Baboon placental heparin-binding epidermal growth factor-like growth factor

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Abstract

Placental extravillous trophoblast remodeling of the uterine spiral arteries is important for promoting blood flow to the placenta and fetal development. Heparin-binding EGF-like growth factor (HB-EGF), an EGF family member, stimulates differentiation and invasive capacity of extravillous trophoblasts *in vitro*. Trophoblast expression and maternal levels of HB-EGF are reduced at term in women with preeclampsia, but it is uncertain whether HB-EGF is downregulated earlier when it may contribute to placental insufficiency. A nonhuman primate model has been established in which trophoblast remodeling of the uterine spiral arteries is suppressed by shifting the rise in estrogen from the second to the first trimester of baboon pregnancy. In the present study, we used this model to determine if placental HB-EGF is altered by prematurely elevating estrogen early in baboon gestation. Uterine spiral artery remodeling and placental expression of HB-EGF and other EGF family members were assessed on day 60 of gestation in baboons treated with estradiol (E₂) daily between days 25 and 59 of gestation (term=184 days). The percentages of spiral artery remodeling were 90, 84 and 70% lower ($P < 0.01$), respectively, for vessels of 26–50, 51–100 and >100 µm diameter in E₂-treated compared with untreated baboons. HB-EGF protein quantified by immunocytochemical staining/image analysis was decreased three-fold ($P < 0.01$) in the placenta of E₂-treated versus untreated baboons, while amphiregulin (AREG) and EGF expression was unaltered. Therefore, we propose that HB-EGF modulates the estrogen-sensitive remodeling of the uterine spiral arteries by the extravillous trophoblast in early baboon pregnancy.

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Introduction

Placental extravillous trophoblast invasion and remodeling of the uterine spiral arteries are thought to be critical for ensuring optimal maternal blood flow to the placenta and thus development of the fetus (Norwitz *et al.* 2001, Frank & Kaufmann 2006). Failure of extravillous trophoblasts to invade and convert the spiral arteries to dilated conduits may hamper perfusion of the villous placenta and has been linked to preeclampsia (Brosens *et al.* 1972, DiFederico *et al.* 1999) and fetal growth restriction (Khong *et al.* 1986, Ishihara *et al.* 2002). Etiology of the defect in placentation that predisposes to preeclampsia or fetal growth restriction is unknown, but events occurring early in gestation that interfere with extravillous trophoblast survival, differentiation and/or uterine artery transformation are likely to be involved.

Although experimental animal models have been established which replicated the sequelae of maternal hypertension, proteinuria, and renal dysfunction

symptomatic of human preeclampsia (McCarthy *et al.* 2011), these paradigms have not induced a defect in extravillous trophoblast remodeling of the uterine spiral arteries, a hallmark feature of preeclampsia. However, shifting the normal surge in maternal serum E₂ levels from the second to the first trimester of baboon pregnancy suppressed extravillous trophoblast remodeling of the uterine spiral arteries and uteroplacental blood flow dynamics (Aberdeen *et al.* 2012, Bonagura *et al.* 2012). These authors have proposed, therefore, that the low levels of E₂ in the first trimester permit rapid transformation of the uterine arteries and the elevated levels of E₂ in the second trimester has a physiologically important role in suppressing and thus controlling the extent to which these vessels are remodeled. Prematurely elevating E₂ in early baboon pregnancy decreased placental vascular endothelial growth factor (VEGF) expression within the placental basal plate, that is, anchoring villi, trophoblastic shell and junctional zone (Bonagura *et al.* 2012), while VEGF gene delivery selectively to the placental basal plate

restored spiral artery remodeling to normal (Babischkin *et al.* 2019). Therefore, it was proposed that VEGF mediates the estrogen-induced reduction in trophoblast spiral artery remodeling.

