Expression, localization and hormonal control of angiopoietin-1 in the rhesus macaque endometrium: potential role in spiral artery growth

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Angiopoietin-1 (Ang-1) is an important angiogenic factor that has not been thoroughly studied in the primate endometrium. We evaluated the endometrial expression of Ang-1 and its receptor, Tie2, during induced menstrual cycles in rhesus macaques. Tie2 expression was confined to the vascular endothelium without marked change during the cycle. However, Ang-1 expression varied considerably during the cycle. In the proliferative phase, Ang-1 was only expressed in the basal zone glands, and this expression was estradiol (E2) dependent. In the early- to mid-secretory phase, Ang-1 expression spread to the upper glands, luminal epithelium and the vascular smooth muscle cells (VSMC) of spiral arteries. In the late secretory phase, the signal disappeared from the glands but remained elevated in the VSMC of spiral arteries. Notably, there was a significant correlation between VSMC proliferation and Ang-1 expression in the VSMC of the spiral arteries. Progesterone (P) withdrawal in the early secretory phase induced a decline in Ang-1 expression in the glands and VSMC of spiral arteries along with a complete suppression of VSMC proliferation. These data suggest, for the first time, that Ang-1 may play a key role in the P-dependent growth of the unique spiral arteries in the primate endometrium.

Key words: angiopoietin/endometrium/rhesus monkey/spiral artery/Tie2

Introduction

The primate endometrium undergoes remarkable vascular remodelling during the menstrual cycle and pregnancy. These processes involve both angiogenesis and arteriogenesis. The spiral arteries develop from the radial arteries of the myometrium and course through the endometrium, where they develop their coiled structure and vascularize primarily the upper endometrial zones. These spiral arteries are unique to the primate endometrium, and their growth is primarily driven by progesterone (P) in the secretory phase of the menstrual cycle and pregnancy (Bartelmez, 1957; Ramsey, 1982; Rogers and Abberton, 2003). At the end of a non-fertile cycle, when P levels fall, the spiral arteries severely constrict, leading to ischemia of the upper endometrial zones and their subsequent menstrual breakdown (Markee, 1950). During pregnancy, the trophoblasts invade the spiral arteries and replace their internal lining, thereby regulating the vascular resistance and blood flow to the placenta and fetus (Zhou et al., 1998; Blankenship and Enders, 2003). Lack of such changes in the spiral arteries has been postulated to be a major etiological factor for several diseases associated with pregnancy, including pre-eclampsia, which represents a major cause of maternal and fetal morbidity and mortality (Zhou et al., 1998). Despite the clinical importance of these vessels, their cellular and molecular control mechanisms are poorly understood.

Pericytes are essential for the maturation of newly formed vessels during angiogenesis. Subsequently, the vessels acquire a coat of vascular smooth muscle cells (VSMC) which facilitates vascular function (Carmeliet, 2000). However, the mechanisms of VSMC recruitment by endometrial capillaries are unknown. Several reports show the roles of a novel family of angiogenic factors, the angiopoietins (Angs), in regulating the recruitment of VSMC precursors during arterial development in different systems (Hanahan, 1997; Yancopoulos et al., 2000; Loughna and Sato, 2001). Angs regulate endothelial cell functions by activating or inhibiting the activation of Tie2, a tyrosine kinase receptor (Yancopoulos et al., 2000; Loughna and Sato, 2001). Two members of this family of ligands, Ang-1 and Ang-2, have been studied extensively in several models of angiogenesis. Ang-1 promotes angiogenesis, especially including vessel maturation and recruitment of VSMC (Suri et al., 1996; Yancopoulos et al., 2000). Ang-1 or Tie2 knockouts in mice result in embryonic lethality because of severe vascular abnormalities characterized by disturbance in endothelial cells and pericyte interactions (Suri et al., 1996). Although Ang-1 and Ang-2 share 60% homology in protein structure, Ang-2 is a naturally occurring competitive antagonist of Ang-1 and is believed to play a role in destabilizing the vessels before remodelling (Maisonneuve et al., 1997; Witzenbichler et al., 1998). Ang-1 binding stimulates autophosphorylation of the kinase domain of Tie2. In contrast, Ang-2 does not stimulate Tie2 autophosphorylation and blocks Ang-1-mediated Tie2 activation (Maisonneuve et al., 1997; Davis et al., 2003). Moreover, mice overexpressing Ang-2 show phenotypes similar to...
mice in which Ang-1 or Tie2 have been knocked out (Suri et al., 1996; Maisonnier et al., 1997). These studies indicate that Ang-Tie2 interactions play critical roles in remodelling and maturation of embryonic vessels. The expression pattern of Angs and Tie2 during ovarian folliculogenesis and cutaneous wound healing also suggest important roles of the Tie2 pathway in development and remodelling of neovessels in adult vasculature (Wong et al., 1997; Kampfer et al., 2001).

