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Review Article

Uterine angiogenesis during implantation and decidualization in mice

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Increased uterine vascular permeability and angiogenesis are hallmarks of implantation and placentation. These events are profoundly influenced by vascular endothelial growth factor (VEGF). Although VEGF and its receptor Flk-1 are primarily important for uterine vascular permeability and angiogenesis before and during the attachment phase of the implantation process, VEGF together with the angiopoietins and their receptor Tie-2 directs angiogenesis during decidualization after implantation. Uterine expression of HIF and ARNT follows the localization of VEGF expression with increasing angiogenesis during the postimplantation period, although their expression does not correlate with VEGF expression during the pre-implantation period. Upstream of VEGF, estrogen promotes uterine vascular permeability but inhibits angiogenesis, whereas progesterone stimulates angiogenesis with little effect on vascular permeability. Furthermore, COX-2-derived prostaglandins participate in uterine vascular permeability and angiogenesis during implantation and decidualization. (Reprod Med Biol 2006; **5**: 81–86)

Key words: angiogenesis, decidualization, implantation, uterus, vascular permeability.

INTRODUCTION

ANGIOGENESIS IS A hallmark event during implantation and decidualization. Under physiological conditions, angiogenesis, the process by which new blood vessels develop from pre-existing vessels, primarily occurs in the uterus and ovaries of adult women during the reproductive cycle and pregnancy.[1] Indeed, increased vascular permeability and angiogenesis are crucial to successful implantation, decidualization, and placentation. A number of studies provided indirect and descriptive evidence for the potential roles of estrogen and progesterone in these processes in various species.[1–3] These studies primarily examined the changes in the whole uterus of the expression of a number of gene products known to regulate vascular permeability and angiogenesis, including VEGF and its receptors, without investigating its angiogenic status. Thus, *in vivo* roles for estrogen and progesterone in uterine angiogenesis are not fully appreciated. The present review of the uterine angiogenesis during implantation and decidualization process focuses on the molecular basis of angiogenic factors, steroid hormones and prostaglandins for uterine receptivity.

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ANGIOGENIC FACTORS AND THEIR RECEPTORS REQUIRED IN UTERINE ANGIOGENESIS

GENES ENCODING MURINE vascular endothelial growth factor (VEGF) isoforms and their receptors (Flk-1, Flt-1 and Nrp1) are differentially expressed in the mouse uterus in a spatiotemporal manner during implantation and the predominant $VEGF_{164}$ isoform interacts with Flk-1 and Nrp1.[2,3] These results suggest that the VEGF system is important for uterine vascular permeability and angiogenesis during implantation.

Vascular endothelial growth factor

Vascular endothelial growth factor, originally discovered as a vascular permeability factor, is also a potent mitogen for endothelial cells and a key regulatory growth factor for vasculogenesis and angiogenesis.[4] Targeted disruption of even one allele of the *Vegf* gene

results in embryonic death *in utero* during midgestation with aberrant blood vessel formation.[5,6] Differential splicing of the *Vegf* gene generates several VEGF isoforms in both humans and mice; $VEGF_{121}$ and $VEGF_{165}$ are the predominant isoforms in humans, whereas $VEGF_{120}$ and $VEGF_{164}$ are the most abundant isoforms in mice.[2,7] In the mouse uterus, $Vegf_{164}$ is the predominant isoform.[2] $Vegf_{164}$ mRNA accumulation primarily occurred in epithelial cells on days 1 and 2 of pregnancy. On days 3 and 4, the subepithelial stroma in addition to epithelial cells exhibited accumulation of this mRNA. After the initial attachment reaction on day 5, luminal epithelial and stromal cells immediately surrounding the blastocyst exhibited distinct accumulation of $Vegf_{164}$ mRNA. On days 6–8, the accumulation of this mRNA occurred in both mesometrial and antimesometrial decidual cells. These results suggest that $VEGF_{164}$ is available in mediating vascular changes and angiogenesis in the uterus during implantation and decidualization.[2]