In vitro studies have shown that heparin-binding EGF-like growth factor (HB-EGF), a member of the EGF family that includes amphiregulin (AREG), prevents apoptosis within and stimulates differentiation and invasive capacity of extravillous trophoblast (Leach *et al.* 1999, Jessmon *et al.* 2009). HB-EGF is abundantly expressed by trophoblasts of the placental chorionic villi and basal plate and expression was significantly reduced at term in women with preeclampsia (Leach *et al.* 2002, Armant *et al.* 2015). However, it is uncertain whether HB-EGF is downregulated earlier in development when it may contribute to placental insufficiency or if dysregulation occurred later in gestation as a result of advanced disease. There appears to be a regulatory relationship between HB-EGF and VEGF, since HB-EGF increased VEGF expression *in vitro* by HTR-8/SVneo trophoblast (Liu *et al.* 2019) and granulosa (Karakida *et al.* 2011) cells; conversely, VEGF increased HB-EGF expression by umbilical vein vascular endothelial (Arkonac *et al.* 1998) and ocular (Inoue *et al.* 2018) cells. We hypothesize that dysregulation of HB-EGF, as well as VEGF, contributes to the reduced extravillous trophoblast invasion and transformation of the spiral arteries that occurs in human preeclampsia and that is induced by elevating estrogen in early baboon pregnancy. Therefore, placental expression of HB-EGF and related growth factors of the EGF signaling system and the level of spiral artery remodeling were assessed at the end of the first-third of gestation in baboons in which E₂ levels were prematurely elevated.

Materials and Methods

Animals

Female baboons (*Papio Anubis*), originally obtained from the Southwest National Primate Research Center (San Antonio, TX) and weighing 13–15 kg, were used in this study. Animals were housed individually in large primate cages and received standard monkey chow (Harlan Primate Diet, Madison, WI, USA) and fresh fruit twice daily, multiple vitamins daily, and water *ad libitum*. Females were paired with male baboons for 5 days at the time of ovulation, as estimated by menstrual cycle history and perineal turgescence, and day 1 of gestation was designated as the day preceding deturgescence. Baboons were cared for and used strictly in accordance with U.S. Department of Agriculture regulations and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Academy Press, 2011). The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine.

A 2- to 4-mL blood sample was obtained from a maternal peripheral saphenous vein every 1–2 days during the study period from baboons after ketamine HCl sedation (10 mg/kg

body weight, im). Placentas were removed by cesarean section on day 60 of gestation (length of gestation is 184 days) from isoflurane-anesthetized baboons untreated ($n=5$) or treated daily on days 25–59 with E₂ benzoate (25 µg/kg body weight/day, sc injection in 1.0 mL sesame oil, $n=5$). At the time of cesarean section, blood samples (1 mL) were obtained from the left and right uterine veins. Serum E₂ levels were quantified using an automated chemiluminescent immunoassay system (Immulite; Diagnostic Products Corp., Los Angeles, CA, USA), as described previously (Albrecht *et al.* 2000). Serum HB-EGF levels were quantified by Quantikine ELISA (R&D Systems).

Quantification of uterine spiral remodeling

At the time of cesarean section on day 60, eight randomly selected areas of placental basal plate tissue were obtained and fixed in 10% formalin, embedded in paraffin, sectioned (5 µm), microwaved in sodium citrate for antigen retrieval, and processed for hematoxylin and eosin histology and cytotrophoblast/epithelial cell-specific cytokeratin immunocytochemistry (15 µg/mL, mouse monoclonal, Cat No. 349205, BD Biosciences, San Jose, CA, USA), as described previously (Bonagura *et al.* 2012). Extravillous trophoblasts invade veins and lymphatic vessels, as well as arteries, in the first trimester of pregnancy (Windsperger *et al.* 2017, Moser *et al.* 2018, Huppertz 2019). Although cell-specific histochemical methods were not employed in the present study to identify these different vessel types, trophoblast intramural invasion of arteries, the criteria used to confirm vessel remodeling in baboons, exceeds that in veins and lymphatic vessels, whereas trophoblast intraluminal localization predominates in veins and lymphatic vessels (Windsperger *et al.* 2017, Moser *et al.* 2018). Moreover, trophoblast invasion of lymphatic vessels and veins appears more prevalent in early pregnancy, while trophoblast invasion and remodeling of the spiral arteries continues and becomes extensive in the latter stage of the first trimester, a time when vessel remodeling was analyzed in baboons of the current study. Therefore, the term spiral arteries is used throughout the present study.

The baboon placenta consists of approximately 20 cotyledons/placentomes (Houston & Hendrickx 1968, Ramsey & Donner 1980) each supplied via a single spiral artery. Light microscopy (Nikon Eclipse E 1000 M; Nikon) and an image analysis system (IP Laboratory, version 3.63; Scanalytics, Fairfax, VA, USA) were used to quantify trophoblast invasion/remodeling in at least 12 of these spiral arteries randomly identified in each animal and categorize them into groups with diameters of 26 to 50, 51 to 100, and >100 µm. The number of arteries exhibiting trophoblast invasion was quantified by identifying spiral arteries in which at least half of the vessel wall was occupied by cytokeratin-positive trophoblasts as illustrated in Fig. 1B. The level of uterine artery remodeling is expressed as the number of vessels exhibiting EVT invasion divided by the total number of vessels counted.

