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ORIGINAL PAPER

Placental villous vascular endothelial growth factor expression and vascularization after estrogen suppression during the last two-thirds of baboon pregnancy

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Abstract We have recently shown that placental cytotrophoblast vascular endothelial growth factor (VEGF) expression and vessel density were increased by elevating estrogen and decreased by suppressing estrogen in early baboon pregnancy. The present study determined whether the elevation in estrogen which occurs in the last two-thirds of baboon pregnancy also has a role in the regulation of placental villous VEGF expression and angiogenesis. Placentas were obtained on day 170 of gestation (term, 184 days) from baboons untreated or treated with the aromatase inhibitor CGS 20267 or CGS 20267 plus estradiol daily on days 30–169. Serum estradiol levels in CGS 20267-treated baboons were decreased ($P < 0.001$) by 95%, however, placental cytotrophoblast VEGF mRNA levels (means ± SE, attomoles/μg RNA) were similar in untreated (25,807 ± 5,873), CGS 20267-treated (23,900 ± 1,940) and CGS 20267 plus estradiol-treated (26,885 ± 2,569) baboons. VEGF mRNA levels in the syncytiotrophoblast (2,008 ± 405) and inner villous stromal cell (1,724 ± 287) fractions of untreated baboons also were not altered by CGS 20267. However, whole villous VEGF mRNA levels in CGS 20267-treated baboons (18,590 ± 2,315) were 4-fold greater ($P < 0.001$) than in untreated animals and restored to normal by estradiol. Percent vascularized area (15.88 ± 0.88%) and vessel density (1,375 ± 71/mm^2) of the villous placenta in untreated animals were not altered by estrogen deprivation. We propose that villous cytotrophoblasts lose their responsivity to estrogen and that placental villous cytotrophoblast VEGF expression and angiogenesis are regulated by estrogen in a cell- and gestational age-specific manner, and that factors other than estrogen maintain VEGF expression in the last two-thirds of pregnancy.

Keywords VEGF · Placenta · Estrogen · Primate · Vascularization

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Introduction

During human and nonhuman primate pregnancy an extensive vascular system develops within the villous placenta, via vasculogenesis and angiogenesis, to promote fetal growth and development. Vascular endothelial growth factor (VEGF) has a pivotal role in regulating angiogenesis [1]. VEGF [2–7] and the fms-like tyrosine kinase (flt-1) and the kinase-insert domain containing (KDR/flk-1) receptors [5, 8, 9] are expressed in the human and nonhuman primate villous placenta. Relatively little is known, however, about the regulation of placental VEGF expression and angiogenesis during human pregnancy.

Estrogen regulates the expression of VEGF and angiogenesis in reproductive organs. For example, estrogen rapidly increased VEGF mRNA in vivo in the uterus of nonpregnant rats [10, 11], sheep [12], and baboons [13, 14] and in vitro in human endometrial cells [15]. Furthermore,

estrogen promoted microvascular permeability and other aspects of angiogenesis in the rat [16, 17], sheep [18], and baboon [14] uterus.

We have used the baboon as a nonhuman primate model and shown a developmental increase in placental villous cytotrophoblast VEGF mRNA levels and vascularization in association with the rise in estrogen of advancing pregnancy [4]. Moreover, suppressing estrogen levels by administration of an aromatase inhibitor decreased, while prematurely elevating estrogen increased, placental villous cytotrophoblast VEGF expression and blood vessel density during early baboon pregnancy [19, 20]. However, comparable studies have not been conducted to determine the potential role of endogenous estrogen on placental villous VEGF expression and angiogenesis during the last two-thirds of primate pregnancy, a time when angiogenesis is extensive to promote placental-fetal exchange [21–23]. Therefore, in the present study a highly specific aromatase inhibitor was used in the pregnant baboon to assess the potential effect of suppressing placental estrogen formation/levels on VEGF expression and vascularization within the villous placenta during the last two-thirds of pregnancy.

Results

Since the results obtained for serum estradiol levels, placental and fetal body weights, placental VEGF mRNA levels, and vessel density were similar on day 170 of gestation in baboons in which CGS 20267 was initiated on either day 30 or day 60, an overall mean was calculated for purposes of analysis and presentation of the data.