There have been reports on the expression and regulation of Angs and Tie-2 in the human endometrium, but these studies have not led to agreement on the role of Angs in this tissue (Rogers and Abborton, 2003). For example, Li et al. (2001) using in situ hybridization (ISH) could not detect expression of Ang-1 in any cell type during the cycle, whereas Hewett and colleagues reported abundant Ang-1 mRNA expression in glands and stroma during the proliferative phase (Hewett et al., 2002). By immunohistochemistry, Krikun et al. (2000) showed weak Ang-1 expression in the stroma and endothelial cells without noticeable changes during the cycle, while Hewett et al. (2002) reported that Ang-1 protein was present in all cell types and was markedly up-regulated during the proliferative phase. On the other hand, Hirchenhain et al. (2003), with different antibodies, found up-regulation of Ang-1 during the secretory phase. Arterial expression of Ang-1 was not described in any of these studies.

In this study, to provide precise control of hormonal conditions, we treated ovariectomized rhesus macaques sequentially with estradiol (E2) and P with Silastic implants to induce menstrual cycles, as previously reported (Nayak et al., 2000; Germeyer et al., 2005). We obtained full-thickness endometrial samples to examine Ang-1 and Tie-2 expression in all endometrial zones in a variety of hormonal conditions including the premenstrual/menstrual, proliferative and secretory phases of the induced cycle, after hormone deprivation (without E2 and P) and P withdrawal after a short term (3 days) exposure to P. We observed that Ang-1 mRNA was expressed in the glandular epithelium, luminal epithelium and the VSMC of spiral arteries and that its expression changed during the cycle in a cell-type-specific manner. Notably, Ang-1 expression in the VSMC of the spiral arteries was highly correlated with their P-dependent proliferative activity. These results implicate a potential role for Ang-1 in spiral artery growth in the primate endometrium.

Materials and methods

Experimental animals

Animal care and handling were provided by the veterinary staff of the Oregon National Primate Research Center and the California National Primate Research Center, in accordance with the National Institutes of Health Policy on the care and use of non-human primates under approved protocol by the respective Primate Center Institutional Animal Care and Use Committee. Thirty-two sexually mature (6–12 years) rhesus macaques (Macaca mulatta) were ovariectomized. Each animal was treated sequentially with 3 cm Silastic capsules (Dow Corning Corp., Midland, MI, USA) containing E2 for 14 days and then E2 plus P (6 cm Silastic capsules) for additional 14 days to induce artificial cycles, as described previously (Nayak et al., 2000; Germeyer et al., 2005). To create an artificial proliferative phase, menstruation was induced by withdrawal of the P implant, whereas the E2 implant remained in place. Usually, menstruation begins after 2 days of P withdrawal. The uteri were obtained from different groups of animals after different days of P withdrawal: premenstrual/menstrual phase (2–3 days after P withdrawal, n = 4), early proliferative phase (5–6 days after P withdrawal, n = 4), mid-proliferative phase (8–10 days after P withdrawal, n = 4) and late proliferative phase (14 days after P withdrawal, n = 3). To induce the secretory phase, P implants were reintroduced into a second set of animals on day 14 of the proliferative phase, and uteri were obtained after different days of P treatment: early secretory phase (3 days after E2 + P treatment, n = 3), mid-secretory phase (7–8 days after E2 + P treatment, n = 3) and late secretory phase (14 days after E2 + P treatment, n = 3). In another set of animals, we obtained endometrial samples 10–14 days after removal of both the E2 and P implants at the end of the artificial cycle (hormone deprived, HD; n = 3). In a final group (early P withdrawal, n = 3), we evaluated the effects of P withdrawal on Ang-1 expression after a shorter term treatment with P. In this group, animals were treated for 14 days with E2, followed by 3 days of E2 plus P to induce early progestational changes in the rhesus endometrium. After this brief P treatment, the P implants were removed (with the E2 implant remaining in place), and the endometrium was collected 2 days later. This ‘early P withdrawal group’ permits assessment of whether the changes in Ang-1 expression seen in the early secretory phase were E2 or P dependent.

