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Decidualized Human Endometrial Stromal Cells Mediate Hemostasis, Angiogenesis, and Abnormal Uterine Bleeding

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Abstract

Factor VII binds trans-membrane tissue factor to initiate hemostasis by forming thrombin. Tissue factor expression is enhanced in decidualized human endometrial stromal cells during the luteal phase. Long-term progestin only contraceptives elicit: 1) abnormal uterine bleeding from fragile vessels at focal bleeding sites, 2) paradoxically high tissue factor expression at bleeding sites; 3) reduced endometrial blood flow promoting local hypoxia and enhancing reactive oxygen species levels; and 4) aberrant angiogenesis reflecting increased stromal cell-expressed vascular endothelial growth factor, decreased Angiopoietin-1 and increased endothelial cell-expressed Angiopoietin-2. Aberrantly high local vascular permeability enhances circulating factor VII to decidualized stromal cell-expressed tissue factor to generate excess thrombin. Hypoxia-thrombin interactions augment expression of vascular endothelial growth factor and interleukin-8 by stromal cells. Thrombin, vascular endothelial growth factor and interleukin-8 synergistically augment angiogenesis in a milieu of reactive oxygen species-induced endothelial cell activation. The resulting enhanced vessel fragility promotes abnormal uterine bleeding.

Keywords

Tissue factor; endometrium; hemostasis; angiogenesis; bleeding

TISSUE FACTOR EXPRESSION BY DECIDUALIZED HUMAN ENDOMETRIAL STROMAL CELLS PROMOTES HEMOSTASIS

Infertile human menstrual cycles terminate in sloughing of the functional endometrial layer in the menstrual fluid. Restoration begins in the follicular phase of the next cycle as increased circulating estradiol (E₂) levels induce proliferation of epithelial and stromal cells. After ovulation, rising plasma progesterone levels block mitosis and initiate differentiation of the E₂-primed cells. In the luteal phase, the glandular epithelial cells secrete implantation-promoting products into the uterine lumen, the luminal epithelial cells become receptive to blastocyst attachment, and the stromal cells decidualize. The decidualization reaction is initiated around blood vessels and under the glands and then spreads throughout the luteal

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phase and gestational endometrium. Decidualization involves striking phenotypic changes including enhanced expression of proteins that promote hemostasis.¹⁻³

In situ hybridization and immunohistochemistry localized tissue factor (TF) messenger RNA (mRNA) and protein to decidualized stromal cells of luteal phase and gestational endometrium.⁴⁻⁶ A cell membrane-bound glycoprotein (molecular weight 46 kd) member of the class-2 cytokine receptor family, TF consists of a hydrophilic extracellular domain, which acts as a receptor for factor VII and its active form VIIa, a membrane-spanning hydrophobic domain, and a cytoplasmic tail. Binding of circulating factor VII to perivascular cell-bound TF8 initiates a complex series of changes that mediate the proteolytic cleavage of prothrombin to thrombin, which promotes hemostasis by forming fibrin.⁸⁻⁹

Blastocyst-derived extravillous trophoblasts (EVTs) penetrate the underlying decidua, then breach its capillaries to provide the embryo with oxygen and nutrients prior to placentation.¹⁰ Subsequently, EVT's traverse the decidua, where they breach and remodel spiral arteries and arterioles into high-capacitance vessels that increase blood flow to the intervillous space required for growth and development of the feto-placental unit.¹¹⁻¹² Expression of TF by decidual cells, the major cell type encountered by invading EVT's, counteracts the threat of hemorrhage by promoting local hemostasis during endovascular trophoblast invasion and subsequent vascular remodeling. Decidualized human endometrial stromal cells (HESCs) continue to express TF throughout pregnancy to provide hemostatic protection against the occurrence of hemorrhage during delivery.¹⁴

DECIDUALIZED HESC-EXPRESSED TF PROMOTES ANGIOGENIC RESTORATION OF ENDOMETRIAL VASCULATURE

Withdrawal of circulating progesterone leads to matrix metalloproteinases (MMPs)—mediated degradation of the peridecidual cell extracellular matrix (ECM) and basal lamina underlying endometrial epithelial and endothelial cells.¹⁵⁻¹⁶ These MMPs are derived primarily from resident stromal and epithelial cells¹⁷⁻¹⁸ as well as a marked infiltrate of neutrophils and uterine natural killer (uNK) cells that secrete cytokines to modulate MMP production in the epithelial and stromal cells.^{18,19} The resulting disruption of the structural integrity of the uterine stroma and luminal epithelium destabilizes the vasculature prior to sloughing of the functional endometrial layer.