Serum estradiol

Maternal peripheral serum estradiol levels in untreated baboons increased from 0.01 ng/ml early in gestation (Fig. 1) to over 2.00 ng/ml on days 60–85, declined to approximately 1.20 ng/ml at midgestation (i.e., day 100), then increased thereafter reaching levels of approximately 4.00 ng/ml late in gestation (i.e., day 170). Within 1–2 days of the onset of CGS 20267 administration, maternal peripheral serum estradiol concentrations declined to and remained at a level throughout the remainder of gestation (mean ± SE, 0.096 ± 0.003 ng/ml) that was less then 5% ($P < 0.001$) of that in the untreated animals (Fig. 1). Concomitant treatment of pregnant baboons with CGS 20267 plus estradiol benzoate resulted in maternal serum estradiol levels with a pattern similar to that of, but levels that were approximately 2-fold greater than those in, untreated baboons (Fig. 1).

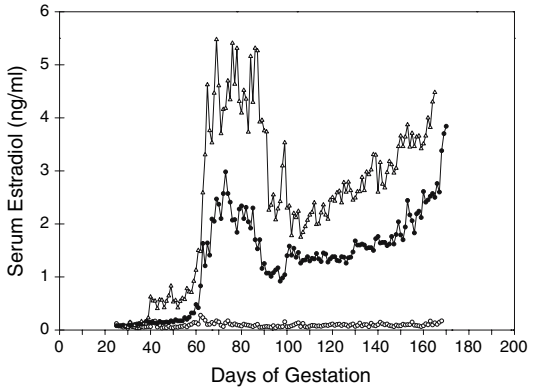

Fig. 1 Maternal peripheral serum estradiol concentrations in baboons untreated (•, each data point is the mean of n = 4 animals) or treated daily with CGS 20267 (0.1–0.2 mg/day, sc, ○, n = 6) or CGS 20267 plus estradiol (0.1–0.2 mg/day, sc, Δ, n = 3) between days 30 (60) and 169 of gestation (term = 184 days)

Placental and fetal body weights

Placental and fetal body weights in untreated baboons were 188 ± 6 g and 872 ± 24 g, respectively, on day 170 of gestation (Table 1). The administration of CGS 20267 or CGS 20267 plus estradiol did not significantly alter placental or fetal body weight.

RT-PCR of VEGF

Figure2 shows a representative quantitative analysis of VEGF mRNA levels by competitive RT-PCR in placental cytotrophoblasts obtained on day 170 from an untreated baboon. The expected 323-bp VEGF target and 256-bp VEGF competitive reference standard (CRS) products generated by PCR are shown (Fig. 2A). No PCR product was detected when either RNA or RT enzyme was omitted from the reaction. The slope of the log of the CRS to target areas plotted as a function of increasing amounts of CRS indicated that amplification efficiency was linear (Fig. 2B). The concentration of VEGF target mRNA was determined as outlined in the Materials and Methods Section.

Table 1 Placental and fetal body weights in baboons[a]

Treatment	N	Placenta (g)	Fetus (g)
Untreated	4	188 ± 6	872 ± 24
CGS 20267	6	208 ± 10	830 ± 20
CGS 20267 plus estradiol	3	176 ± 16	785 ± 43

[a] Values represent the means (±SE) on day 170 of gestation (term = 184 days) in baboons untreated or treated daily with CGS 20267 (0.1–2.0 mg/day, sc) on day 30–169 (n = 3) or days 60–169 (n = 3) or CGS 20267 plus estradiol benzoate (0.1–2.0 mg/day, sc) on days 30–169 (n = 2) or days 60–169 (n = 1)

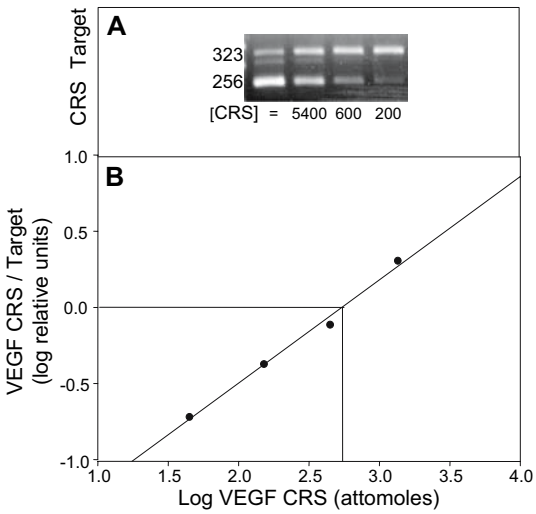

Fig. 2 Representative competitive RT-PCR of VEGF in placental cytotrophoblasts of an untreated baboon on day 170 of gestation. Total RNA (75 ng) was mixed with 3-fold serial dilutions of VEGF CRS as depicted in Panel A. Samples were reverse transcribed and amplified for 26 cycles in the presence of specific primers. (**A**) The 323-bp target and 256-bp CRS products were separated via agarose gel electrophoresis and stained with ethidium bromide. (**B**) Intensities of the amplified products were analyzed by densitometry and the log of the ratios of VEGF CRS and target areas was plotted as a function of CRS added to each PCR reaction. Lines were constructed by linear regression analysis and VEGF mRNA levels determined from the equivalence points (i.e. intersection of the vertical with regression lines)