Semiquantitative immunohistochemistry of HB-EGF

Ten-micron sections were prepared from blocks of paraffin-embedded placental tissue and mounted on microscope

slides. Immunohistochemistry was performed using a DAKO Autostainer Universal Staining System (DAKO), as previously described (Leach *et al.* 2002, 2004, Imudia *et al.* 2008). Rehydrated sections were labeled for 1 h at 25°C with either goat polyclonal antibody against human HB-EGF (8 µg/mL, Cat No. AF-259 E, R&D Systems), amphiregulin (AREG, 10 µg/mL, Cat No. AF 262, R&D Systems) or EGF (10 µg/mL, Cat No. MAB 236, clone 10825, R&D Systems) or 10 µg/mL non-immune goat IgG (Jackson ImmunoResearch Laboratories). Tissues were then incubated for 1 h at 25°C with 0.1 µg/mL rabbit anti-goat IgG (Jackson ImmunoResearch). To visualize and quantify antigen, an Envision System peroxidase anti-mouse/rabbit kit (DAKO) was used in conjunction with image analysis, according to our published procedure (Leach *et al.* 2002). Images obtained with a DM IRB inverted microscope (Leica) interfaced with an Orca digital camera (Hamamatsu, Hamamatsu City, Japan) were processed using Simple PCI software (Hamamatsu) to determine the mean gray level over designated regions of each image, according to our published procedure (Leach *et al.* 2002). Average values

obtained with IgG substituted for primary antibody were subtracted from each sample to determine the specific stain intensity. In a preliminary experiment, antibody was titrated using a human trophoblast HTR-8/SVneo cell line (Kilburn *et al.* 2000) to determine optimal concentration that gave robust staining within the linear portion of the concentration curve.

Statistical analysis

The levels of spiral artery remodeling and placental HB-EGF protein were analyzed by ANOVA followed by Student–Newman–Keuls test. Serum E₂ and fetal body and placental weights were analyzed by unpaired Student's *t* test.

Results

Serum E₂ and HB-EGF levels

Maternal saphenous and uterine vein serum E₂ levels on day 60 of gestation were approximately five-fold (0.74 ± 0.07 ng/mL) and two-fold (1.21 ± 0.16 ng/mL) greater ($P < 0.01$), respectively, in the E₂-treated compared to the untreated baboons (Table 1), but considerably lower than the level (>2.0 ng/mL) typical of the second trimester of normal baboon pregnancy (Albrecht *et al.* 2000). However, HB-EGF was not measurable in serum or plasma of the maternal saphenous or uterine veins of untreated and E₂-treated baboons. Fetal body and placental weights on day 60 were unaltered by estrogen treatment (Table 1).

Uterine spiral artery remodeling

Figure 1 shows representative hematoxylin-eosin and cytokeratin stained extravillous trophoblasts within the walls of uterine spiral arteries in the decidua basalis on day 60 of baboon pregnancy. The striking invasion and remodeling by cytokeratin-stained extravillous trophoblasts of a spiral artery in an untreated baboon is shown in Fig. 1B. In contrast, the absence of cytokeratin-stained trophoblasts within and tightly coiled nature of several spiral arteries of an E₂-treated baboon are illustrated in Fig. 1D.

Figure 2 shows the level of trophoblast invasion and remodeling of the uterine spiral arteries as quantified by image analysis on day 60 of gestation in the baboons of this study. In untreated baboons, the number of spiral arteries remodeled by trophoblasts, expressed as a ratio of the total number of vessels counted, was $5.4 \pm 1.1\%$, $20.3 \pm 5.9\%$ and $67.3 \pm 7.1\%$, respectively, for arteries of 26–50, 51–100 and >100 µm in diameter. The percentages of spiral artery remodeling in the E₂-treated animals were $0.1 \pm 0.0\%$, $3.3 \pm 1.3\%$ and $20.8 \pm 6.4\%$ or 90%, 84% and 70%, lower ($P < 0.01$) respectively, for the three different categories of vessels when compared with that in the untreated baboons.