Endometrial samples from all animals were collected by hysterectomy or during necropsy, as described previously (Nayak et al., 2000; Germeyer et al., 2005). Full-thickness cross-sections (from luminal epithelium to the myometrium) from two uterine quarters were cut and prepared for immunohistochemistry (ICC) and ISH studies. Tissues for ICC were microwave stabilized, mounted in Optimal Cutting Temperature (OCT) (Miles Inc., Elkhart, IN, USA) and frozen in liquid propane. The samples for ISH were similarly frozen without microwave treatment. Endometrium from the remaining quarters were separated from the myometrium and used for protein and RNA extraction. Serum samples were collected during tissue collection to confirm normal circulating levels of E2 and P delivered by the implants (Nayak et al., 2000).

ICC

ICC for Ki-67 (to detect proliferating cells), alpha smooth muscle actin (αSMA; to detect VSMC) and Tie2 was performed in full-thickness frozen sections (7 μm) of endometrium, as described previously (Nayak et al., 2000; Germeyer et al., 2005). Briefly, cryostat sections were thaw-mounted on SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA, USA) and microwaved for 2 s on ice (Nayak et al., 2000). The microwave-treated sections were lightly fixed in 0.2% picric acid and 2% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min. To inhibit endogenous peroxidase activity, the sections were incubated with a solution containing glucose oxidase (1 unit/ml), NaAzide (1 mM) and glucose (10 mM) in PBS for 45 min. Sections were then incubated with blocking serum for 20 min and then with the mouse monoclonal primary antibody for Ki-67 (1:300, BioGenex, San Ramon, CA, USA) or αSMA (1:1000, DakoCytomation California Inc., Carpinteria, CA, USA) or Tie-2 (1:60, gift from Regeneron Pharmaceuticals, Tarrytown, NY, USA) overnight at 4°C. The primary antibody was replaced with either PBS with 1.0% bovine serum albumin (BSA) or mouse non-immune immunoglobulin G (IgG) as two different negative controls. After rinsing and blocking, the sections were incubated with a biotinylated second antibody (anti-mouse) for 30 min at room temperature. Final brown immunostaining was achieved by peroxidase/3,3'-diaminobenzidine (DAB) with the ABC kit from Vector Laboratories (Burlingame, CA, USA). The slides were rinsed several times in deionized water and lightly counterstained with hematoxylin to facilitate identification of cell types.

Analysis of the abundance of Ki-67 positive nuclei in VSMC of endometrial arteries was assessed on 10–20 arteries selected randomly in the upper and lower functionalis zones. The total number of VSMC nuclei and the number of VSMC nuclei that were clearly Ki-67 positive were counted, as described previously, for endothelial cells (Nayak and Brenner, 2002; Germeyer et al., 2005). The percent of Ki-67-positive VSMC nuclei were then calculated, and differences in the percentage of positive cells among the animals in each treatment group were analysed using one-way analysis of variance and t-test.

