Growth and development of blood vessels in maternal placenta during early pregnancy in sheep[1]

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Data from our study may help to identify factors that can be used therapeutically to restore normal placental vascular function and blood flow to rescue compromised pregnancies. In addition, these data will help to compare the patterns of vascularization and expression of angiogenic factors, and timing of angiogenesis initiation in compromised pregnancies vs. normal pregnancy in future studies.

Summary

Placental vascular development (angiogenesis) is critical for placental function and thus for normal embryonic/fetal growth and development. Specific environmental factors or use of assisted reproductive techniques may result in poor placental blood vessel growth and development (angiogenesis), which may contribute to embryonic losses and/or fetal growth retardation. To provide a description of normal placental angiogenesis, uterine tissues were collected on days 14, 16, 18, 20, 22, 24, 26, 28, and 30 after mating and on day 10 after estrus (nonpregnant controls). To determine vascular development in the endometrium, we used histochemistry and/or immunohistochemistry followed by image analysis. Compared to controls, several measurements of vascularity increased ($P<0.001$) including vascular labeling index (LI; proportion of proliferating cells), the tissue area occupied by capillaries, area per capillary (capillary size), total capillary circumference per unit of tissue area and expression of factor VIII (marker of blood vessels), but capillary number decreased ($P<0.001$) in endometrium. These data indicate that endometrial angiogenesis, manifested by increased vascularity is initiated very early in pregnancy. This more complete description of early placental angiogenesis will provide the foundation for determining whether placental vascular development is altered in compromised pregnancies.

Introduction

During pregnancy, vascular development or angiogenesis parallels the growth of uterine and placental tissues to support fetal growth and development (Reynolds and Redmer, 2001; Reynolds et al. 2005a,b,c, 2006). Inadequate vascular growth during early pregnancy may be associated with inadequate uterine and umbilical blood flow, which directly affects transport of nutrients to the embryo/fetus. The consequences of inadequate placental vascular development include compromised implantation, spontaneous abortion/embryonic loss, defective formation of the placenta, and

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altered fetal growth and development resulting in intrauterine growth restriction (IUGR) potentially leading to reduced lifelong health and productivity of the offspring (Wallace et al. 2002; Reynolds et al. 2005a,b,c, 2010; Sherer and Abulafia, 2001; Torry et al. 2004; Demir et al. 2007).

Most of embryonic loss occurs in early pregnancy reaching ≥30% of embryos lost in most mammalian species and possibly ≥50% in humans (Reynolds and Redmer, 2001). These are astonishing figures and highlight the early part as a critical period of pregnancy. High embryonic loss may appear as a consequence of a variety of negative effects, and thus establishes the need to analyze angiogenesis in both normal pregnancies and compromised pregnancies, such as those generated from assisted reproductive technologies (ART), or affected by environmental factors such as malnutrition or other stress.

Angiogenesis is the formation of new blood vessels from preexisting vasculature, and it is a critical process for the growth and development of all tissues, including the placenta (Reynolds and Redmer, 1992, 1998; Borowicz et al. 2007; Reynolds et al. 2010). Growth of the tissue is associated with the high metabolic demands (Reynolds and Redmer, 1998). The placenta, which serves as the organ of exchange between maternal and fetal systems, represents a very fast growing tissue with a high metabolic demand that requires a dynamic angiogenic process from early to late pregnancy to support its growth and function (Mayhew 2002; Torry et al. 2004; Reynolds and Redmer, 1995, 2001; Redmer et al. 2004; Reynolds et al. 2005a,b,c, 2010). Angiogenesis is regulated by numerous angiogenic factors including vascular endothelial growth factor (VEGF) family, fibroblast growth factor 2 (FGF2, also known as basic fibroblast growth factor), angiopoietins (ANGPT), nitric oxide (NO) system and other factors (Reynolds and Redmer, 1998; Reynolds et al. 2005a,b,c, 2010). Moreover, placental angiogenesis is abnormal at mid to late period in compromised pregnancies (Reynolds et al. 2005a,b,c, 2006, 2010; Meyhew et al. 2004; Burton et al. 2009). Very limited information is available concerning changes in vascular development and expression of angiogenic factors during early pregnancy.

We hypothesized that proliferation of vascular cells, and vascular growth and development will begin to change very early in pregnancy. Therefore, the purpose of the present study was to determine the pattern of vascular cell proliferation and vascular growth. These data will serve as an important reference to study placental developmental defects in compromised pregnancies in the future.