Ovarian steroids indirectly regulate the cyclical angiogenic restoration of the vasculature lost in the menstrual fluid by inducing epithelial and/or stromal cells to release angiogenic effectors.²⁰⁻²¹ Among the several putative angiogenic factors identified in human endometrium,²²⁻²⁶ most studies have focused on vascular endothelial growth factor (VEGF)-A, the primary mediator of angiogenesis,²⁷ which is expressed cyclically by endometrial stromal and glandular epithelial cells.²⁸⁻³⁰ The premenstrual endometrium develops focal hypoxia in response to prostanoid and endothelin-induced vasoconstriction followed by reflex dilatation and ischemia.³¹⁻³² Hypoxia acts via the cis-acting hypoxia inducible factor (HIF)-1 α site on the VEGF gene promoter to augment VEGF mRNA transcription and stability in several cell types,³³⁻³⁵ prompting evaluation of ovarian steroids and hypoxia on VEGF expression in the predominant human endometrial cell types. We found that hypoxia markedly enhanced VEGF synthesis in primary cultures of both glandular epithelial and stromal cells. Consistent with the absence of estrogen and progestin response elements from the VEGF gene promoter,²⁷ the VEGF output by glandular epithelial or stromal cells was unaffected by incubation of glandular epithelial or stromal cell with E₂, a progestin, or E₂ plus progestin either under normoxia or under hypoxia.³⁶ Hypoxia-induced VEGF expression is a putative regulator of an initial wave of

angiogenesis, which occurs in the perimenstrual period prior to the elevation in circulating E₂ levels and involves early follicular phase repair of endometrial vessels.³⁷

The luteal phase endometrium exhibits a peak in VEGF expression^{38,39} that coincides with a second wave of angiogenesis manifested by increased endothelial cell proliferating activity and microvascular density as well as coiling of the spiral arteries.^{40–42} During the luteal phase, the endometrium also experiences enhanced vascular permeability associated with stromal edema,⁴³ which would provide access of circulating factor VII to decidual cell-expressed TF to generate thrombin. Figure 1 shows that incubation of in vitro decidualized HESCs with thrombin induces both time- (Figure 1A) and concentration-dependent (Figure 1B) increases in VEGF output. Thrombin (0.5 U/mL) elevated VEGF levels by several-fold after 48 hours (Figure 1C). By contrast, the human endometrial glandular epithelial cells were unresponsive to thrombin (Figure 1B). Mediation by the constitutively expressed protease-activated receptor-1 (PAR1) thrombin receptor in HESCs⁴⁴ is indicated in Figure 1D, which demonstrates that thrombin receptor activating peptide, a PAR1 agonist,⁴⁵ also upregulated the VEGF output in decidualized HESCs. Extrapolation of these in vitro results to the luteal phase endometrium suggests that induction of TF expression during progestin-induced decidualization of HESCs leads to steady state formation of thrombin. By acting as an autocrine/paracrine enhancer of VEGF expression in HESCs such HESC-derived thrombin can mediate angiogenesis in the luteal phase endometrium.