Western Immunoblots

Tissue lysates from proliferative and secretory phase endometrium were prepared and subjected to western blot, as described previously (Lathi et al., 2005). Briefly, protein concentration in each sample was determined using the Bradford protein assay (BioRad, Hercules, CA, USA). Samples (30 μg) were separated on a 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (Gradipore, San Diego, CA, USA) and semidy electrobotted onto a nitrocellulose membrane (Schleicher & Schuell Bioscience, Keene, NH, USA). The membrane was blocked in Tris-buffered saline-T (TBS-T) (20 mM Tris–HCl and 500 mM NaCl, pH 7.60, 0.1% Tween) containing 5% non-fat dry milk, washed in TBS-T, incubated overnight at 4°C with the primary Ang-1 rabbit polyclonal antibody (1:500, gift from Regeneron Pharmaceuticals) in TBS-T containing 5% non-fat dry milk. Non-immune IgG was used as the negative control. After several washings, antibody binding was
detected using a goat anti-rabbit- peroxidase conjugate and enhanced chemiluminescence Plus (ECL Plus) detection system (Amersham Bioscience, Little Chalfont, Buckinghamshire, UK) and exposed to X-ray films (Eastman Kodak, Rochester, NY, USA).

**RNA extraction and RT–PCR**

Total RNA from endometrial samples was extracted using Trizol (Invitrogen, Carlsbad, CA, USA), following the manufacturer’s protocol. The RNA was treated with deoxyribonuclease and purified by RNasey Spin Columns (QIAGEN, Valencia, CA, USA), as described previously (Tulac et al., 2003; Germeyer et al., 2005). About 2 μg of purified RNA was reverse transcribed using Omniscript kit (QIAGEN), according to the manufacturer’s instructions. The reverse transcription product was amplified in a standard PCR using the same Ang-1 and Tie-2 primer pairs used by Hazzard et al. (2000). The amplified product was isolated in agarose gel and subcloned into pGEM-T vector (Promega Corp., Madison, WI, USA). At least two clones with the right-sized inserts were miniprepped (5′-3′; Perfect Preps, Boulder, CO, USA) and were sequenced on an ABI 373 XL sequencer (Applied Biosystems, Foster City, CA, USA) to confirm the sequences of each PCR product.

**Real-time PCR**

Real-time PCR for Ang-1 was performed in the Mx4000 Q-PCR system (Stratagene, La Jolla, CA, USA) using the QuantiTect SYBR Green PCR Kit (QIAGEN), following the manufacturer’s instructions (Tulac et al., 2003; Lathi et al., 2005). The same Ang-1 primer pairs and procedures for generation of RT products were used, as indicated above. Eighteen seconds were used as a reference gene using commercially available primer pairs (Lathi et al., 2005). The RT products were diluted 200 times for 18 s based on best PCR amplification efficiency. All assays were optimized for primer concentration and PCR product specificity based on melting curve analysis using the Mx4000 software and gel electrophoresis, as described previously (Tulac et al., 2003). The thermal cycling conditions were an initial activation step at 95°C for 10 min, followed by 40 cycles of denaturation (95°C for 30 s), annealing (60°C for 1 min) and amplification (72°C for 1 min). PCR products were analysed by thermal dissociation (55–95°C) with a fluorescence measurement at every 1 degree increment. Appropriate controls including no-template and no-RT controls were included for each sample. Final PCR products were verified by agarose gel electrophoresis for each sample and negative controls. Amplification efficiency and correlation coefficients for Ang-1 and 18 s were determined from serially diluted RT products using the Mx4000 software. No primer–dimer formation for Ang-1 and 18 s were observed during the 40 PCR amplification cycles. Relative expression of Ang-1 (gene of interest) in different hormonal conditions during the secretory phase was calculated compared with its relative expression during the late proliferative phase after correction with 18 s (reference gene). The relative expression ratio (R) of the gene of interest was calculated based on the corresponding efficiencies of amplification for each gene and the differences in threshold cycle values (Ct) using the mathematical model, as described previously (Tulac et al., 2003; Lathi et al., 2005). Ct values were calculated by the Mx4000 software based on fluorescence intensity values after normalization with an internal reference dye and baseline correction.