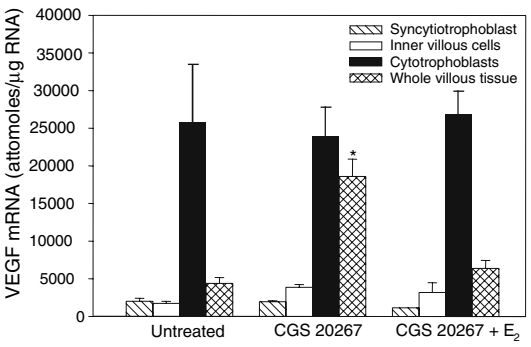

Fig. 3 VEGF mRNA levels in placental syncytiotrophoblast, inner villous core cells, and cytotrophoblasts isolated by Percoll gradient centrifugation and whole villous tissue on day 170 of gestation from baboons untreated or treated daily with CGS 20267 or CGS 20267 plus estradiol (E_2) benzoate between days 30 (60) and 169 of gestation (see footnote of Table 1). Values represent the means (± SE) of 3 to 6 baboons for each cell fraction and each treatment group (except syncytiotrophoblast from baboons treated with CGS 20267 plus E_2, n = 2). *Different at $P < 0.001$ from untreated control within respective tissue fraction (ANOVA and Newman–Keuls multiple comparisons test)

Cytotrophoblasts were a major source of VEGF mRNA in the villous placenta on day 170 of gestation (Fig. 3). Cytotrophoblast VEGF mRNA levels (mean ± SE, attomoles/µg total RNA) on day 170 were similar in baboons untreated (25,807 ± 5,873), or treated on days 30 (60)-169 with CGS 20267 (23,900 ± 1,940) or CGS 20267-plus estradiol (26,885 ± 2,569, Fig. 3). VEGF mRNA levels (attomoles/µg RNA) on day 170 in the syncytiotrophoblast (2,008 ± 405) and inner villous cells (1,724 ± 287) of untreated baboons were approximately 10-fold lower than in cytotrophoblasts and also not altered by CGS 20267 (1,953 ± 137 and 3,853 ± 385, respectively) or CGS 20267 plus estradiol (1,139 and 3,169 ± 1,321, respectively) administration (Fig. 3). However, VEGF mRNA levels in whole villous tissue of untreated baboons (4,382 ± 778) were increased ($P < 0.001$) over 4-fold by the administration of CGS 20267 (18,590 ± 2,315) and restored to normal by treatment with CGS 20267 plus estrogen (6,383 ± 1,048).

VEGF immunocytochemistry

VEGF protein was expressed in relatively high level within placental villous cytotrophoblasts and in apparently lower levels in the syncytiotrophoblast and cells within the inner villous core, including the vascular endothelium, in untreated baboons in late gestation (Fig. 4A). The qualitative pattern of VEGF staining did not appear to be different in placentas obtained from baboons treated with CGS 20267 (Fig. 4B). Validation of VEGF immunostaining was confirmed by absence of staining using primary antibody preabsorbed with recombinant human VEGF (Fig. 4C).

Placental vascularization

Mean ± SE percent vascularized area of the villous placental from untreated baboons on day 170 was 15.88 ± 0.88% (Fig. 5). However, the level of placental villous vascularization was not altered by the administration of CGS 20267 (14.40 ± 1.18%) or CGS 20267 plus estradiol (16.45 ± 3.34%). Vessel density, i.e., number of vessels/mm^2 whole villous tissue, in untreated baboons (1,375 ± 71) also was not changed by CGS 20267 (1,273 ± 77) or CGS 20267 plus estradiol (1,510 ± 112) treatment (Fig. 6).

Approximately 65% of the blood vessels in the villous placenta of untreated baboons on day 170 of gestation were less then 100 µm^2 and 20% were 101–200 µm^2 in size, while the remaining vessels were greater than 201 µm^2 (Fig. 7). The size distribution and number of vessels within each size class were also not changed by the administration of CGS 20267 or CGS 20267 plus estradiol.