Figure 1 Photomicrographs of hematoxylin-eosin (panels A and C) and cytokeratin (panel B) stained extravillous trophoblasts within walls of uterine spiral arteries in the decidua basalis on day 60 of gestation in baboons untreated (panels A and B) and E₂-treated (panels C and D) baboons. Magnification bar = 100 µm. Arrows, spiral arteries.

Table 1 Maternal estradiol and fetal body and placental weights in baboons.

	<i>n</i>	Estradiol (ng/mL)		Fetal body weight (g)	Placental weight (g)
		Saphenous vein	Uterine vein		
Untreated	5	0.16 ± 0.02	0.70 ± 0.06	12.2 ± 0.3	30.7 ± 1.7
Estradiol	5	0.74 ± 0.07*	1.21 ± 0.16*	11.6 ± 0.6	30.3 ± 0.5

Mean (± s.e.) estradiol and fetal body and placental weights on day 60 of gestation in baboons untreated or administered estradiol benzoate (25 µg/kg body weight/day, sc) daily on days 25–59 of gestation (term = 184 days).

**P* < 0.01 versus untreated baboons.

Placental HB-EGF protein expression

HB-EGF protein, as assessed by immunohistochemistry, in untreated baboons on day 60 of pregnancy was abundantly localized in the anchoring villi, cytotrophoblastic shell and decidua basalis of the placental basal plate (Fig. 3A and A') and the trophoblast layer of the chorionic villi and inner villous core (Fig. 3B and B'). However, in E₂-treated baboons HB-EGF protein expression was negligible in the anchoring villi, cytotrophoblastic shell and decidua basalis (Fig. 3C and C') and markedly reduced in the trophoblast layer covering the chorionic villi, although staining appear to be maintained in the inner villous core (Fig. 3D and D').

HB-EGF protein immunostaining intensity, quantified by image analysis, was 60% lower (*P* < 0.01) in the placental basal plate of E₂-treated (8.6 ± 1.9 units) compared with that in untreated (23.5 ± 3.5 units) baboons (Fig. 4A). In contrast, AREG and EGF expression in the placental basal plate was not significantly different in untreated and E₂-treated animals (Fig. 4A). In the placental chorionic villi, HB-EGF expression was 40% lower (*P* < 0.05) in E₂-treated (14.9 ± 2.3 units) than in untreated (25.3 ± 4.9 units) baboons (Fig. 4B). However, AREG and EGF immunostaining values in the chorionic villi were similar in untreated and E₂-treated animals (Fig. 4B).

Figure 2 Percent of spiral artery remodeling (expressed as number of arteries exhibiting trophoblast invasion and remodeling divided by the total number of vessels counted) grouped by vessel diameter on day 60 in baboons untreated or treated daily on days 25–59 with E₂ benzoate. *Value different (*P* < 0.01) from untreated animals.

Discussion

The present study shows that in baboons in which serum E₂ levels were prematurely elevated in the first trimester of pregnancy, there was a decrease in placental basal plate and villous trophoblast expression of HB-EGF, along with suppression of trophoblast invasion and remodeling of the uterine spiral arteries. In contrast to the decline in placental HB-EGF, placental AREG and EGF expression was unaltered. HB-EGF expression in the placental basal plate is also significantly reduced at term in women with preeclampsia (Leach *et al.* 2002), a condition associated with failed physiologic conversion of the uterine spiral arteries (Brosens *et al.* 1972, Khong *et al.* 1986). HB-EGF is well established as a trophoblast survival factor (Armant *et al.* 2006, Wolff *et al.* 2007, Imudia *et al.* 2008, Leach *et al.* 2008), while both HB-EGF and EGF stimulate trophoblast invasion *in vitro* (Bass *et al.* 1994, Leach *et al.* 2004). The decrease in placental HB-EGF expression observed at term in women with preeclampsia may reflect an earlier attenuation of this growth factor which hampers trophoblast function and contributes to the pathology that arises during the second half of gestation in this disorder. Collectively, based on the present and previous findings we propose that dysregulation of HB-EGF early in development predisposes trophoblasts to cell death and reduced vessel invasion and thus modulates the estrogen-induced suppression of trophoblast remodeling of the uterine arteries in the baboon and improper placentation in human preeclampsia.

