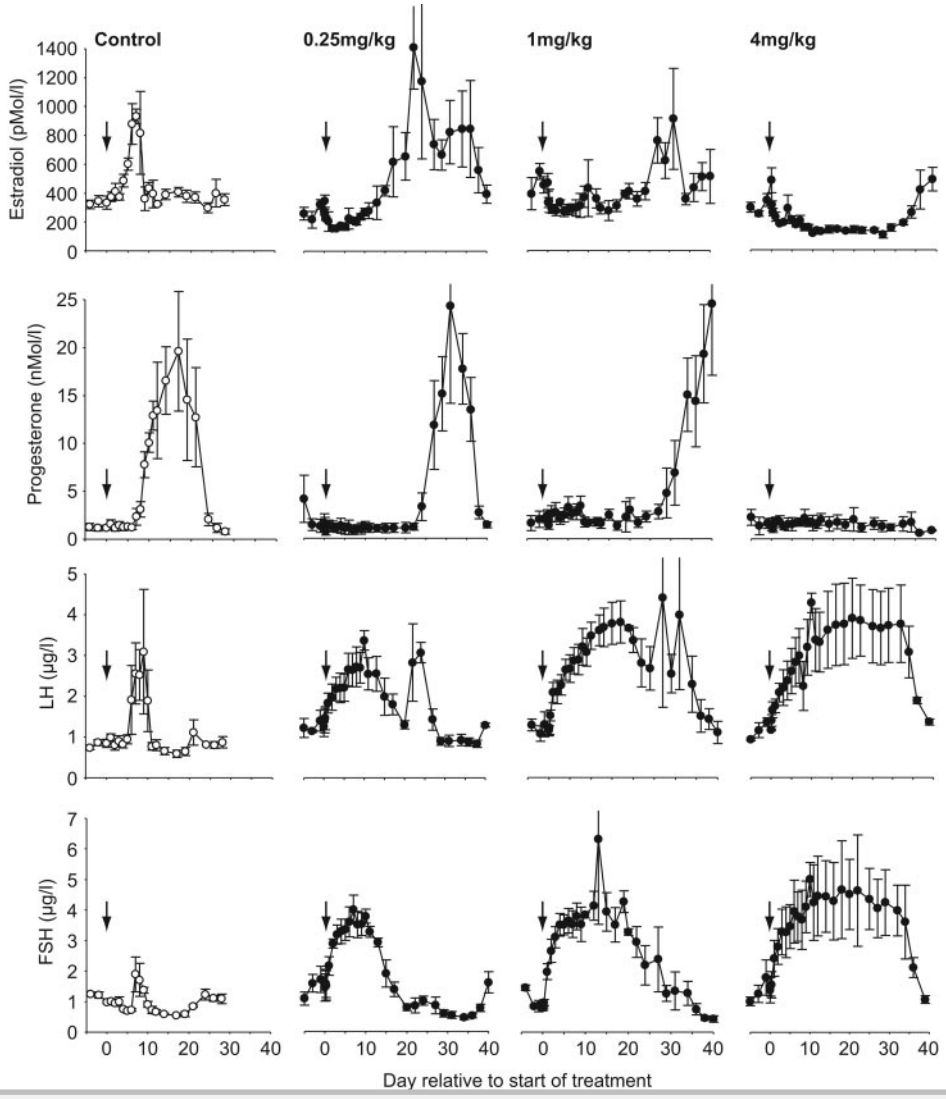

FIG. 2. Serum concentrations of estradiol, progesterone, LH, and FSH after treatment with vehicle/Fc or 0.25, 1, or 4 mg/kg VEGF $Trap_{R1R2}$ in the midfollicular phase. Values are means ± SEM plotted with reference to the day of injection (d 0). Note the timely rise in estradiol, followed by the preovulatory LH/FSH surge and sustained rise in luteal progesterone levels in controls. VEGF $Trap_{R1R2}$ treatment results in the failure of the characteristic increases in estradiol and progesterone and the induction of a persistent increase in LH and FSH. Note the dose-related duration of response followed by recovery initiated by the rise in estradiol.

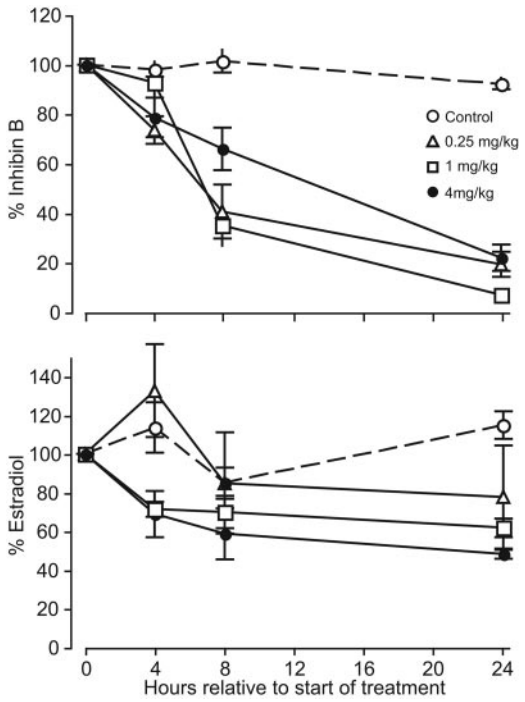

FIG. 3. Percentage change from pretreatment values in inhibin B and estradiol after treatment with vehicle/Fc or 0.25, 1, or 4 mg/kg VEGF $Trap_{R1R2}$ in the midfollicular phase. Note the more rapid decline in inhibin B, which was significantly lower by 8 h, whereas estradiol was not significantly suppressed until 24 or 48 h.