Procedures

Animals and tissue collection. The Institutional Animal Care and Use Committee at NDSU approved all animal procedures in this study. Gravid uteri were obtained from crossbred Western Range (primarily Rambouillet, Targhee, and Columbia crossbreeds) ewes (n = 6 to 8 per day) on days 14, 16, 18, 20, 22, 24, 26, 28, and 30 after mating (day of mating = day 0), and also from mid-luteal, nonpregnant (day 10 after estrus; n = 8) control ewes. For immunohistochemical staining, at tissue collection, specimen pins were inserted completely through the uterus and fetal membranes at the level of the external intercornual bifurcation to maintain specimen morphology; cross sections of the entire gravid uterus (0.5 cm thick) were obtained using a Stadie-Riggs microtome blade, immersion-fixed in Carnoy's solution, and embedded in paraffin.

Histochemistry and immunohistochemistry. Histochemical and immunohistochemical procedures were used as described before (Borowicz et al. 2007; Grazul-Bilska et al. 2008, 2009). Briefly, paraffin-embedded tissues were sectioned at 5 μm, mounted onto glass slides, rinsed several times in PBS containing Triton-X100 (0.3%, v/v), and treated for 20 min with blocking buffer [PBS containing normal goat serum (2%, vol/vol)]. The tissue sections were then incubated with specific primary antibody for proliferating cell nuclear antigen (PCNA; 1:500 dilution; monoclonal mouse; Zymed, San Francisco, CA) or factor VIII (1:100; rabbit polyclonal antibody, Sigma, St. Louis, MO) overnight at 4˚ C. Primary PCNA or factor VIII antibodies were detected by using biotin-

labeled secondary anti-mouse or anti-rabbit antibodies, respectively, and the ABC method (Vector Laboratories Burlingame, CA). For PCNA staining, the sections were then counterstained with hematoxylin and periodic acid-Schiff's reagent (H plus PAS). Control sections were incubated with normal mouse or rabbit serum in place of PCNA or factor VIIII primary antibody, respectively.

Image analysis. Image analysis was performed as described in detail before (Grazul-Bilska et al. 2009; Borowicz et al. 2007). Images of randomly chosen areas of intercaruncular (ICAR) and caruncular (CAR; 5-10 per uterine section/sheep; 0.025 mm^2 per field) stained for PCNA or factor VIII were taken at 600x (PCNA) or 400x (factor VIII) magnification, using an Eclipse E600 Nikon microscope and digital camera. Vascular labeling index (LI; proportion of proliferating cells within blood vessels), vascularity, and relative expression of factor VIII were determined by using computerized image analysis (Image-Pro Plus, version 5.0; Media Cybernatics, Houston, TX). The following vascularity measurements were determined for endometrial CAR and ICAR: the number of proliferating nuclei along with the total number of nuclei within blood vessels per tissue area to determine vascular LI, capillary area density (CAD; total area occupied by capillaries expressed as a proportion per unit of tissue area), capillary number density (CND; total number of capillaries per unit of tissue area), capillary surface density (CSD; total capillary circumference per unit of tissue area), area per capillary (APC; average cross-sectional area per capillary, which represents average capillary size) and the percentage of the total tissue area that exhibited positive staining for factor VIII.

Statistical analysis. Data were analyzed using the general linear models (GLM) procedure of SAS, with the main effect of day of pregnancy (SAS, 2008), and are presented as means ± SEM. When the F-test was significant ($P<0.05$), differences between specific means were evaluated by using the least significant differences test (Kirk 1982).