Discussion

The present study shows that placental villous cytotrophoblast VEGF mRNA levels, which typically reach maximal

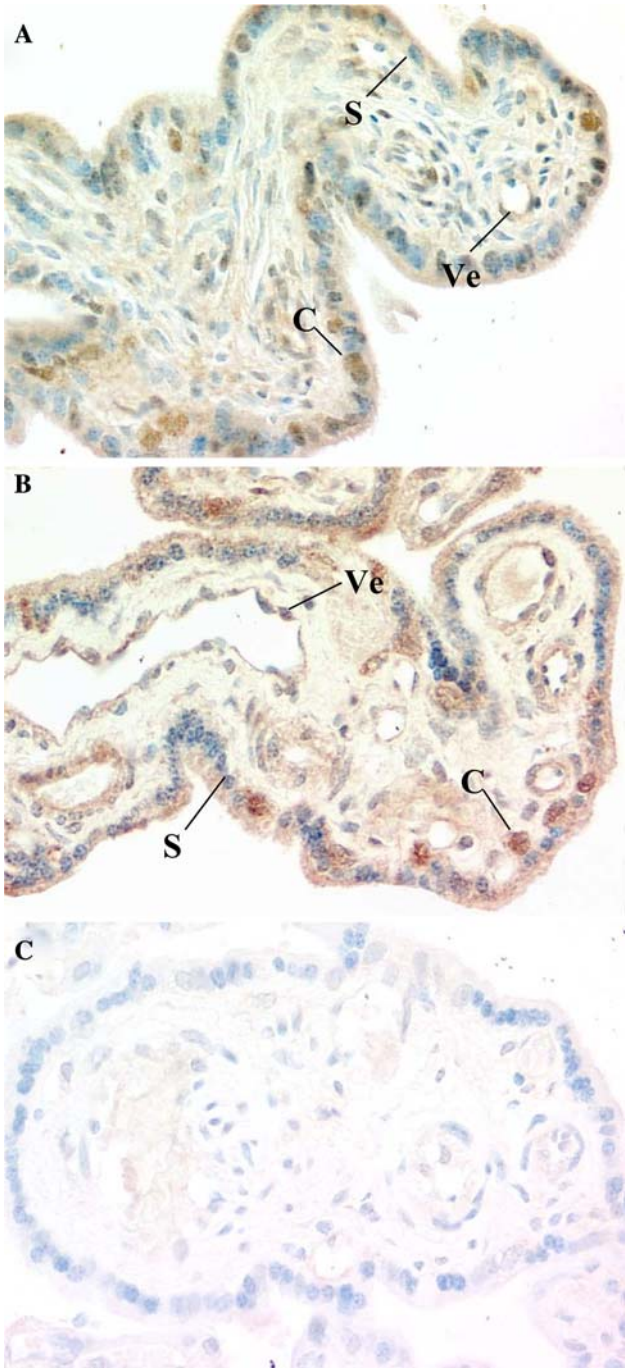

Fig. 4 Representative photomicrographs of VEGF immunocytochemistry (brown precipitate) in the villous placenta on day 170 of gestation in baboons untreated (**A**) or treated on days 30–169 with CGS 20267 (**B**). (**C**) VEGF immunocytochemistry when the primary VEGF antibody was preabsorbed with recombinant human VEGF. C, cytotrophoblast nucleus; S, syncytiotrophoblast nucleus; Ve, vascular endothelium. Magnification, ×200

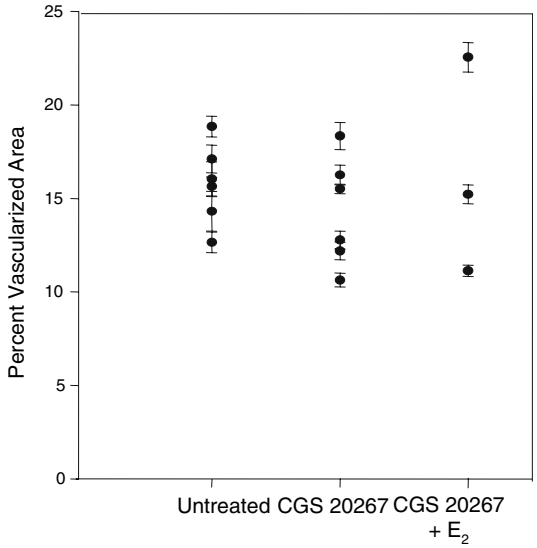

Fig. 5 Percent vascularized area, i.e. ratio of total vessel area and total villous area examined, of placental villous tissue obtained on 170 of gestation from baboons untreated or treated daily with CGS 20267 or CGS 20267 plus estradiol (E_2) benzoate as detailed in the footnote of Table 1. Each data point represents the mean (± SE) determined on 20–40 different tissue sections from a single placenta