ABNORMAL UTERINE BLEEDING DURING LONG-TERM PROGESTIN-ONLY CONTRACEPTION IS ASSOCIATED WITH HIGH-STROMAL TF LEVELS

Long-term progestin-only contraceptives (LTPOCs) provide safe, discrete, and highly effective contraception. Formulations include Depo-Provera, an injectable form of medroxyprogesterone acetate whose actions persist for 3 months; Mirena, an intrauterine device that releases levonorgestrel for 5 years is currently available, whereas Norplant that releases levonorgestrel from subdermal rods has been removed from the market; Implanon, which releases etonogestrel from implantable rods over a 3-year period is widely used in Europe, Asia, and Australasia. Because Implanon is easy to insert and remove, it is ideal for use by women with limited access to trained medical personnel. In general, LTPOCs are safe to use during lactation and by patients in which estrogen use is contraindicated. Abnormal uterine bleeding (AUB) occurs in the majority of users. Such AUB as a source of personal annoyance and discomfort as well as religious and social taboo contributes to the high discontinuation rate of LTPOCs.^{46,47} Unlike menstrual bleeding, which represents an organized response to circulating progesterone withdrawal and stems primarily from spiral arterioles,^{32,48,49} LTPOC-associated AUB occurs sporadically and originates from irregularly distributed, superficial, abnormally enlarged, “fragile capillaries and venules.” These vessels are surrounded by a collapsed stromal ECM and exhibit focal and transient episodes of bleeding on hysteroscopic examination.^{42,50–52}

In endometrial biopsies obtained from adjacent bleeding and nonbleeding sites by camera-directed hysteroscopy after 1 year of Norplant contraception, microscopic examination revealed the presence of enlarged, thin-walled blood vessels at the bleeding sites.⁵³ Because TF is the primary initiator of hemostasis,⁷ we expected TF expression to be selectively elevated in decidualized HESCs at nonbleeding versus bleeding sites of Norplant-treated endometria but made the opposite observation, that is immunoreactive TF levels in endometrial biopsies obtained 1 year after Norplant were selectively enhanced in decidualized stromal cells at bleeding versus nonbleeding sites. We observed TF levels at the bleeding sites that were comparable to those of normal luteal phase decidualized HESCs.⁵³ Previously, we showed that decidualization-enhanced TF expression involves both the epidermal growth factor receptor (EGFR) and the progesterone receptors (PRs),⁵⁴

suggesting that expression of these receptors would be selectively enhanced at the bleeding sites. Instead, immunostaining and Western blotting revealed no significant differences at bleeding or nonbleeding sites in levels of the EGFR and/or the progesterone receptor A (PRA) or progesterone receptor B (PRB) isoforms during Depo-Provera or Norplant contraception.^{53,55} Norplant releases levonorgestrel from subdermal rods, whereas the Mirena intrauterine system releases levonorgestrel directly into the uterus, resulting in a much more decidualized endometrium.⁵⁶ Figure 2B confirms that in an endometrial biopsy obtained 3 months after insertion of Mirena, the stromal cells are highly decidualized, cuboidal cells with edematous cytoplasm displaying a similar morphology as the decidual cells in first trimester decidua (Figure 2A). Despite the frequent occurrence of AUB during Mirena contraception, the decidualized stromal cells also exhibit prominent staining for TF at the cell membranes (Figure 2B), which is similar to that seen in the first trimester decidual cells (Figure 2A).

LTPOC-ASSOCIATED AUB INVOLVES IMPAIRED ENDOMETRIAL ANGIOGENESIS

Administration of LTPOC results in reduced vasomotion and impaired uterine blood flow, as measured by laser Doppler fluxmetry, which provides sensitive dynamic quantification of perfusion in microvascular beds.⁵⁸ Consistent with reduced endometrial perfusion and enhanced hypoxia/reperfusion (HX/RP), Implanon administration elevated levels of endometrial lipid peroxidation as indicated in Figure 3 by increased expression of the reactive oxygen species (ROS), 8-Hydroxy-2'-deoxyguanosine, a marker of oxidative DNA damage, and nitrotyrosine, a marker of oxidative protein damage.⁵⁸ Norplant treatment also induces oxidative damage of the endometrium, as indicated by increased levels of the activated phosphorylated forms of stress-activated kinases, stress-activated protein kinase/c-Jun N-terminal kinase and p38 kinase.⁵⁹ Experiments with primary cultured HESCs and human endometrial endothelial cells (HEECs) found that medroxyprogesterone acetate, the active ingredient in Depo-Provera, reduced expression in HESCs of angiopoietin-1 (ang-1), a vessel-stabilizing factor. This would leave unopposed HEEC-expressed ang-2, which promotes vessel branching and enhances endothelial cell permeability.⁵⁸ In contrast to reduced levels of ang-1, immunoreactive levels of VEGF, the primary angiogenic agent, are reported to be elevated in the endometrial stroma of women administered Norplant⁶⁰ or Implanon.⁴¹ Although physiological levels of VEGF promote angiogenesis, overexpressed VEGF induces endothelial vascular "leakiness" and perivascular ECM dissolution that culminates in bleeding.⁶¹ The central scheme presented in Figure 4 indicates that reduced uterine blood flow resulting from LTPOC administration elicits HX/RP and the generation of ROS promotes AUB by direct physical vascular damage or by promoting aberrant angiogenesis via enhanced expression of VEGF and reciprocal inhibition of ang-1 and elevation of ang-2 levels.

