Angiogenesis and its control in the female reproductive system

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The rapid, controlled and cyclical nature of angiogenesis in the female reproductive tract suggests that interference with this process should provide a novel approach to manipulation of reproductive function. Many factors involved in the regulation of angiogenesis have been identified, and the possibility of stimulating or inhibiting these paracrine control mechanisms is being addressed using current advances in the development of angiogenic and anti-angiogenic compounds. Studies with animal models indicate that the normal processes of folliculogenesis, ovulation and corpus luteum function in the ovary, and the control of menstruation and implantation in the endometrium could be profoundly influenced by manipulation of angiogenesis. Novel therapeutic agents targeted to the angiogenic pathway may also have a wide range of applications in pathological processes in the reproductive tract such as cancer, endometriosis, fibroid growth, and ovarian hyperstimulation syndrome.

Angiogenesis is the development of new blood vessels by endothelial cell proliferation and outgrowth from pre-existing vessels. It is regulated by a complex series of growth factor interactions, including stimulatory, modulatory and inhibitory regulators and is associated with changes in the extracellular matrix required to allow migration of the newly-forming vessels. Apart from during tumour growth and wound healing, the adult vascular endothelium is generally quiescent. The notable exceptions are within the female reproductive system; the ovaries, uterus and placenta. These tissues undergo cyclic changes in angiogenesis necessary to supply the nutrients and hormone precursors essential for the establishment and maintenance of pregnancy. During the menstrual cycle, this physiological angiogenesis in the corpus luteum and endometrium is distinctive in that, in the absence of conception, it is followed by tissue regression and re-initiation of cyclicity. This tight physiological control contrasts to the situation in tumours, and raises the possibility that endogenous inhibitors of angiogenesis play an integral role in the reproductive tract. Thus, these tissues provide an outstanding system in which to study the normal physiology of angiogenesis. Understanding the mechanisms of control of angiogenesis in the reproductive system and the harnessing of major...
advances in the development of angiogenesis inhibitors and stimulators may reveal new approaches to regulation of reproductive function and treatment of pathological conditions of the female reproductive tract. In addition, insight should be gained into the changes which precipitate the relatively uncontrolled angiogenesis responsible for the growth of solid tumours and how this may be inhibited.

This chapter describes briefly the potential angiogenic control mechanisms within the female reproductive tract and how these might be targeted, reviews recent data investigating the consequences of angiogenesis inhibition in experimental models, and examines the projected clinical applications. Since mechanisms involved in regulation of cyclic ovarian and uterine angiogenesis have much in common, progress with respect to the ovary is highlighted and the reader referred to recent reviews for the uterus.

Angiogenic control mechanisms

Follicular angiogenesis commences at, or shortly before, the development of a recognisable thecal layer and increases in follicles undergoing maturation to the antral stage. Blood vessels increase in number and size as the follicle develops, but do not penetrate the granulosa cell layer while the basement membrane surrounding it remains intact. The extent to which angiogenesis governs follicular atresia on the one hand, and selection of the dominant follicle on the other, is of great interest; in some species, a relationship has been demonstrated, but differences are not obvious in the human ovary.

During the process of ovulation, the basement membrane loses its integrity and the thecal blood vessels access the granulosa cell layer. Subsequently, formation of the corpus luteum is associated with intense angiogenesis. By the mid-luteal phase, when the corpus luteum (CL) is fully functional, blood flow is among the greatest of any tissue in the body. Proliferation of endothelial cells continues at a moderate level, decreasing towards the end of the cycle. By the mid-luteal phase, the microvascular tree is established. It appears that the rescue of the CL in early pregnancy is not associated with a further spurt of intense angiogenesis since chorionic gonadotrophin (hCG) does not influence endothelial cell proliferation.

Angiogenesis is regulated in the reproductive tract and elsewhere by at least 20 angiogenic growth factors and inhibitors identified to date. A key player is vascular endothelial growth factor (VEGF) also known as vascular permeability factor. VEGF is a member of the platelet-derived growth factor family, being a dimeric protein existing as 5 different homodimeric isoforms generated by alternative splicing from a single gene. The secreted forms are composed of 121 and 165 amino acids and act upon...
vascular endothelial cells in which its receptor tyrosine kinases (Flt-1 and KDR) are expressed selectively. VEGF is expressed in the ovary in a regulated manner\textsuperscript{11,12} and its importance in ovarian angiogenesis has been established following studies in which its action has been inhibited during the ovulatory cycle (see below).