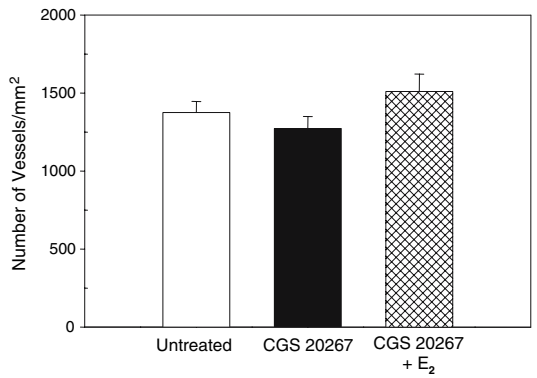

Fig. 6 Vascular density determined in the villous placenta of the same baboons on which placental vascularization data are shown in Fig. 5. Values represent the means (± SE) of 3 to 6 placentas per group

values near term [4], were unaltered late in pregnancy in baboons in which endogenous estrogen production/levels were suppressed by administration of an aromatase inhibitor or increased above normal levels by administration of estradiol throughout the last two-thirds of gestation. In contrast, we have recently shown that placental villous cytotrophoblast VEGF mRNA levels were upregulated by prematurely elevating estrogen [19, 20] and downregulated by suppressing estrogen [20] levels during the first third of baboon pregnancy. In addition, VEGF mRNA levels in extravillous cytotrophoblasts were decreased by elevating estrogen levels in early baboon pregnancy [24]. In contrast to the effects of estrogen on villous and extravillous cytotrophoblast expression in early gestation, neither syncytiotrophoblast or inner villous stromal cell VEGF mRNA levels were changed by estrogen administration or deprivation in

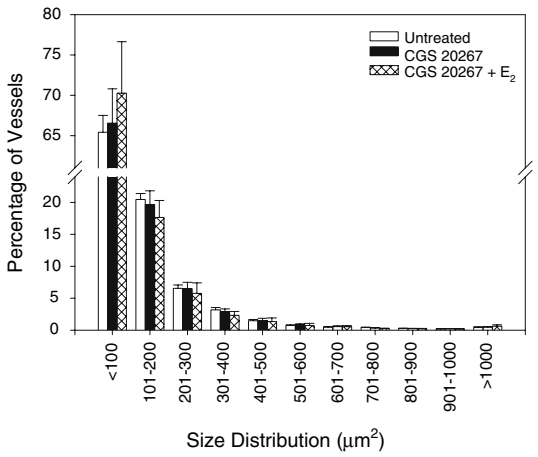

Fig. 7 Size distribution of blood vessels determined in the villous placenta from baboons untreated or treated with CGS 20267 or CGS 20267 plus estradiol (E_2). Values are the means (± SE) of 3 to 6 placentas per group

early [19, 20] or late (current study) baboon gestation. Collectively, based on the results obtained in the present and our recent studies, several important concepts emerge regarding the regulation of placental VEGF and angiogenesis by estrogen during primate pregnancy. First, it is evident that estrogen promotes placental villous cytotrophoblast and suppresses placental extravillous cytotrophoblast VEGF expression during early pregnancy, but villous cytotrophoblasts lose their responsivity to and dependence upon endogenous or exogenous estrogen with respect to VEGF expression in later stages of gestation. Thus, we propose that estrogen regulates expression of VEGF in a placental cell- and gestational age-specific manner. This apparent cell-selective and divergent regulation of VEGF by estrogen in the placenta has also been observed in other estrogen responsive systems. For example, estrogen increased VEGF gene transcription in estrogen receptor α- and β-positive MCF-7 breast cells, but decreased VEGF expression in estrogen receptor α-negative, β-positive MDA breast cells [25, 26]. Interestingly, villous cytotrophoblasts are estrogen receptor α- and β-positive while extravillous cytotrophoblasts are estrogen receptor α-negative and β-positive [27, 28]. The cell-specific regulation of VEGF in the primate placenta, therefore, may reflect presence/absence of estrogen receptor subtypes, as well as functional co-activators/repressors, that modulate estrogen-regulated target gene transcription [29]. The gestational age-specific loss in cytotrophoblast cell responsivity to estrogen in the second half of baboon pregnancy may result from a temporal change in expression of estrogen receptor species and/or co-regulators, although this remains to be determined.