**ISH**

ISH of full-thickness frozen sections of endometrium was conducted with a [35]SUTP (NEN Life Science Products, Boston, MA, USA)-labelled sense and antisense riboprobes generated from the rhesus monkey-specific Ang-1 and Tie-2 cDNAs (Hazzard et al., 2000). Techniques for ISH and riboprobe synthesis and labelling were previously published (Nayak and Brenner, 2002; Germeyer et al., 2005). Briefly, riboprobes were generated with the MAXiscript in vitro transcription kit from Ambion (Austin, TX, USA) and purified by mini Quick Spin TM RNA Columns (Roche Applied Science, Indianapolis, IN, USA), following the manufacturer’s instructions. About 10 μm thick frozen sections were mounted on Super Frost Plus slides (Fisher Scientific) and fixed in 4% paraformaldehyde in PBS for 10 min at 4°C. The tissue sections were rinsed in 2× saline sodium citrate (SSC), acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min and then air-dried. At this point, at least one slide per tissue group was treated with RNase A (20 μg/ml RNase A, 0.5 M NaCl, 0.01 M Tris and 1 mM EDTA (pH 8.0)) as a negative control. Sections were then incubated at 55°C overnight in 10 mM dithiothreitol (DTT), 0.3 M NaCl, 20 mM Tris, pH 8.0, 5 mM EDTA, 1× Denhardt’s solution, 10% dextran sulphate and 50% formamide containing the appropriate concentration of the sense and antisense probe (about 5 million cpm/ml). After hybridization, all slides were treated with RNase A at 37°C for 30 min to inactivate non-hybridized probe. Sections were dehydrated in alcohol, vacuum dried, coated with NTB2 autoradiographic emulsion (Eastman Kodak Co., Rochester, NY, USA), stored at 4°C for 7 days, developed in aqueous D-19 (Eastman Kodak Co.), lightly counterstained with hematoxylin, dehydrated in alcohol, cleared with xylene and coverslipped with Permount (Fisher Scientific).

Silver grains were counted over VSMCs of arteries in sections hybridized with Ang-1 radiolabelled probe. Identification of VSMCs in these preparations was confirmed by ICC of αSMA in adjacent sections from the same block. Equal numbers of arteries (8–12) from lower and upper zones were randomly selected for grain count as described previously for other endometrial cell types (Nayak and Brenner, 2002; Germeyer et al., 2005). The counts were made with MetaMorph (Universal Imaging Corp., Downingtown, PA, USA) on images captured by a CoolSNAP color CCD digital camera (Roper Scientific Inc., Tucson, AZ, USA). The abundance of silver grains was expressed as the number of grains per cell. These counts were then expressed as a percentage of the maximum signal in all the sections analysed.

**Statistical analysis**

Data from real-time PCR. ISH grain counts and percent of Ki-67 positive VSMCs were analysed by one-way ANOVA, followed by appropriate post hoc tests (Nayak and Brenner, 2002; Germeyer et al., 2005). P values below 0.05 were considered significant. Correlations between different attributes and coefficients of simple determination ($R^2$) were calculated using the StatView software (SAS Institute, Cary, NC, USA).

**Results**

**Ang-1 expression in the rhesus macaque endometrium**

The relative abundance of Ang-1 protein and transcripts in the rhesus macaque endometrium were analysed by western blot and real-time PCR, respectively. Figure 1A is a representative western blot of Ang-1 protein (∼68 kDa) showing that this protein was markedly up-regulated during the early- to mid-secretory phase. Consistent with the western blots, real-time PCR analysis indicated that Ang-1 transcripts were significantly increased during the early (P < 0.05)- and the mid (P < 0.01)-secretory phase (Figure 1B). These increases were ∼7- and ∼3-fold in the early- and mid-secretory phase, respectively, compared with the late secretory phase. Ang-1 mRNA was significantly lower (∼12-fold, P < 0.01) during the mid-secretory phase levels. The increased signal during the early- and mid-secretory phases was also significantly (P < 0.05) reduced after early P withdrawal (Figure 1B). There was no significant difference in Ang-1 mRNA expression between the late proliferative and early P withdrawal groups (Figure 1B).