Particular attention is being given to the inter-relationship of VEGF with a novel family of angiogenic regulators, the angiopoietins, which consist of about 500 amino acids and act via another receptor tyrosine kinase, Tie-2, on the endothelial cells. Angiopoietins are relatively highly expressed in the ovary, uterus and placenta\textsuperscript{12}. Since they appear to play a major role in stabilisation of blood vessels on the one hand and endothelial cell death on the other, the angiopoietins are attractive candidates as regulators of these divergent processes as they occur in the female reproductive tract. When active angiogenesis is occurring in the presence of VEGF, Ang-2 acts in concert to enhance angiogenesis, while Ang-1 is involved in the process of maturation and stabilisation of new blood vessels\textsuperscript{12}. Paradoxically, in circumstances of reduced VEGF expression, Ang-2 may act to destabilise blood vessels and induce vascular regression by competitive inhibition of Ang-1\textsuperscript{12,13}.

The temporal changes in the pattern of expression of VEGF and angiopoietin mRNA and in the rat ovary have shown that VEGF is...
expressed in the pre-ovulatory follicle and in the hormone-producing cells of the corpus luteum. Ang-2 is present in theca of the pre-ovulatory follicle and in the endothelial cell sprouts of the developing corpus luteum, suggesting an angiogenic role in concert with VEGF at this time. Intriguingly, Ang-2 is highly expressed in regressing corpora lutea, when VEGF is declining, implying its involvement in the luteolytic process via destabilisation of blood vessels. Similarly, quantitative PCR in the bovine revealed the Ang-2:Ang-1 mRNA ratio increases during luteolysis. While luteolysis in the normal cycle is associated with endothelial cell loss, stabilisation and maturation of luteal blood vessels may be crucial to the maintenance of the corpus luteum in early pregnancy. The mechanisms involved in these divergent processes are not understood. If luteolysis is associated with an increase in Ang-2 expression and blood vessel destabilisation perhaps ‘rescue’ of the corpus luteum is associated with an increased expression of Ang-1 resulting in recruitment of pericyte cells which stabilise the blood vessels. Control of luteal function differs markedly between species and it is not yet known whether similar inter-relationships between positive and negative angiogenic mechanisms exist in the primate corpus luteum.

Two further natural inhibitors of angiogenesis have been discovered recently, both being fragments of larger molecules; angiotatin is 98% identical to an internal fragment of plasminogen, while endostatin corresponds to the C-terminal fragment of collagen XVIII. Both proteins inhibit tumour growth, but their role in reproductive physiology is unknown. Since it appears that an ‘angiogenic switch’ regulates the divergent states of follicular development, luteal rescue and luteolysis, and endometrial function, it is of importance to localise and quantify the temporal changes in the angiotatin/endostatin system in these situations to explore the hypothesis that production of these proteins is increased in the regressing state and suppressed in association with rescue.

Changes in the extracellular matrix (ECM) are an essential part of the angiogenic process and are regulated by metalloproteinases and their inhibitors. Cell adhesion to the ECM is primarily through integrins, a family of heterodimeric transmembrane proteins. These mediate cell adhesion to the ECM leading to intracellular signalling events that regulate cell survival, proliferation and migration. During angiogenesis, it is thought that a number of integrins expressed on the surface of activated endothelial cells regulate critical adhesive interactions with a variety of ECM proteins.

A complete understanding of these complex regulatory mechanisms within the ovary and uterus will allow systematic targeting in pathologies which have a vascular component. While basic studies strive to achieve this goal, targeting of putative key regulators has been conducted both in the laboratory and clinic during the last decade, particularly in the area of solid tumour biology.
Manipulation of angiogenesis

Development of anti-angiogenic agents

Over 300 endogenous, natural or synthetic anti-angiogenic agents have been described. The initial development involved screening for such activity in compounds developed for other indications. A second group required the design of specific antagonists to known positive angiogenic factors and their receptors, while a third class have been identified from tumour sources as having negative effects on angiogenesis (Table 1).

Numerous studies have shown that inhibition both by broad-spectrum or systematic targeting of the angiogenic pathway can have profound suppressive effects upon tumour growth in animal models. These include the effects of neutralisation of VEGF in an ovarian cancer model. Most of the clinical trials on angiogenesis inhibitors have employed first generation compounds such as the fumagillin analogue, TNP-470, but in common with many of these compounds its mechanism of action is multi-factorial and is still under investigation.