Flk-1

Flk-1 is the major transducer of VEGF signals that induce chemotaxis, actin reorganization and proliferation of endothelial cells.[4,8,9] Targeted deletion of the *Flk-1* gene in mice produces defects in hematopoietic and endothelial cell development, leading to embryonic death by day 9.5.[10] In the mouse uterus, very low to undetectable levels of expression of *Flk-1* mRNA were noted on the first two days of pregnancy.[2] On days 3 and 4, these *Flk-1* were distinctly expressed in cells in the stromal bed. The expression of *Flk-1* mRNA was evident in stromal cells close to, but not immediately surrounding, the implanting blastocyst on day 5. On days 6–8, *Flk-1* mRNA accumulation occurred in cells at both the mesometrial and antimesometrial decidual beds. However, the expression was more intense at the mesometrial pole, the presumptive site of placentation and heightened angiogenesis. On day 8, some embryonic cells also exhibited clear accumulation of this mRNA. *Flk-1* was absent in the primary decidual zone (PDZ), which is avascular.[2,11]

Flt-1

Vascular endothelial growth factor effects are primarily mediated by two tyrosine kinase receptors; VEGFR1 (fms-like tyrosine kinase 1 [Flt-1]) and VEGFR2 (fetal liver kinase 1 [Flk-1]/kinase insert domain-containing receptor [KDR]).[12–15] Although Flt-1 activation does not stimulate endothelial cell mitosis, targeted disruption of the *Flt-1* gene produces impaired endothelial cell assembly into blood vessels and embryonic lethality.[16] In the peri-implantation mouse uterus, expression levels of *Flt-1* detected by northern blot hybridization and *in situ* hybridization were less abundant than those of *Flk-1*.[11]

Nrp1

Another multifunctional VEGF receptor has been identified as Neuropilin-1 (Nrp1). Nrp1 was originally described as a neuronal transmembrane receptor that participates in axonal guidance in the developing nervous system[17,18] and is a receptor for the collapsin/semaphorin family of proteins.[19,20] It is now known that Nrp1 functions as a receptor for at least five different ligands; collapsin-1/semaphorin-IIII/D, semaphorin-E, semaphorin-IV, $VEGF_{165}$ and placental growth factor (PlGF). These ligands are involved in different biological processes, such as nervous system development, vasculogenesis and angiogenesis.[20,21] *Nrp1* is expressed in human endothelial cells as a $VEGF_{165}$-specific receptor. When coexpressed in endothelial cells with Flk-1, Nrp1 enhances the binding of $VEGF_{165}$ to Flk-1 and $VEGF_{165}$-mediated chemotaxis severalfold higher than that of Flk-1 alone.[22] In contrast, inhibition of $VEGF_{165}$ binding to Nrp1 inhibits its binding to Flk-1 and its mitogenic activity in endothelial cells. *Nrp1*-deficient mice show peripheral nervous system abnormalities and die in midgestation as a result of yolk sac vascular insufficiency and developmental anomalies of the cardiovascular system.[23] Mice overexpressing *Nrp1* also show cardiovascular abnormalities including increased number of blood vessels and abnormal hearts.[24]

The expression pattern of *Nrp1* mRNA was similar to *Flk-1*. During the first two days of pregnancy, very low to undetectable levels of expression of Nrp1 mRNAs were noted in the uterus.[2] On days 3 and 4, these genes were distinctly expressed in cells in the stromal bed. The expression of *Nrp1* mRNA was evident in stromal cells close to, but not immediately surrounding, the implanting blastocyst on day 5. On days 6–8, *Nrp1* mRNA accumulation occurred in cells at both the mesometrial and antimesometrial decidual beds. However, the expression was more intense at the mesometrial pole, which is the presumptive site of placentation and heightened angiogenesis. On day 8, some embryonic cells also exhibited clear accumulation of this mRNA. *Nrp1* was absent in the PDZ.[2,11] This absence was also reflected in reduced levels of *Nrp1* mRNA, as determined

by northern hybridization, in whole uterine RNA samples on days 6 and 7 when the PDZ was prominent. However, it is interesting to note that the distribution of *Nrp1* mRNA was more widespread than that of Flk-1, suggesting that Nrp1 is present in stromal cells other than endothelial cells.