**Ang-1 mRNA cellular localization during the menstrual cycle**

Ang-1 mRNA cellular localization was examined in ISH preparations from different stages of the cycle (Figures 2 and 3). Full-thickness ISH preparations showed that during the proliferative phase, the Ang-1 mRNA was only expressed in the glands of the basalis zone (Figure 2A). During the early secretory phase, the signal was expressed in most middle zones glands (in addition to the basal glands) (Figure 3A). In the mid-secretory phase, glands in all zones of endometrium including the luminal epithelium showed strong signals, with a gradient in expression from highest at the basal zone to least in the luminal epithelium (Figure 2B). By the late secretory phase, the signal became non-detectable in the glands (Figure 2C). During the proliferative phase, the VSMCs of most spiral arteries in the endometrium showed no detectable Ang-1 signal (Figure 2D and G).
declined significantly (<i>P</i> < 0.05) in the mid-secretory phase, increased through the mid-secretory phase and then also decreased (<i>P</i> < 0.05) in the early-secretory phase, further up-regulated (<i>P</i> < 0.05) in the mid-secretary phase, and, unlike the Ang-1 signals in the glands, was maintained at a high level through the late secretory phase (Figure 5).

**Strong correlation of Ang-1 mRNA and Ki-67 expression in spiral arteries**

Proliferating VSMC in spiral arteries were identified by ICC for Ki-67 and α-smooth muscle actin (α-SMA) in adjacent sections. VSMC proliferation in these arteries was low or non-detectable in the proliferative phase and in HD animals (Figure 5). Following P treatment, there was a dramatic increase in the percentage of proliferating VSMC during the early- to late-secretory stage (Figures 2K and L and 5). This increase in proliferation coincided with the P-dependent increase in Ang-1 expression in the spiral arteries. Also, in the VSMC of spiral arteries, the Ki-67 labelling index was statistically significantly correlated with the Ang-1 grain counts (<i>R</i><sup>2</sup> = 0.853, <i>P</i> < 0.001). However, there was no significant correlation between the Ki-67 labelling index in VSMC of spiral arteries and Ang-1 grain counts in upper (<i>R</i><sup>2</sup> = 0.083, <i>P</i> < 0.092) or basal (<i>R</i><sup>2</sup> = 0.013, <i>P</i> < 0.501) zone glands (data not shown).

**Effect of P withdrawal after early secretory phase on Ang-1 mRNA expression and VSMC proliferation**

To further support the view that the increases in glandular and spiral artery Ang-1 mRNA were P dependent, we treated animals for only 3 days with <i>E</i><sub>2</sub> + <i>P</i>, then withdrew <i>P</i> and left the <i>E</i><sub>2</sub> implant in place. Real-time PCR analysis revealed that there was a significant overall decrease of Ang-1 mRNA in the endometrium in this early P-withdrawal group compared with the early secretory phase (Figure 1B). The ISH data revealed that this fall was because of a decline in expression of Ang-1 by the upper zone glands (Figure 3B) and the VSMC of spiral arteries (Figure 3D), though there was persistent expression of a low signal in the glands of the basalis (Figure 3B). VSMC proliferation in the spiral artery wall, as evidenced by ICC of Ki-67, was also dramatically suppressed after early P withdrawal compared with the early secretory phase (Figure 3H). These comparisons provide additional evidence that the increases in Ang-1 mRNA in the upper glands and VSMC as well as the increases in proliferation of the VSMC that occur during the early secretory phase depend on <i>P</i>, not <i>E</i><sub>2</sub>.

**Tie-2 cellular localization**

Figure 6 presents representative photomicrographs showing expression of Tie-2 mRNA (ISH, Figure 6A–D) and protein (ICC, Figure 6E–H) in the endometrium. Tie-2 mRNA and protein expression were confined to the endothelium of arteries and veins, not in the glands or stroma, and there were no marked changes in expression throughout the cycle.