Specific angiogenic peptide or receptor antagonists are being developed for clinical application. A humanised monoclonal antibody to VEGF is currently on clinical trial and the VEGF receptor is also being targeted using a soluble truncated Flt-1 receptor or tyrosine kinase inhibitors, the latter having the advantage of oral activity. Combination treatments such

<table>
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<th>Table 1 Examples of anti-angiogenic agents</th>
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<tr>
<td><strong>First generation</strong></td>
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<tr>
<td>Suramin</td>
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<tr>
<td>Fumagillin derivative (TNP-470)</td>
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<tr>
<td>Thalidomide</td>
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<tr>
<td>Medroxyprogesterone</td>
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<td>Anti-oestrogens</td>
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<td><strong>Inhibition of positive regulators</strong></td>
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<td>VEGF inhibitors</td>
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<td>VEGF immunoneutralising antibodies</td>
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<td>Truncated soluble receptor</td>
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<td>Tyrosine kinase receptor antagonists, e.g. Sugen 5416</td>
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<tr>
<td>VEGF receptor immunoneutralising antibodies</td>
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<td>VEGF diphtheria toxin</td>
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<td>Ribozymes</td>
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<tr>
<td>Angiopoietin/Tie receptor antagonists</td>
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<td>Metalloproteinase inhibitors (e.g. Marimastat)</td>
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<td>Integrin inhibitors (e.g. Vitaxin)</td>
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<td><strong>Administration of negative regulators</strong></td>
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<td>Angiogenin</td>
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<td>Angiostatin</td>
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<td>Thrombospondin</td>
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<td>Endostatin</td>
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as VEGF inhibitor followed by Ang-2 may prove to be exceptionally effective in preventing angiogenesis\textsuperscript{13}.

The involvement of the ECM in angiogenesis has led to development of metalloproteinase inhibitors and neutralising antibodies capable of inhibiting specific integrins. The therapeutic application of such approaches is currently under investigation in the clinic.

Initial studies in experimental tumour models using negative regulators of angiogenesis, such as recombinant angiostatin, were encouraging in that they demonstrated that tumours may be treated successfully with these agents without generating resistance\textsuperscript{15,16,22}. In addition, prolonged tumour dormancy was observed post-treatment. The challenge of extending these results to man is eagerly awaited.

As increasing numbers of specific antagonists are developed, it should become possible to target systematically key steps in the angiogenic pathway at selected periods of the reproductive cycle using suitable animal models and their effects upon angiogenesis in the ovary, uterus and placenta determined. This potential for inhibition of physiological angiogenesis is only just beginning to be explored and three of the first experiments are reviewed below.

\textit{Rodent studies}

In the reproductive system, the pioneering study underlining the importance of angiogenesis was carried out in the mouse using TNP-470\textsuperscript{24}. Females were treated at various times after mating and it was shown that TNP-470 prevents pregnancy by interfering with the process of decidualisation and placental and yolk sac formation. In mice with regular oestrous cycles treated for 16 days, mating was abolished, there was a marked decrease in endometrial stromal and glandular cell proliferation, uterine blood vessel development was severely restricted, and the ovaries contained fewer and smaller corpora lutea than cyclic controls.

The importance of angiogenesis in the immature rat ovary was shown in studies in which VEGF was blocked using a truncated Flt receptor\textsuperscript{25}. Follicular development was induced by gonadotrophin treatment and resulted in multiple ovulations and elevated serum progesterone in control rats, whereas those treated with antagonist starting 4–6 h prior to gonadotrophin administration showed small antral follicles and only a few corpora lutea. The latter were relatively avascular and often demonstrated central ischaemic necrosis. While the uteri of the gonadotrophin-treated rats demonstrated morphological evidence of steroid stimulation, such changes were largely blocked in antagonist-treated animals, presumably secondary to the comparative lack of steroid stimulation, but also in part as a result of inhibition of uterine VEGF.
Primate studies

The mechanisms which regulate luteal function in rodents and primates are markedly different. In the non-fertile cycle of the rodent, the corpus luteum is active for less than a day, while that of primates is functional for 2 weeks prior to its regression. In addition, in many non-primates, regression of the corpus luteum is mediated by a specific luteolysin, a phenomenon not apparent in the human. In the rat model, since VEGF antagonist treatment was initiated prior to the onset of follicular hyperstimulation, it was not possible to dissociate the luteal inhibition from the consequences of inhibition of angiogenesis in the developing follicle. Experiments outlined below targeted the corpus luteum specifically by treating animals during the normal cycle at the time of ovulation.

When macaques or marmosets were treated with TNP-470, there were no apparent effects upon angiogenesis or luteal function in contrast to the results in the mouse. The reason for this difference is unclear, but could be related to dose or route of administration of the compound or real species differences. The effects of ‘first generation’ compounds may be comparatively weak relative to the more specific antagonists being developed. These primate results emphasise the need for caution in extrapolating results from the rodent to the human.