THROMBIN DYSREGULATES ENDOMETRIAL ANGIOGENESIS AND DISRUPTS THE VASCULAR SUPPORT STRUCTURE

The endometria of women on LTPOC display elevated TF expression by decidualized HESCs^{6,57} and overexpressed VEGF in the stromal compartment.^{41,60} According to the model shown in Figure 5, which presents putative decidualized HESC/HEEC interactions, such VEGF would promote abnormally high endothelial cell permeability,⁶¹ further promoting thrombin formation by enhancing transudation of factor VII to decidual cell-expressed TF. As VEGF, thrombin is angiogenic and enhances endothelial cell permeability.^{62,63} In addition to augmenting VEGF expression, hypoxia and thrombin drive interleukin-8 (IL-8) expression in decidualized HESCs.⁵⁷ Because VEGF, thrombin, and IL-8 each

promote angiogenesis via separate endothelial cell receptors,^{27,62,64} the consequent synergistic stimulation of abnormal angiogenesis and impaired vessel maintenance would contribute to the enlarged, structurally compromised, “fragile” vessels that are prone to bleed. Moreover, as indicated by the Central scheme in Figure 4, LTPOC-impaired uterine blood flow is associated with the focal hypoxia that enhances VEGF^{36,65,66} and IL-8 expression⁶¹ in decidualized HESCs to further exacerbate abnormal angiogenesis.

Administration of LTPOCs in women⁶⁸ and in a mouse model⁶⁹ promotes neutrophil infiltration of the endometrium. Mirena and Norplant contraception are associated with elevated levels in the endometrium of IL-8,^{57,70} the primary neutrophil chemoattractant and activator.⁷¹ Therefore, enhanced IL-8 production by decidualized HESCs would contribute not only to aberrant angiogenesis as depicted in Figure 5 but also to the neutrophil excess that characterizes the LTPOC-derived endometrium.⁶⁸ Neutrophils express proteases such as neutrophil elastase and neutrophil MMP-8 and -9 that target ECM components.⁷² The consequent degradation of the basal laminar component-enriched decidualized ECM would produce the thin atrophic stroma seen in LTPOC users and disrupt the integrity of the perivascular ECM support scaffolding. This disruption of the ECM integrity in a milieu of thrombin-mediated aberrant angiogenesis would exacerbate LTPOC-induced AUB.

SUMMARY AND CONCLUSIONS

The absence of any TF-deficient condition in humans emphasizes the crucial role played by TF, in species survival. Elevated TF expression by decidualized HESCs (decidual cells) protects against hemorrhage during EVT invasion of endometrial vessels and during preterm and term delivery. Thrombin augments VEGF expression in progestin-decidualized HESCs and may mediate luteal phase angiogenesis. In general, LTPOCs reduce endometrial blood flow to promote local hypoxia and enhance oxidative damage-inducing ROS. Enhanced vascular permeability in such fragile vessels increases circulating factor VII availability to decidualized HESC-expressed TF to generate excess thrombin. Hypoxia–thrombin interactions enhance HESC-expressed VEGF and IL-8. Synergy between thrombin, VEGF, and IL-8 augments angiogenesis in a milieu of ROS-induced endothelial cell activation. Moreover, a neutrophil excess in the LTPOC-derived endometrium expresses proteases that degrade the endometrial perivascular support structure to intensify vessel fragility and promote AUB.