Secondly, the maintenance of cytotrophoblast VEGF expression after estrogen suppression in late baboon pregnancy suggests that other factors stimulate/maintain, possibly in a compensatory fashion, VEGF expression in the second half of gestation. Hypoxia has a fundamentally important role in upregulating VEGF expression in many tissues, including the placenta [30–32]. Importantly, estrogen-induced VEGF expression in the uterus is linked to hypoxia-inducible factor 1α via a hypoxia response element in the VEGF promoter [33]. It is possible, therefore, that in the current study placental trophoblast VEGF expression was decreased shortly after estrogen deprivation in baboons, thereby compromising angiogenesis and oxygen perfusion and creating a hypoxemic state, leading to restoration of VEGF formation and blood vessel development despite chronic estrogen suppression. Additional studies using more acute experimental paradigms need to be performed in baboons to assess the latter possibility.

Thirdly, although the administration of estrogen stimulated angiogenesis in the villous placenta in early baboon pregnancy [20] and the uterus of nonpregnant sheep [18] and baboons [14], chronic estrogen deprivation or estrogen administration had no effect on blood vessel density in the villous placenta in the final two-thirds of baboon gestation (present study). Chronic estrogen suppression also did not modify uteroplacental blood flow dynamics in the second half of baboon pregnancy [34]. The maintenance of normal placental and fetal growth/weights in estrogen-deficient baboons of the present study is consistent with sustained uteroplacental angiogenesis and blood perfusion. Therefore, the regulation of placental blood vessel growth and uteroplacental blood flow by estrogen also appears to involve gestational age-specific mechanisms. It is possible that the continued expression of VEGF by cytotrophoblasts and elevated expression of VEGF by cells other than trophoblasts within whole villous tissue of estrogen-deprived baboons provide an autocrine/paracrine system to promote angiogenesis and thus maintain blood vessel development in the developing placenta. The identity of the cell source of elevated VEGF present in whole villous tissue after estrogen suppression is unknown, but may be macrophages or other cell types that express VEGF [2, 35], possibly in a compensatory manner after estrogen deprivation. Since angiogenesis is regulated by an interplay between angiostimulatory and angioinhibitory factors, other angioregulatory molecules may also be upregulated or downregulated in the absence of estrogen to sustain placental angiogenesis. It is unlikely that VEGF-B, VEGF-C, or VEGF-D are involved, because little or no expression of these VEGF species has been demonstrated within the human placenta [9]. However, other angiogenic factors expressed in the placenta, e.g., basic fibroblast growth factor and placental growth factor [32, 36], may have particularly important roles in addition to VEGF in regulating blood vessel maturation in placental villi with advancing primate pregnancy.

In contrast to the maintenance of placental villous cytotrophoblast VEGF expression and blood vessel density in estrogen-deprived baboons of the present study, we have recently shown that several important developmental events, including placental villous trophoblast 11β-hydroxysteroid dehydrogenase-1 and -2 localization and expression leading to activation of the fetal pituitary adrenocortical axis [37], fetal adrenocortical maturation [38], and fetal ovarian development [39] were suppressed by CGS 20267 and restored by concomitant CGS 20267 plus estradiol administration in the second half of baboon pregnancy. The efficacy of estrogen deprivation in preventing the latter fundamentally important aspects of development, and the absence of an effect on placental villous trophoblast VEGF expression and vascularization in baboons of the current study, point to the specificity of estrogen in regulating, in a gestational age-selective fashion, these physiologically significant processes. Although unlikely, it is possible that a difference in responsivity of these systems to estrogen exists, whereby the mechanisms underpinning VEGF expression and angiogenesis are more sensitive and thus maintained by the very low levels of estrogen remaining after CGS 20267-treatment.

In summary, the present study shows that cytotrophoblast VEGF mRNA levels and blood vessel development were maintained in the villous placenta near term of baboons in which serum estradiol levels were suppressed or elevated throughout the last two-thirds of gestation. Based upon these results, and our previous study which showed that placental villous cytotrophoblast VEGF mRNA levels were suppressed by estrogen deprivation during early pregnancy, we propose that villous cytotrophoblasts lose their responsivity to estrogen and that placental villous cytotrophoblast VEGF expression and angiogenesis are regulated by estrogen in a cell- and gestational age-specific manner, and that factors other than estrogen maintain VEGF expression during the last two-thirds of pregnancy.