Luteal angiogenesis is suppressed by neutralisation of VEGF. Marmosets were treated with a monoclonal antibody to human VEGF starting at the time of ovulation and continuing for 3 days (early luteal phase group) or 10 days (mid-luteal group). Treatment effects were determined by measuring plasma progesterone concentrations, endothelial cell proliferation index using bromodeoxyuridine (BrdU) incorporation, and endothelial cell number using the specific cell marker, factor VIII. Neutralisation of VEGF during the early luteal phase inhibited the intense angiogenesis associated with luteal formation (Fig. 2a, b). This resulted in blockade of development of the normally extensive capillary bed since, in the mid-luteal phase treated animals, the endothelial cell numbers were reduced (Fig. 2c, d). Luteal function, as judged by secretion of progesterone, was markedly compromised by the treatment, being reduced by 60% in comparison with controls and demonstrating the principle of manipulation of ovarian function by this approach. Further studies using this model should allow the identification of the essential components of the angiogenic pathway and provide a vehicle for translating these findings into safe and effective clinical application.

Information on stimulation of angiogenesis within the reproductive system, e.g. by recombinant protein or by gene transfer, is currently limited. However, the feasibility of this approach has been demonstrated in clinical trials in patients with critical limb ischaemia and myocardial ischaemia.
Clinical studies and possible applications

Anti-angiogenic compounds have been used primarily for treatment of cancer, but are also envisaged for non-malignant pathologies which have a neovascular component (e.g. rheumatoid arthritis, psoriasis and retinal neovascularisation). Results from the use of 31 agents which have entered clinical trials at over 140 centres have been reviewed. In addition to treatment efficacy, such studies allow evaluation of side-effects and these have turned out to be more wide-spread than anticipated. This may suggest that, in tissues in which there appears to be no angiogenesis, angiogenic regulators have a more general ‘housekeeping’ role. The introduction of drugs having a selective site of action may dissociate specific from non-specific adverse effects on the vasculature or overcome them altogether. Paradoxically, such observations could shed light on novel physiological sites and/or pathways of vascular regulation. In any event, it is important that future trials incorporate a multidisciplinary team approach which includes a reproductive component. Ultimately, as with any other drug, a risk-benefit assessment will have to be undertaken prior to commencement of treatment.
To date, there have been no reports on the effects of regulators of angiogenesis on the reproductive system in women, although it is acknowledged that it offers numerous potential targets both for manipulation of fertility and treatment of pathologies involving a vascular component\(^1\)\(^-\)\(^6\)\(^,\)\(^24\)\(^,\)\(^25\). Manipulation of angiogenesis indicates a potentially powerful approach to either promoting or inhibiting the normal processes of folliculogenesis, ovulation and corpus luteum function in the ovary, and to the control of menstruation and implantation in the endometrium. It is unlikely that for fertility control, one would aim to suppress angiogenesis for prolonged periods as the risk of disruptive side-effects would be unacceptable. Targeting anti-angiogenic treatment to the follicular phase would probably compromise follicular development by inhibition of the thecal vasculature. Treatment during the early luteal phase would suppress progesterone production and probably inhibit implantation. Despite the fact that the functioning corpus luteum is essential for the establishment of pregnancy, there is no agent currently in use which can prevent the supporting effect of hCG during the conceptual cycle. Thus, the use of angiogenesis inhibitors during the postovulatory period could have potential for development.

One clinical condition in which inhibition of follicular angiogenesis would be indicated is ovarian hyperstimulation syndrome (OHSS) which occurs in a proportion of patients undergoing ovulation induction. The pathophysiology of OHSS is complex, but increased ovarian angiogenesis and permeability are major factors\(^28\). An effective treatment for this potentially fatal condition has proved elusive and it is likely that the introduction of anti-angiogenic approaches will offer a major clinical advantage.

Angiogenesis is implicated to varying degrees in numerous pathological processes of the endometrium including dysfunctional uterine bleeding, endometrial response to exogenous hormonal treatments, bleeding associated with intra-uterine devices, uterine leiomyomata, endometriosis and endometrial carcinoma\(^7\). The pathophysiology of the vasculature in these conditions is currently the focus of active research. For example, VEGF has been implicated in endometriosis\(^5\). Inhibition of angiogenesis should have potential in the treatment of these conditions, possibly as an adjunct to existing therapies operating via a separate pathway.

Administration of angiogenic agents to increase blood vessel development could be of use in stimulation of follicular development, for the treatment of inadequate luteal function, early pregnancy failure, pre-eclampsia and intra-uterine growth retardation.

Studies in the female reproductive tract are gathering momentum as the potential for control of angiogenesis in normal and pathological conditions come under scrutiny. This is an exciting field in which advances are likely in the near future and this review has given some indication as to the prospects for further developments.
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