ANGIOPOIETINS AND THEIR RECEPTOR TIE-2

VASCULAR ENDOTHELIAL GROWTH factor effects are complemented and coordinated by another class of angiogenic factors; the angiopoietins.[25] Vascular endothelial growth factor acts during the early stages of vessel development.[5,6,10] whereas angiopoietin-1 (Ang-1) acts later to promote angiogenic remodeling, including vessel maturation, stabilization, and leakiness.[26–28] In contrast to agonistic functions of Ang-1, Ang-2 behaves as an antagonist. Thus, Ang-1 and Ang-2 are naturally occurring positive and negative regulators of angiogenesis, respectively. They interact with an endothelial cell-specific tyrosine kinase receptor Tie-2.[29] A recent report has shown that Ang-2 is required for postnatal angiogenic remodeling, and Ang-2 in collaboration with VEGF participates in the development of lymphatic vasculature.[30] Two additional members of the angiopoietin family have been identified recently. Ang-3, which is expressed in mice, appears to function as an antagonist to Ang-1 activation of Tie-2 in a manner similar to Ang-2.[31] In contrast, Ang-4, the human counterpart of Ang-3, functions as an agonist to Tie-2.[31] However, definitive biological functions of Ang-3 and Ang-4 remain unclear.

Although VEGF and its receptor Flk-1 are primarily important for uterine vascular permeability and angiogenesis before and during the attachment phase of the implantation process, VEGF together with the angiopoietins and their receptor Tie-2 directs angiogenesis during decidualization after implantation.[32] On days 1–5, the expression levels of *Ang-1*, *Ang-2* and *Ang-3* were very low to undetectable in any uterine cell types. On day 6, although *Ang-1* expression was restricted to the secondary decidual cells and undifferentiated stromal cells away from the implanting embryo, *Ang-2* expression was primarily observed in decidual cells close to the implanting embryo along the mesometrial and antimesometrial poles. *Ang-3* expression was low on this day. On day 7, *Ang-1* expression became further restricted to a thin layer of undifferentiated stromal cells along the myometrium. The pattern of *Ang-2* expression on day 7 was similar to that observed on day 6 but at much higher levels. *Ang-3*, whose expression was not detected on earlier days, became prominent on day 7 and followed the pattern of *Ang-2* expression. On day 8, a very low level of expression of *Ang-1* was detected only in a small number of stromal cells at the mesometrial pole just underneath the myometrium. In contrast, patterns of *Ang-2* and *Ang-3* expression similar to those observed on day 7 persisted through day 8. However, the expression pattern of *Tie-2*, a receptor for the angiopoietins, was similar to that observed for *Flk-1* on days 1–8.

EXPRESSION OF HYPOXIA-INDUCIBLE FACTORS IN THE PERI-IMPLANTATION UTERUS IS REGULATED IN A CELL-SPECIFIC AND OVARIAN STEROID HORMONE-DEPENDENT MANNER

OXYGEN HOMEOSTASIS IS essential for cell survival and is primarily mediated by hypoxia-inducible factors (HIF). These factors are intimately associated with vascular events and induce *Vegf* expression by binding to the hypoxia response element in the *Vegf* promoter. HIF isoforms function by forming heterodimers with the aryl hydrocarbon nuclear translocator (ARNT) (HIF-β) family members. There is very limited information on the relationship among HIF, ARNT, and VEGF in the uterus during early pregnancy, although the roles of HIF in regulating VEGF and angiogenesis in cancers and vasculogenesis during embryogenesis are well documented.[33]

Using molecular and physiological approaches, we have recently shown that uterine expression of HIF and ARNT does not correlate with *Vegf* expression during the pre-implantation period in mice.[34] In contrast, their expression follows the localization of uterine *Vegf* expression with increasing angiogenesis during the postimplantation period. This disparate pattern of uterine HIF, ARNT and *Vegf* expression during the pre-implantation, on days 1–4 of pregnancy, suggests that HIF has multiple roles in addition to the regulation of angiogenesis during the peri-implantation period. Using pharmacological, molecular and genetic approaches, we also observed a novel finding that although progesterone primarily upregulates uterine *HIF-1* expression, estrogen transiently stimulates that of *HIF-2*. The definitive role of hypoxia in uterine angiogenesis warrants further investigation.