Results

Proliferating cells marked by PCNA, and the network of blood vessels marked by H plus PAS staining or factor VIII were localized in CAR and ICAR regions of endometrial tissues in nonpregnant and pregnant animals. By day 18 after mating, the endometrial luminal epithelium was beginning to flatten, becoming low cuboidal to squamous, compared with that observed on earlier days of pregnancy (days 14 and 16), which were primarily columnar. In addition, by day 18, the subepithelial capillary plexus, which we have previously described (Reynolds and Redmer, 1992; Reynolds et al. 2005a), was beginning to form, and by day 28 it was quite well developed. Extensive vascularization of the endometrial CAR and ICAR tissues began as early as day 18 and was extensive by day 28 after mating. In addition, in some of the ewes, microscopic fetal and maternal placental villi were evident by day 30 and were beginning to vascularize. In contrast, on days 14 and 16 after mating, these pregnancy-induced changes in endometrial morphology or vascular development were not yet evident. The pattern of changes in vascularity and vascular development was similar in CAR and ICAR, therefore, data for vascular measurements including LI, CAD, CND, CSD and APC were combined for CAR and ICAR. Several measurements of vascular growth in CAR and ICAR areas including LI in the blood vessel (Fig. 1A), CAD (Fig. 1B), CND (Fig. 1C), CSD (Fig. 1D), APC (Fig. 1E), and expression of factor VIIII (Fig. 1F) changed dramatically ($P<0.0001$-0.05) compared to nonpregnant controls or from day 14 to day 30 of pregnancy. Compared to nonpregnant controls, vascular LI increased ($P<0.0001$) 7 to 20-fold on days 14 to 20, and 23 to 34-fold on days 22 to 30 of pregnancy (Fig. 1A). Vascular LI for the non-pregnant controls was 0.71 ± 0.12% in CAR and ICAR. Compared to nonpregnant controls, CAD increased ($P<0.001$) 1.3-fold on day 16 and 1.5 to 1.9-fold on days 20 to 30 (Fig. 1B). Compared to nonpregnant controls, CND remained unchanged on days 14 to 20, and then decreased ($P<0.0001$) on days 22 to 30 of pregnancy (Fig. 1C). Compared to nonpregnant controls, CSD