10.8, 34, and 140 mg/liter for the 0.25, 1, and 4 mg/kg groups, respectively. The clearance at the different doses ranged from 5.8 to 8.7 ml/d/kg, and the steady-state volume of distribution was approximately 34.8 ml/kg.

The differential duration of suppression in ovarian function observed at each dose of VEGF $Trap_{R1R2}$ was temporally correlated with clearance of unbound VEGF Trap from the circulation. The mean estradiol and inhibin B values for each of the three dose groups are plotted in relation to levels of VEGF Trap in Fig. 4. At each dose, inhibin B was suppressed to the detection limit of the assay, whereas estradiol was maintained around early follicular levels until plasma concentrations of VEGF $Trap_{R1R2}$ fell below approximately 1 mg/liter.

The duration of suppression in ovarian function produced by administration of the VEGF Trap was clearly dose related. The time to the first posttreatment ovulation was significantly longer ($P < 0.001$) in the 4 mg/kg group than either the 1 or 0.25 mg/kg groups, and the 1 mg/kg dose also resulted in a significantly longer ($P < 0.002$) period of suppression than 0.25 mg/kg (Fig. 5).

A single exception to the above pattern was observed in one macaque that received a 4 mg/kg injection. The initial 10-d posttreatment period was associated with hormonal profiles indistinguishable from that of others in this dose group. However, estradiol levels began to increase rapidly around d 11. This uncharacteristically rapid escape from the effect of VEGF inhibition was associated with an early and abrupt fall in serum-free Trap levels to 1.5 mg/liter at d 10, compared with around 20 mg/liter at this time point in the other animals in this group. The sample taken at d 10 from this atypical case was assayed for antibodies to the VEGF $Trap_{R1R2}$ and found to be positive, 6478 mIU/ml. This macaque had been injected with 12.5 mg/kg VEGF $Trap_{R1R2}$ in the pilot study 16 months previously.

Characteristics of posttreatment recovery of ovarian cycles

Evaluation of the hormonal profiles on recovery of ovarian function suggested that posttreatment menstrual cycles were characteristically normal in terms of length as well as the pattern and magnitude of pituitary gonadotrophin and ovarian steroid levels. This impression was confirmed by quantitative analyses, which showed that peak preovulatory estradiol levels and area under the curve for progesterone during subsequent luteal phase were not statistically different in the immediate pre- and posttreatment cycles (Table 1).

Vaginal bleeding

In control cycles, bleeding was detected during the first week post treatment in one of six cases. Nine of the 12 macaques treated with VEGF $Trap_{R1R2}$ during the midfollicular phase exhibited bleeding during this period, likely reflecting the abrupt and sustained reduction in estradiol levels.

Late follicular phase treatment

Where control injections were given during the late follicular phase, serum estradiol levels continued to rise and a distinct LH/FSH surge occurred 1–5 d later, followed by a sustained rise in serum progesterone. In marked contrast, the anticipated preovulatory rise in estradiol and ovulatory progesterone were blocked in all macaques treated with VEGF Trap (1 mg/kg); rather there was a rapid and sustained decrease in plasma estradiol levels (Figs. 6 and 7), which persisted for an average of 19 d in three of the four treated macaques. Treatment was followed within 1 d by a marked increase in LH and FSH secretion (Figs. 6 and 7). In contrast to the midcycle gonadotrophin surge seen in normal and control cycles, the LH/FSH increase produced by administration of VEGF $Trap_{R1R2}$ was characterized by a marked increase in the FSH to LH ratio. In the remaining animal, the suppression in estradiol was maintained for only 8 d, and the duration of the LH/FSH rise was similarly abbreviated.

The peak of unbound VEGF $Trap_{R1R2}$ (39 mg/liter) in the serum, as well as other pharmacokinetic parameters (not shown), closely resembled those observed after midfollicular phase injection of 1 mg/kg VEGF Trap. As was the case for midfollicular phase injections, recovery of ovarian function, as evidenced by an increase in estradiol levels followed by normalization of gonadotrophin levels, occurred only after circulating levels of unbound VEGF $Trap_{R1R2}$ fell below about 1 mg/liter. The more rapid recovery of estradiol secretion observed in one animal was not accompanied by accelerated clearance of free VEGF $Trap_{R1R2}$.

In all four cases, the luteal phase progesterone rise failed to occur at the anticipated time, and progesterone remained at follicular phase levels until after the first posttreatment ovulation, which occurred 32 ± 0.9 d after treatment, a significant delay, compared with control cycles ($P < 0.001$) in

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