During pre-implantation, the expression of *HIF* (*HIF-1α*, *–2α* and *–3α*) was very low to undetectable in the uterus on day 1 of pregnancy. However, *HIF-1α* was distinctly expressed in the luminal epithelium on day 4

of pregnancy as opposed to the expression of *Vegf* in the stroma. Interestingly, distinct but patchy expression of *HIF-2α* was noted in the stroma, whereas the expression of *HIF-3α* was undetectable. HIF must heterodimerize with ARNT for transcriptional activation of *Vegf*.[35] All three *ARNT* (*ARNT1*, *ARNT2* and *ARNT3*) were expressed at very low to undetectable levels on these days of pregnancy, except *ARNT1*, which was expressed at a low to modest level both in the luminal epithelium and stroma on day 4 of pregnancy. In contrast, the stromal expression of *HIF-2α* that correlates with *ARNT1* on day 4 of pregnancy suggests that HIF-2α regulates *Vegf* transcription after heterodimerization with ARNT1. Immunoreactive HIF-1α and ARNT1 proteins were primarily localized to the uterine epithelium on day 4 of pregnancy, suggesting that HIF-1α effects are probably restricted to the epithelium at this time. The unavailability of suitable antibodies to other HIF and ARNT has precluded to determine the localization of these proteins in the uterus. Nonetheless, the mRNA localization of *HIF-2α* in the stroma in the presence of little or no expression of *ARNT2* and *ARNT3*, and a very low level of *ARNT1* expression with restricted localization of its protein in the epithelium raises questions regarding a role for HIF-2α in stromal *Vegf* expression on day 4 of pregnancy. It is possible that a yet unidentified ARNT isoform is expressed in the stroma at this time. Nonetheless, the localization of both the mRNA and protein for HIF-1α and ARNT1 in the epithelium on day 4 of pregnancy suggests that HIF-1α has a different role in the uterus, because *Vegf* is expressed in the stroma but not in the epithelium at this time.

In the postimplantation uterus, both the luminal epithelium and stroma exhibited *HIF-1α* expression similar to that of *Vegf*, whereas *HIF-2α* expression was restricted to only stromal cells surrounding the blastocyst on day 5. In contrast, the expression of *HIF-3α* was very low without any cell-specific localization. On day 8, *HIF-1α* expression showed further increases in the decidual bed, but the most robust expression was noted for *HIF-2α*. The expression of *HIF-3α* was, again, very low and diffuse. All three *HIF* showed expression in the developing embryo. The cell-specific accumulation of *HIF-1α* and *HIF-2α* mRNA closely correlated with the levels determined by northern hybridization of whole uterine RNA samples. When expression patterns of *HIF* were compared with those of *ARNT*, *ARNT1* and *ARNT3*, similar expression patterns were shown in the stroma as that of *HIF-1α* and *HIF-2α* on day 5, but the expression of *ARNT2* was primarily restricted to the luminal epithelium. These results suggest that HIF-1α and HIF-2α can partner with ARNT1 or ARNT3 in the stroma, but only HIF-1α can partner with ARNT2 in the epithelium on day 5. On day 8 of pregnancy, the localization of *ARNT* was similar to that of *HIF-1α* and *HIF-2α*, but the expression intensity was low to modest in the decidual bed. The expression of *ARNT* in the developing embryo was similar to that of *HIF*. Collectively, these results suggest that ARNT1 and ARNT3 are perhaps the major partners of HIF-1α and HIF-2α in the uterine stroma that is operative for the *Vegf* expression during the postimplantation period.