Placental extravillous VEGF mRNA and protein levels are also markedly decreased in baboons in which uterine spiral artery remodeling is suppressed by E₂ administration in early baboon pregnancy (Bonagura *et al.* 2012). Cross-talk between HB-EGF and VEGF pathways appears to exist, since VEGF increased HB-EGF expression by vascular endothelial (Arkonac *et al.* 1998) and ocular (Inoue *et al.* 2018) cells, while HB-EGF increased VEGF expression by HTR-8/SVneo trophoblast (Liu *et al.* 2019), granulosa (Karakida *et al.* 2011) and breast cancer (Yotsumoto *et al.* 2013) cells. Moreover, placental VEGF expression was decreased in fetal HB-EGF-knockout mice (Liu *et al.* 2019). Both VEGF and HB-EGF are potent endothelial mitogens and these growth factors may act synergistically in promoting proliferative and chemotactic responses (Gerritsen *et al.* 2003).

Figure 3 Immunohistochemical labeling of HB-EGF in anchoring villi (AV), cytotrophoblastic shell (CS) and decidua basalis (DB) of the placental basal plate (A, C and E) and the chorionic villi (B, D and F) on day 60 of gestation in baboons untreated (A and B) or treated with E_2 (C and D). No labeling was apparent in placental sections when non-immune IgG was substituted for anti-HB-EGF antibody (E and F). The areas within the boxes of panels A–D are magnified as inserts in panels A' and C' to show a portion of the cytotrophoblastic shell and in panels B' and D' to show edges of chorionic villi.

E_2 either stimulates or inhibits the expression of VEGF and HB-EGF, depending on the specific tissue type (Wang *et al.* 1994, Zhang *et al.* 1994, Albrecht *et al.* 2004, Lee *et al.* 2004, Kanda & Watanabe 2005). Consensus estrogen response elements do not appear to exist on the HB-EGF promoter, although induction of HB-EGF expression by E_2 in the uterus required activation of the estrogen receptor (Wang *et al.* 2002). Moreover, a link between HB-EGF/EGF receptor AKT-mTOR signaling with estrogen receptor α has been shown in the brain (Pandey *et al.* 2020) and E_2 signaling to Erk-1/-2 stimulated HB-EGF release by breast cells (Filardo *et al.* 2000). The E_2 -induced decrease in placental basal plate VEGF and HB-EGF in early baboon pregnancy may reflect the presence of estrogen receptor corepressors (Hu & Lazar 2000, Yoon & Wong 2006). The decrease in placental basal VEGF and HB-EGF expression with early E_2 treatment does not appear to be a consequence of the suppression of uterine spiral artery remodeling and blood flow. Thus, a decline in uterine artery blood flow does not emerge in E_2 -treated baboons until the second half of gestation (Aberdeen *et al.* 2012) and in contrast to the E_2 -induced decrease in placental basal plate VEGF and HB-EGF expression, AREG and EGF were unaltered and VEGF expression by trophoblasts in the chorionic villi was increased (Albrecht *et al.* 2004). Additional study is required to establish the potential interaction of VEGF and HB-EGF in modulating the action of E_2 in regulating trophoblast spiral artery transformation in early pregnancy. The baboon provides a valuable nonhuman primate translational model to explore the relationship of signaling proteins to early placental development using *in vivo* experimental paradigms not possible to employ through human investigation.

Figure 4 Quantitative assessment by image analysis of immunohistochemical staining of EGF family growth factors in placentas obtained on day 60 of gestation from baboons that were untreated or treated with E_2 . Baboon placentas labeled with antibodies against HB-EGF, AREG, and EGF were imaged over the placental basal plate (A) and chorionic villi (B) and the level of staining (means $\pm$ S.E.) for each protein (mean gray levels) was determined by image analysis, as described in the Methods section. * $P < 0.01$ and ** $P < 0.05$ versus values in untreated animals.

In summary, the present study shows that placental basal plate expression of HB-EGF was decreased in baboons in which uterine spiral artery trophoblast invasion was suppressed by prematurely elevating E_2 levels in early pregnancy. Therefore, we suggest that dysregulation of HB-EGF early in pregnancy predisposes trophoblasts to reduced invasion and improper placentation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

D R A and E D A conceived the study and wrote the paper. G W A performed the animal experiments and tissue analyses. B A K performed tissue analyses. G J P performed serum hormone analyses and collaborated with E D A on study design.

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