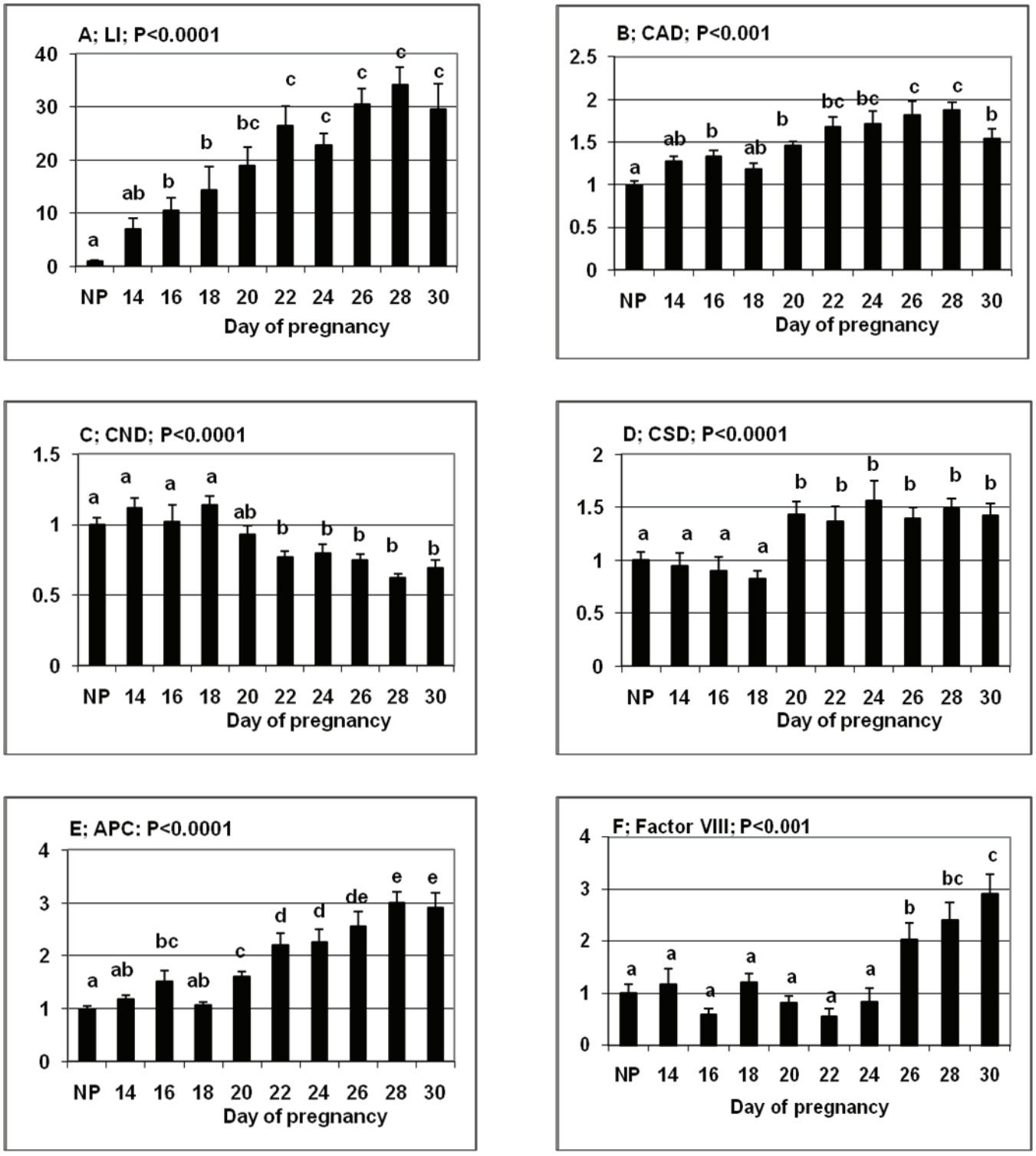

Fig. 1. Measurements of vascularity including vascular labeling index (LI; A), capillary area density (CDA; B), capillary number density (CND; C), capillary surface density (CSD; D), area per capillary (APC; E), and factor VIIII (F) for nonpregnant (NP) controls and on days 14 to 30 of pregnancy. [a,b,c,d,e]P<0.0001-0.001; values ± SEM with different superscripts differ within specific measurement. Data are expressed as a fold-change compared to NP control arbitrary set as 1.

increased (P<0.0001) 1.4 to 1.6-fold on days 20 to 30 of pregnancy (Fig. 1D). Compared to nonpregnant controls, APC increased (P<0.0001) 1.5-fold on days 16 and 22, and 2.2 to 3-fold on days 22 to 30 of pregnancy (Fig. 1E). Area per capillary in non-pregnant controls was 50 ± 3 μm^2. Compared to nonpregnant controls, expression of factor VIII increased (P<0.001) 2 to 3-fold on days 26 to 30 of pregnancy (Fig. 1F).

Labeling index was positively correlated with CAD (r^2 = 0.454; P<0.002), APC (r^2 = 0.577; P< 0.0001) and expression of factor VIII (r^2 = 0.486; P<0.001), and negatively correlated with CND (r^2 = -0.472; P<0.001).

Discussion

Uterine and placental growth, including vascular development is critical for successful pregnancy and production of healthy offspring. For the placenta of many mammalian species, a close relationship exists between fetal weight, placental size and vascularity, and uterine and umbilical blood flows (Reynolds et al. 2005a,b,c). Therefore, the study of normal uterine and placental development, as presented here, is critical to establish the patterns of vascular growth and expression of angiogenic factors that will help to determine the causes and consequences of pregnancy failures in compromised pregnancies, and also to establish therapeutic strategies to rescue compromised pregnancies (Reynolds et al. 2006, 2010; Burton et al. 2009).

The present study demonstrated that angiogenesis is initiated very early in pregnancy, since an increase in vascular LI was first observed on day 14 of pregnancy, and several measurements of angiogenesis including CAD, CSD and APC, and expression of several angiogenic factors increased by day 16 of pregnancy. The most dramatic increase was observed for vascular LI during days 14 to 20 of pregnancy and this was maintained until day 30. This enhanced vascular cell proliferation likely allows for rapid blood vessel growth, which was reflected by the increase of CAD, CSD and APC seen in endometrium. Thus, the time of the major increase in vascular cell proliferation corresponds to the period of maternal recognition of pregnancy, initial attachment/implantation of fetal membranes to uterine epithelium and initiation of placental growth and development (Bowen and Burghardt, 2000; Spencer et al. 2004, 2007, 2008; Bazer et al. 2009). Numerous factors of fetal and uterine origin have been identified in the maternal recognition of pregnancy and implantation in sheep including interferon (IFN)-τ, glycoproteins, steroid hormones, prostaglandins, osteopontin, and growth factors and their receptors (Bowen and Burghardt, 2000; Spencer et al. 2004; Cammas et al. 2006; Weems et al. 2006; Bazer et al., 2009a,b). Some of these factors (e.g., FGF, HIF, prostaglandins, osteopontin) have been recognized as angiogenic factors (Reynolds and Redmer, 2001; Reynolds et al. 2002, 2005a,b, 2006; Bazer et al. 2009a,b). Thus, initiation of angiogenesis seems to be directly associated with maternal recognition of pregnancy and implantation. However, further study should be undertaken to identify which factors are involved in both pregnancy recognition signaling and initiation of angiogenesis.

