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Endocrine Regulation of Menstruation

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In women, endometrial morphology and function undergo characteristic changes every menstrual cycle. These changes are crucial for perpetuation of the species and are orchestrated to prepare the endometrium for implantation of a conceptus. In the absence of pregnancy, the human endometrium is sloughed off at menstruation over a period of a few days. Tissue repair, growth, angiogenesis, differentiation, and receptivity ensue to prepare the endometrium for implantation in the next cycle. Ovarian sex steroids through interaction with different cognate nuclear receptors regulate the expression of a cascade of local factors within the endometrium that act in an autocrine/paracrine and even intracrine manner. Such interactions initiate complex events within the endometrium that are crucial for implantation and, in the absence thereof, normal menstruation. A clearer understanding of regulation of normal endometrial function will provide an insight into causes of menstrual dysfunction such as menorrhagia (heavy menstrual bleeding) and dysmenorrhea (painful periods). The molecular pathways that precipitate these pathologies remain largely undefined. Future research efforts to provide greater insight into these pathways will lead to the development of novel drugs that would target identified aberrations in expression and/or of local uterine factors that are crucial for normal endometrial function. (*Endocrine Reviews* 27: 17–46, 2006)

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Abbreviations: Ang, Angiopoietin; AR, androgen receptor; COX, cyclooxygenase; Dkk-I, Dickkopf-1; EBAF, endometrial bleeding associated factor; EGF, epidermal growth factor; EG-VEGF, endocrine gland VEGF; EP, E-series prostanoid; ER, estrogen receptor; FGF, fibroblast growth factor; GR, glucocorticoid receptor; HLA, human leukocyte antigen; HSD, hydroxysteroid dehydrogenase; IGFBP, IGF binding protein; IP, I-series prostanoid; KDR, kinase domain receptor; LBD, ligand-binding domain; LNG, levonorgestrel; LNG-IUS, LNG-releasing intrauterine system; MMP, matrix metalloproteinase; NFκB, nuclear factor κB; PAI, plasminogen activator inhibitor; PCOS, polycystic ovarian syndrome; PDGF, platelet-derived growth factor; PGDH, 15-hydroxyprostaglandin dehydrogenase; PGE_2, prostaglandin E_2; PR, progesterone receptor; RAR, retinoic acid receptor; SP, specificity protein; TF, tissue factor; TIMP, tissue inhibitors of MMP; uNK, uterine natural killer; uPA, urokinase-type plasminogen activator; USC, uterine stromal cell; VEGF, vascular endothelial growth factor.

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I. Introduction

THE UTERUS PLAYS a crucial role in survival of the species in all viviparous animals. Implantation of the fertilized egg is a critical event common to all species, but humans and old-world primates differ from most other animals in that they shed a significant proportion of their endometrium if pregnancy is not established at the opportune time. Human endometrium is thus a dynamic tissue that, to prepare for implantation, undergoes well-defined cycles of proliferation, differentiation, and shedding (menstruation) in response to the prevailing endocrine and paracrine environment. However, the process of menstruation may be accompanied by distressing symptoms such as menorrhagia

(excessive menstrual blood loss) and dysmenorrhea (painful periods) that are still little understood.

Regular menstrual bleeding is the outward manifestation of cyclical ovarian function. The average woman today in developed countries may expect to menstruate over 400 times during her reproductive life span. In contrast, in less well-developed countries and before the availability of reliable contraception, which has allowed women to regulate their own fertility, the majority of women were amenorrheic for most of their lives. This feature was due to later puberty, high numbers of pregnancies, and prolonged lactation.

It is essential to have a detailed knowledge of the mechanisms regulating endometrial events involved in implantation and menstruation if we are to understand the mechanisms responsible for abnormal menstrual bleeding, early pregnancy failure, and infertility. Indeed, only with a better understanding of the local mechanisms involved in endometrial function will progress be made to modulate sex steroid interactions in target cells. This review will focus on endocrine and paracrine regulation of menstruation and the local molecular aberrations associated with menstrual dysfunction.

II. Endometrial Morphology

The human endometrium is a dynamic tissue that, in response to the prevailing steroid environment of sequential ovarian estrogen and progesterone exposure, undergoes well-characterized cycles of proliferation, differentiation, and tissue breakdown on a monthly basis. If pregnancy fails to be established, then the endometrium is shed and regenerates. Menstruation is the reproductive process whereby the upper two thirds of the endometrium (functional layer) is shed and regenerated on a repetitive basis. The endometrium is consequently a site of recurrent physiological injury and repair (1).

Studies undertaken by Markee (2) and Corner and Allen (3) established the role for ovarian steroids, estradiol, and progesterone in regulating the changes in endometrial conformation across the menstrual cycle. Progesterone is essential for the establishment and maintenance of pregnancy consequent upon the transformation of an estrogen-primed endometrium. Sex steroids, acting via their cognate receptors, initiate a cascade of gene expression and events crucial for successful implantation and early stages of pregnancy. Application of knowledge from the human genome, utilizing microarray technologies, has allowed several groups to contribute to a rapidly expanding literature on gene profiles during the "putative window" of implantation (4–6).