**Discussion**

**Hormonal regulation of Ang-1 mRNA**

Our results suggest that the up-regulation of Ang-1 in the basalis during the proliferative phase is estrogen dependent, as such up-regulation did not occur in absence of <i>E</i><sub>2</sub> in HD animals. Similarly, a recent study shows stimulation of Ang-1 expression in the uterine glandular epithelium of ovariectomized rats (Goldman et al., 2004) after treatment with the estrogenic compound, methoxychlor. However, during the secretory phase, the marked increases in expression of Ang-1 in the upper glands, luminal epithelium and spiral arteries are <i>P</i> dependent, not <i>E</i><sub>2</sub> dependent, because P withdrawal (in the presence of <i>E</i><sub>2</sub>) after the early secretory phase led to loss of these signals in the glands, luminal epithelium and arteries (Figure 3). Moreover, as indicated in the methods, the premenstrual/menstrual phase results from...
Figure 2. Cellular localization of angiopoietin-1 (Ang-1) mRNA in the rhesus macaque endometrium. The first three rows (A–I) are Ang-1 in situ hybridization (ISH) preparations, and the fourth (J–L) and fifth (M–O) rows are immunocytochemistry (ICC) preparations for Ki-67 and alpha smooth muscle actin (αSMA), respectively. The photomicrographs in the first row are full-thickness endometrium (original magnification, ×35); a white line is drawn to demarcate separation of the endometrium from the myometrium. Le, luminal (surface) epithelium; Gl, gland; S, stroma and arrow heads, spiral arteries. All other photomicrographs (D–O) are magnified view (original magnification, ×560) of spiral arteries; D–E are darkfield images, and G–I are corresponding bright field images. Note that Ang-1 mRNA is strongly up-regulated in the glands, luminal epithelium and spiral arteries during the mid-secretory phase (B, E and H). During the late secretory phase, the signal remains elevated in the spiral arteries but is non-detectable in glands and luminal epithelium (C, F and I).
P withdrawal from the late secretory phase (in the presence of E2) and the low level of signals in the arteries in these samples further indicates that arterial Ang-1 mRNA expression is P dependent. However, it is not clear why glandular Ang-1 declines during continued P treatment through the late secretory phase while arterial Ang-1 expression persists. The answer may lie in the well-established observation that epithelial P receptors (PR) decline in the glands but are maintained in the stroma during the continuous P exposure typical of the luteal phase (Lessey, 2003; Hess et al., 2005). The stimulatory actions of P on Ang-1 may be mediated by glandular PR and would therefore decline when these epithelial receptors fall during the late secretory
Although the role of P is clear on the expression of Ang-1 in the spiral arterial wall, further studies are required to understand the mechanisms of P action on these vessels. There is uncertainty in the literature as to the specific cell type that expresses PR in the arterial wall (Critchley et al., 1994; Rogers et al., 1996; Nayak et al., 1998; Brenner and Slayden, 2004), probably because of the close spatial relationships between arterial smooth muscle cells and perivascular myofibroblasts. However, most reports state that PR are expressed in the VSMC and/or perivascular stromal cells throughout the cycle (Critchley et al., 1994; Rogers et al., 1996; Nayak et al., 1998; Brenner and Slayden, 2004), and the persistence of PR in these cells may explain the persistence of P-dependent vascular Ang-1 expression throughout the secretory phase. Regardless of the mechanism involved, the continuous expression of Ang-1 by the spiral arteries during the luteal phase points to the importance of this angiogenic factor in the physiology of these unique vessels.

**Cellular localization and hormonal regulation of Tie-2**

Like other tyrosine kinase receptors, Tie-2 is expressed predominantly by vascular endothelial cells (Hanahan, 1997; Yancopoulos et al., 2000; Loughna and Sato, 2001). Our ISH and ICC results are consistent with these observations and show that Tie-2 is exclusively expressed in the vascular endothelium of the rhesus macaque endometrium. Although there is no consensus among different studies in the human endometrium, most studies show expression of Tie-2 in the vascular endothelium, with minimal change during the cycle (Krikun et al., 2000; Li et al., 2001; Hirchenhain et al., 2003). Some authors reported Tie2 expression in the glandular epithelium as well (Krikun et al., 2000; Hewett et al., 2002; Hirchenhain et al., 2003), but Rogers and Abberton (2003) noted that the immunostaining of Tie-2 in the glandular epithelium of human endometrium may be because of non-specific binding of different polyclonal antibodies to a 60–70 kDa protein in glands, which is different from the 140 kDa Tie-2 protein in endothelial cells. We used a monoclonal antibody to localize Tie-2 protein in the monkey endometrium. With this antibody, Tie2 was only detected in the vascular endothelium, and its expression was not influenced by sex steroid hormones.