DIFFERENTIAL REGULATION OF STEROID HORMONES FOR UTERINE VASCULAR PERMEABILITY AND ANGIOGENESIS

THE EXPRESSION OF VEGF and its receptors in the uterus is in response to steroid hormones.[11] For example, estrogen rapidly induces uterine vascular permeability and *Vegf* expression transcriptionally through the nuclear estrogen receptor,[11] and the *Vegf* gene contains estrogen response elements.[36] Progesterone also upregulates uterine *Vegf* expression through activation of the nuclear progesterone receptor but at a slower rate.[36] Because estrogen rapidly stimulates uterine vascular permeability and *Vegf* expression, and because vascular permeability is considered a prerequisite for angiogenesis, it is widely believed that estrogen is a potent stimulator of uterine angiogenesis during normal reproductive processes *in vivo*. However, recent evidence using molecular, genetic, physiological and pharmacological approaches shows that estrogen and progesterone have different effects *in vivo*; estrogen promotes uterine vascular permeability but profoundly inhibits angiogenesis, whereas progesterone stimulates angiogenesis with little effect on vascular permeability.[37] These effects of estrogen and progesterone are mediated by differential spatiotemporal expression of proangiogenic factors in the uterus.[37]

COX-2-DERIVED PROSTAGLANDINS PARTICIPATE IN UTERINE ANGIOGENESIS DURING IMPLANTATION AND DECIDUALIZATION

BECAUSE OF THEIR roles in angiogenesis, cell proliferation and differentiation in other systems, prostaglandins are also likely to participate in uterine vascular permeability and angiogenesis during implantation and decidualization. Indeed, there is now genetic and molecular evidence that COX-2-derived prostaglandins

participate in uterine angiogenesis during implantation and decidualization.[32] Thus, one cause of failure of implantation and decidualization in *Cox-2*(–/–) mice is the deregulated vascular events in the absence of COX-2. The attenuation of uterine angiogenesis in these mice is primarily the result of defective VEGF signaling rather than the angiopoietin system.

We examined the spatiotemporal expression of angiopoietins and their receptor, *Tie-2*, in the uteri of *Cox-2*(–/–) mice with those of wild-type mice at the site of blastocyst on days 6 and 8.[32] On day 6, no significant difference was noted in the expression patterns of angiopoietins between the *Cox-2*(–/–) and wild-type mice, although the decidual response was depressed in *Cox-2*(–/–) mice. The expression pattern of *Tie-2* was also similar between the wild-type and *Cox-2*(–/–) uteri, albeit the level of expression of *Tie-2* was somewhat lower in *Cox-2*(–/–) mice. On day 8, aberrant decidualization and embryo development were more prominent in *Cox-2*(–/–) mice, but the normal expression patterns for angiopoietins were still maintained. However, *Tie-2* expression was modestly depressed in *Cox-2*(–/–) uteri. These results suggest that angiopoietin signaling involved in uterine angiogenesis is not significantly affected during implantation and decidualization in the absence of COX-2.

We also compared uterine expression patterns of $Vegf_{164}$ at the sites of blastocysts on days 6 and 8 after transfers of day 4 wild-type blastocysts into day 4 wild-type or *Cox-2*(–/–) pseudopregnant recipients.[32] $Vegf_{164}$ expression was remarkably downregulated in stromal cells at the blastocyst site in *Cox-2*(–/–) mice on both days 6 and 8. PGI_2 participates in angiogenesis in other systems.[38] A PGI_2 agonist, carbarprostacyclin (cPGI), functions as a ligand for PPARδ and facilitates its heterodimerization with retinoid X receptor (RXR). cPGI (a more stable analog of PGI_2) plus an RXR agonist, 9-*cis*-retinoic acid (9-*cis*-RA), improves poor implantation in *Cox-2*(–/–) mice.[32,39] Administration of cPGI and 9-*cis*-RA also restored the expression of *Vegf* with improved implantation when examined on day 6. When examined on day 8, the number of blood vessels was remarkably reduced at the blastocyst site in the absence of COX-2. Administration of cPGI and 9-*cis*-RA again restored the normal number of blood vessels. These results suggest that COX-2-derived prostaglandins influence uterine angiogenesis primarily by affecting the VEGF system during implantation. Collectively, these results provide evidence that although ovarian steroid hormones influence uterine vascular permeability and angiogenesis during the preimplantation period, COX-2-derived prostaglandins direct these events during implantation and decidualization by differentially regulating VEGF and angiopoietin signaling.[32,37]

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