Materials and methods

Animals

Female baboons (*Papio Anubis*) weighing 12–16 kg were housed individually in air-conditioned rooms under standardized conditions. Baboons received monkey chow (Teklad-Harlan, St. Louis, MO) and fresh fruit twice daily, vitamins daily, and water at will. Females were paired with male baboons for 5 days at the anticipated time of ovulation, which was determined on the basis of menstrual cycle history and perineal turgescence. Pregnancy was determined by palpation and ultrasonography and day 1 represented the day after ovulation. Blood samples (2–4 ml) were obtained from a maternal saphenous vein at 1- to 3-day intervals between days 20 and 170 of gestation (length of gestation is 184 days), after brief sedation with ketamine HCl (10 mg/kg body weight, im), for RIA of estradiol.

Four animals were left untreated and 6 baboons were injected sc with the aromatase inhibitor CGS 20267 (4, 4′-[1,2,4-triazol-1-yl-methylene]-*bis*-benzonitrite, Letrozole; Novartis Pharma AG, Basel, Switzerland) daily on days 30–169 (n = 3) or days 60–169 (n = 3) of gestation at a dose of 0.1 mg/day in sesame oil on days 30–59 of gestation and 0.2 mg/day on days 60–169. Three baboons were injected daily on days 30–169 (n = 2) or 60–169 (n = 1) with CGS 20267 (0.1–2.0 mg/day) and estradiol benzoate (ranging between 0.1 and 0.2 mg/day) to increase serum estradiol to levels above those in untreated animals.

On day 170 baboons were anesthetized with halothane and the fetus and placenta delivered by cesarean section. Animals were cared for and used strictly in accordance with U.S. Department of Agriculture regulations and the *Guide for the Care and Use of Laboratory Animals* prepared by the National Research Council (National Academy Press, 1996). The experimental protocol used in this study was approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine.

RIA of estradiol

Serum estradiol concentrations were determined by RIA, via an automated chemiluminescent immunoassay system (Immulite; Diagnostic Products Corp, Los Angeles, CA), as described previously [40].

Placental tissue

Randomly-selected sections (4 mm^3) of villous tissue were excised from the placenta and stored frozen in liquid nitrogen for mRNA analysis. Enriched fractions of cytotrophoblasts, syncytiotrophoblast, and cells of the inner villous core were obtained as described by our laboratory [41] and others [42] and used for quantification of VEGF mRNA levels by competitive reverse transcription-polymerase chain reaction (RT-PCR). Briefly, villous tissue was minced in Hanks balanced salt solution (HBSS, Life Technologies Inc., Gaithersburg, MD) and digested in HBSS containing 0.1% hyaluronidase (type I-S, Sigma Chemical, St. Louis, MO), 0.1% collagenase (Type H, Sigma Blend), and 0.01% deoxyribonuclease I (1680 Kunitz Units/mg, Sigma). Enriched cell fractions were isolated via 5–70% Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) gradient centrifugation at 1,200 × g. Kliman et al [42] and we (unpublished data) have shown

that highly-enriched cytokeratin-positive cytotrophoblast, placental lactogen-positive syncytiotrophoblast, and α-antichymotrypsin-positive inner villous cell fractions were obtained by these methods.

RT-PCR of VEGF

The mRNA levels for VEGF were quantified by the competitive RT-PCR assay established by Riedy et al. [43] and modified in our laboratory [44]. Placental cell fractions were homogenized in 4 M guanidine isothiocynate, layered over 5.7 M cesium chloride, and total RNA obtained by centrifugation. Oligonucleotide primers were designed (Invitrogen Life Technologies, Carlsbad, CA) based on the human VEGF cDNA sequence [45]:

> Primer 1: downstream, 5′-GGTGAGGTTTGATCCG CATAATCTGCGCATCAGGGGC ACACAGGAT-3′ (position 336–311 linked to 243–224); Primer 2: upstream, 5′-AATTTAATACGACTCACTATAGG GACTGCTGTCTTGGGTGCATTGG-3′ (position T7 polymerase sequence [underlined] linked to 10–30); Primer 3: downstream, 5′-GGTTTGATCCG-CATAATCTGC-3′ (position 331–311); Primer 4: upstream, 5′-CTGCTGTCTTGGGTGCATTGG-3′ (position 10–30).