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Figure 1.

Thrombin effects on VEGF levels in decidualized HESCs and in HEGECs. Confluent HESCs were decidualized for 7 days in 10^{-8} M E_2 (E) + 10^{-7} M medroxyprogesterone acetate (P), and HEGECs were treated with E + P for 3 days, then incubated in a serum-free defined medium containing E + P with or without thrombin (T) or TRAP (TR). The cultures were incubated for 24 or 48 hours as indicated. Secreted VEGF levels were measured by enzyme-linked immunosorbent assay and normalized to cell DNA. A, 24 and 48 hours of HESCs with E + P with or without 0.1 U/mL T (average of 2 experiments). B, Dose-response effects of thrombin after 48 hours. Ordinate, fold-increase effects of thrombin versus E + P alone (mean $\pm$ SEM, $n = 3$). C, HESCs with E + P with or without 0.5 U/mL T (mean $\pm$ SEM, $n = 6$ cell preparations, $*P < .02$). D, Effects of thrombin receptor activating peptide versus T after 48 hours in HESCs with E + P (reprinted with permission from Lockwood et al.36). E_2 , estradiol; HEGECs, human endometrial glandular epithelial cells; HESCs, human endometrial stromal cells; VEGF, vascular endothelial growth factor.

Figure 2.

Immunohistochemistry of tissue factor (TF) in endometrium following Mirena insertion. Immunostaining was carried out on paraformaldehyde-fixed, paraffin-embedded sections. A, First-trimester decidua. Arrows indicate prominent immunostaining for TF at cell membranes of decidualized stromal cells. A blood vessel is indicated by V. B, Endometrial biopsy 3 months after insertion of the Mirena intrauterine system. Note the marked decidualization of and prominent staining for TF at stromal cell membranes (arrows). An atrophic gland, typical of long-term progestin-only contraceptive treatment, is indicated by G. C, Control follicular phase endometrial specimen obtained from the same woman before Mirena contraception. The stromal cells are not decidualized and display a relative absence of TF immunostaining. Magnification, $\times 200$ (reprinted with permission from Lockwood et al⁵⁷).

Figure 3.

Immunostaining for nitrotyrosine and 8-Hydroxy-2'-deoxyguanosine in control and long-term progestin-only contraceptive (LTPOC)-treated endometrium. Immunostaining for nitrotyrosine in human control (A) endometrium and that exposed to high-dose LTPOC (B). Similar staining was conducted for 8-Hydroxy-2'-deoxyguanosine in control (C) and LTPOC-treated endometria (D). Brown peroxidase staining identifies antigens (reprinted with permission from Hickey et al58).

Figure 4.

Central scheme. Long-term progestin-only contraceptives (LTPOCs) result in hypoxia/reperfusion (HX/RP), reactive oxygen species (ROS) generation, and altered angiogenic factor expression leading to damaged vessels and abnormal uterine bleeding (AUB). Other pathways not yet elucidated may exacerbate this mechanism, denoted by “?” (reprinted with permission from Hickey et al58). Ang, angiopoietin; VEGF, vascular endothelial growth factor.

Figure 5.

Model of abnormal uterine bleeding induced during long-term progestin-only contraception (LTPOC). In the LTPOC-derived endometrium, excess transudation of factor VII contacts perivascular decidualized human endometrial stromal cell (HESC)-TF to generate thrombin that stimulates decidualized HESCs to release vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8). Acting via separate receptors on the human endometrial endothelial cell (HEEC) surface, thrombin, VEGF, and IL-8 each promote angiogenesis while enhancing endothelial cell permeability. The hypoxic milieu of the LTPOC endometrium also augments VEGF and IL-8 expression to promote a feed forward cycle of increased thrombin generation, leading to increased VEGF and IL-8 production and further angiogenesis (reprinted with permission from Lockwood et al67).