Although the importance of vasculogenesis and angiogenesis during early pregnancy is well recognized (Pfarrer et al. 2001, 2006; Sherer and Abulafia, 2001; Mayhew 2002; Demir et al. 2007; Reynolds et al. 2010), limited data are available concerning the timing of initiation of placental angiogenesis. Our previous studies have demonstrated that endometrial cellular proliferation and microvascular volume increased from day 12 to day 18 or 24 of pregnancy and remained elevated, and the general pattern of changes in vascular architecture in maternal placenta during early pregnancy in sheep have been reported (Reynolds and Redmer, 1992, Zheng et al. 1996). In the current, more detailed study, we observed increased maternal placental vascular cell proliferation as early as day 14, increased expression of angiogenic factors by days 14 to 16, and changes in vascular architecture as early as day 18 of pregnancy. These minor discrepancies between previous and current experiments are likely due to the more sensitive and precise techniques we used for determination of vascular changes in this study (Borowicz et al. 2007).

For humans, angiogenesis associated with extensive vascular remodeling in endometrium is initiated during the first weeks of pregnancy and is completed around 20 weeks of gestation (Huppertz and Peeters, 2005; Arroyo and Winn, 2008). Increased endometrial endothelial cell proliferation and blood vessel diameter were observed on week 2 of pregnancy in the marmoset (Rowe et al. 2004). In rats, a dramatic increase in endothelial cell proliferation index was observed as early as on day 3 of pregnancy; however, endothelial cell density did not change in endometrium (Goodger and Rogers, 1995). Based on the results presented above, it is reasonable to postulate that maternal recognition of pregnancy and implantation involves initiation of angiogenesis manifested first by increased vascular cell proliferation followed by enhanced vascularization and expression of angiogenic factors in maternal placenta.

The pattern of blood vessel growth during early pregnancy in this study resembles the pattern from mid to late pregnancy described before (Borowicz et al. 2007) except that during early pregnancy the number of blood vessels per unit tissue area decreased perhaps reflecting the large increase in capillary size. As pregnancy progresses, both area per capillary and number of blood vessels per tissue area increased moderately (Borowicz et al. 2007). Additionally, during early pregnancy, the expression pattern of changes for some of angiogenic factors differs (e.g., NOS3, ANGPT1, ANGPT2, HIF) and for some resembles (e.g., VEGF, VEGFR1, VEGFR2, ANGPTR Tie-2, FGF2) the pattern from mid to late pregnancy (Borowicz et al. 2007). This indicates a different role of specific angiogenic factor in regulation of blood vessel growth and function at different stages of pregnancy in sheep. However, this subject should be further investigated.

Implications

In summary, in this study, we have shown changes in vascular architecture, vascular cell proliferation and expression of factor VIIII. Since the pattern of changes in vascular development parallels expression of several angiogenic factors (Grazul-Bilska et al., 2009b), it indicates a complex regulation of these angiogenic processes. In addition, we know the increase of blood flow is closely associated with angiogenesis (Reynolds et al. 1984; Reynolds and Redmer, 1995). Thus, angiogenesis is crucial in developing and building the placental life line between the maternal and fetal systems and when affected, fetal growth and development are also affected (Carter and Charnock-Jones, 2001; Wulff et al. 2003; Arroyo et al. 2008; Fraser and Duncan, 2009). In fact, abnormalities of placental angiogenesis and expression of angiogenic factors are associated with a variety of compromised pregnancies, including those resulting from environmental stress, maternal iron deficiency, undernutrition or overnutrition, pre-eclampsia, gestational diabetes, application of assisted technologies, and other factors (Reynolds et al. 2006, 2010; Arnold et al. 2008; Stillerman et al. 2008). Therefore, data from our study may help to identify factors that can be used therapeutically to restore normal placental vascular function and blood flow to rescue compromised pregnancies. In addition, these data will help to compare the patterns of vascularization and expression of angiogenic factors, and timing of angiogenesis initiation in compromised pregnancies vs. normal pregnancy in future studies.

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