Since primers 3 and 4 are upstream of the alternative splice site that generates the different isoforms of VEGF, a single 323-base pair (bp) PCR product was generated. The competitive reference standard (CRS) was prepared using primers 1 and 2 and had a 67-bp deletion (length: 256 bp) compared to wild-type target mRNA. Total RNA (3.2 μg) from baboon placenta was reverse transcribed in 1 mM deoxy(d)NTPs, 1 mM dithiothreitol, 200 U SUPERSCRIPT RNase H-reverse transcriptase (RT, Invitrogen), 40 U RNAguard ribonuclease inhibitor (Pharmacia Biotech, Piscataway, NJ), and 250 ng random primers. The RT mixture (5 μl) was added to a PCR reaction mixture containing 0.2 mM dNTPs, 1.25 U cloned *Thermus aquaticus* DNA polymerase (Amplitaq, Perkin-Elmer Corp/Cetus, Norwalk, CT) and 20 pmol each of primers 1 and 2. PCR was performed in a thermal cycler (MJ Research Inc., Cambridge, MA) and samples amplified in 25 sequential cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 5 min. Products were separated by electrophoresis in a 2% agarose gel and visualized with ethidium bromide. The CRS was synthesized from 150 ng of cDNA template using the MEGAscript T7 in vitro transcription kit (Ambion, Inc., Austin, TX) and quantitated via UV absorption spectrophotometry at an optical density of 260 nm.

To quantify VEGF mRNA, a constant amount of total RNA (75–300 ng) was added to an RT mixture containing 3-fold serial dilutions of the VEGF CRS (5,400–200 attomoles). Negative controls, in which either the RT enzyme or RNA was omitted from the RT reaction, were performed to test for genomic DNA. Upon completion of the RT, 5 μl of the RT reaction was added to a PCR mixture containing 20 pmol each of primers 3 and 4 (26 cycles). PCR products were fractionated in a 2% agarose gel containing ethidium bromide, visualized with UV, and photographed using type 665 positive/negative film (Polaroid Corp, Cambridge, MA). Negatives were scanned using a model 620 Video Densitometer and 1-D Analyst II data analysis software (Bio RAD Laboratories, Hercules, CA). The intensity of amplified product was represented as the relative area under each sample band. The logarithm (log) of the ratio of VEGF CRS area to VEGF target area was plotted as a function of the concentration of VEGF CRS added to each PCR reaction. The concentration of VEGF target mRNA was determined where the ratio of the log of CRS and target was equal to 0 (i.e., the equivalence point).

Immunocytochemistry of VEGF and von Willebrand factor

The localization of VEGF and von Willebrand factor protein was determined by immunocytochemistry as detailed previously [4]. Briefly, paraffin embedded placental villous tissue was sectioned (4 μm), boiled in 0.01 M sodium citrate, pretreated with Protease (Biomeda, Foster City, CA) and incubated in H_2O_2 to inhibit endogenous peroxidase. Tissues were incubated 24 h at 4°C with polyclonal goat antibody to VEGF (AF-293-NA, 1:500 dilution; Santa Cruz Laboratories, Santa Cruz, CA) or rabbit antibody to von Willebrand factor (1:1,500 dilution; Dako, Carpinteria, CA). Tissue sections were incubated 1 h with either biotinylated antigoat or antirabbit immunoglobulins (Vector Laboratories, Inc., Burlingame, CA), avidin-biotin-peroxidase complex (ABC Elite, Vector Laboratories, Inc.) and developed using diaminobenzidine (VEGF) or diaminobenzidine and 2.5% nickel sulfate (von Willebrand factor). Negative controls for immunocytochemistry included preabsorption of the primary antibody with a 10-fold excess of human recombinant VEGF protein (Santa Cruz Biotechnology, Inc.).

Placental vascularization

The level of placental vascularization was quantified by computer-assisted image analysis, as described by our laboratory [4]. Quantification of blood vessels, i.e., arterioles, arteries, venules, and veins, in placental villous tissue was performed with an Eclipse E 1,000 M microscope (Nikon, Tokyo, Japan) attached to a color video camera (Dage-MTI,

Michigan City, IN). Color images were digitized by a Power Macintosh G3 computer (Apple Computer, Cupertino, CA) and visualized on a high resolution monitor. Von Willebrand factor-immunoreactive vessels were shaded in with a green pseudo-color, and total villous area circumscribed with a yellow pseudo-color. Image analysis software (IP Lab Scientific Image Processing, Scanalytics, Fairfax, VA) was used for interactive manipulation of the image and data collection. Information on vessel number and area was imported into an Excel worksheet program.

The proportion of villous placenta comprised of blood vessels, i.e., percent vascularized area, was determined by dividing the total vessel area by the total villous area examined. The number of vessels/mm^2 villous tissue, i.e., vessel density, and the size distribution of vessels were also determined. A mean (±SE) value for vessel counts on each placenta was determined on 20–40 different placental villous sections (two views per villous section by five sections per biopsy by two to four biopsies per placenta).

Statistical analysis

Data were expressed as the mean ± SE and were analyzed by two-way ANOVA with post hoc comparison of means by Newman–Keuls multiple comparison test.

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