**Functional correlates of Ang-1 expression and spiral artery growth**

We found a significant correlation between VSMC proliferation and Ang-1 mRNA expression in the spiral arteries, but no such correlation with the Ang-1 produced by the glands. Several lines of evidence suggest that VSMC proliferation could be regulated by paracrine signals between Ang-1, made and secreted by VSMC and pericytes, and its endothelial cell-specific receptor Tie2 (Hanahan, 1997; Yancopoulos et al., 2000; Loughna and Sato, 2001). Mice lacking Ang-1 or Tie2 die in utero from vascular anomalies resulting from a lack of VSMC and pericytes around the blood vessels (Suri et al., 1996). Venous malformations in humans are associated with mutations in Tie2 (Vikkula et al., 1996). Furthermore, VSMC proliferation in spiral arteries (Abberton et al., 1999) and Ang-1 expression (Hewett et al., 2002) are significantly reduced in the endometrium of women with menorrhagia. Ang-1 expression is also reduced in women treated with long-term progestin-only contraceptives which is frequently associated with abnormal uterine bleeding (Krikun et al., 2002). Thus, the reduced Ang-1 level in these women may contribute to the inhibition of VSMC proliferation and destabilization of the spiral arteries leading to excessive endometrial bleeding (Abberton et al., 1999; Hewett et al., 2002; Krikun et al., 2002). Taken together, these results suggest that Ang1-Tie2 signalling may play a critical role in spiral artery growth and stabilization of vessels in the primate endometrium.
A balance between Ang-1 and -2 is believed to be critical in regulating angiogenesis and atherogenesis (Hanahan, 1997; Yancopoulos et al., 2000; Loughna and Sato, 2001). Several studies in the human endometrium (Krikun et al., 2000; Krikun et al., 2002; Hirchenhain et al., 2003) show expression of Ang-2 in the vascular endothelium without any marked changes during the cycle. Presumably, Tie2 activity would be normally suppressed by the constitutive presence of endothelial Ang-2. This suppression could be overcome by local increases in Ang-1 produced in the arterial wall, and such an interaction would induce increased proliferation of the VSMC. In the upper zones, glandular Ang-1 may counteract the suppressive effects of Ang 2 in the capillary endothelium and may stimulate pericyte recruitment to the smaller vessels in these zones. During the proliferative phase, strong Ang-1 expression in the glands of the lower zones may overcome Ang-2 suppression in the basal zone arteries and stabilize these vessels during the healing and repair processes subsequent to menstruation.

As noted in the Introduction, several studies have reported conflicting results on the localization of Ang-1 protein by ICC in the human endometrium (Krikun et al., 2000; Li et al., 2001; Hewett et al., 2002; Hirchenhain et al., 2003). We tried several commercially available antibodies to evaluate Ang-1 protein localization by ICC. Most of these antibodies were recommended by the manufacturer for use only in western blots and, in our hands, were not satisfactory as ICC reagents in the rhesus monkey endometrium. In fact, our Ang-1 western blot results were consistent with our mRNA results by real-time PCR and ISH and with the western blot results in the human endometrium which showed increased expression of Ang-1 during the early secretory phase (Hewett et al., 2002).

In summary, our data indicate that Ang-1 expression is regulated in a zone-specific and cell-type specific manner during the menstrual cycle in the non-human primate endometrium. The signal is confined to the basal zone glands during the proliferative phase, becomes markedly elevated in the upper zone glands and VSMC of spiral arteries during the early-to mid-secretory phase and is maintained in the VSMC of spiral arteries while it declines in glands during the late secretory phase. The strong and continued expression of Ang-1 in the spiral arteries throughout the secretory phase suggests, for the first time, that Angs play an important role in the physiology of these unique vessels.

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References


Ang-1 expression in the rhesus macaque endometrium


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