Menstruation is the response of the endometrium to the withdrawal of progesterone (and estrogen) that occurs with the demise of the corpus luteum in the absence of pregnancy (Fig. 1). The molecular mechanisms by which sex steroids induce these events within the endometrium at the time of menstruation, involves complex interactions between the endocrine and immune system (1). Crucial structural compo-

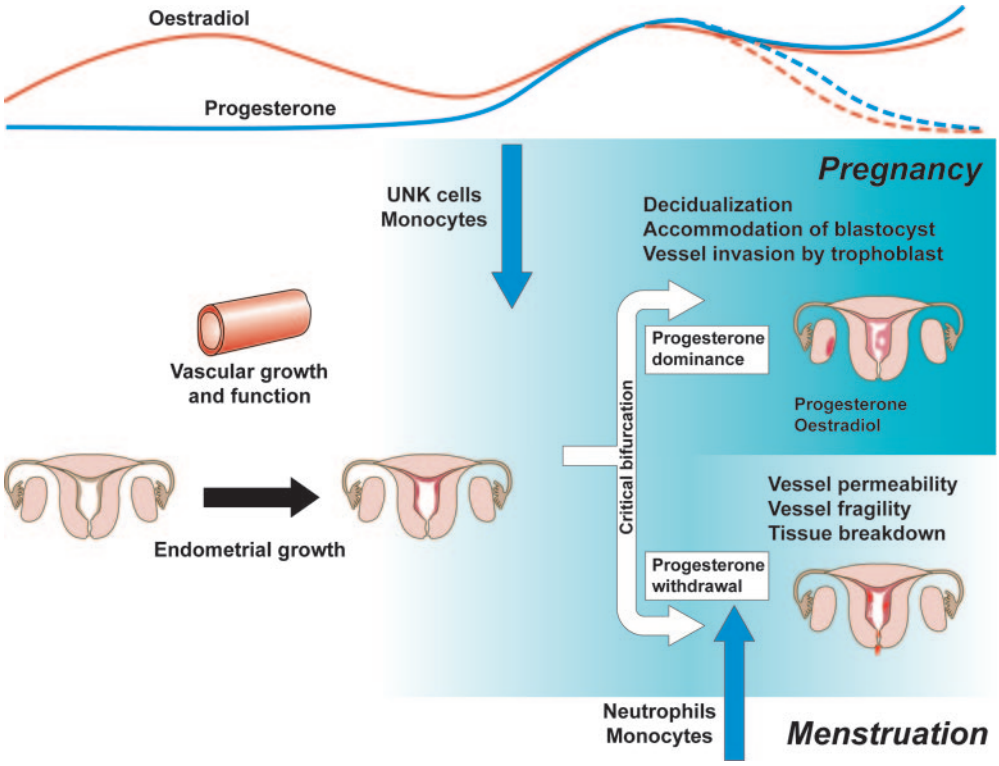

FIG. 1. Illustration of the alternatives for a progesterone-primed endometrium. The progesterone induced changes that characterize the endometrium primed for implantation also bring about progesterone dependency of the tissue. On the demise of the corpus luteum, falling progesterone levels initiate an inflammatory process that starts in the stromal compartment but involves leukocyte immigration and metalloproteinase (MMP) activation. Eventual shedding of the functional layer of the endometrium opens the way for estrogen-dependent regrowth of the tissue.

nents in the endometrium during the menstrual process are the component blood vessels and the dynamic population of leukocytes that influx at this time (Fig. 2). Only with a better understanding of the local mechanisms involved in the regulation of endometrial structure and function will there be the potential to pharmacologically modulate endometrial function in a way that would offer new strategies in the management of female reproductive health.

A. Endometrial structure

There are three classic phases of the menstrual cycle: an estrogen-dominated preovulatory phase, a postovulatory and progesterone-dominated secretory phase, and a menstrual phase following progesterone withdrawal that accompanies demise of the corpus luteum. The series of classic morphological changes that occur in response to cyclical ovarian activity are described and have been reviewed in detail (7, 8). The endometrium is composed of two layers and is a target tissue for steroid hormones. The upper functional layer is shed at menstruation. The endometrium regenerates after menstrual shedding from an underlying basal layer. Estrogen is the steroid responsible for proliferative changes during the follicular phase of the ovarian cycle, and exposure of the endometrium to ovarian progesterone results in differentiation during the secretory phase.

The progesterone-dominated latter half of the menstrual cycle is constituted by an early, mid, and late secretory phase. The pattern of sex steroid receptor expression in the endometrium across the secretory phase reflects the fact that the early secretory phase is regulated by both estrogen and progesterone; the mid secretory phase is regulated by progesterone alone as estrogen receptor (ER) α is down-regulated in the glands and stroma at this time (9); and the late secretory phase is associated with progesterone withdrawal and, consequently, menstruation. Evidence that endometrial genes are regulated has been demonstrated by the observed changes in gene expression from late proliferative to mid secretory (5) and early to mid secretory phase (4) in microarray studies. Interestingly, the failure to make a transition in gene expression has been demonstrated in endometriosis (10) with dysregulation of specific genes during the mid secretory phase in this condition.

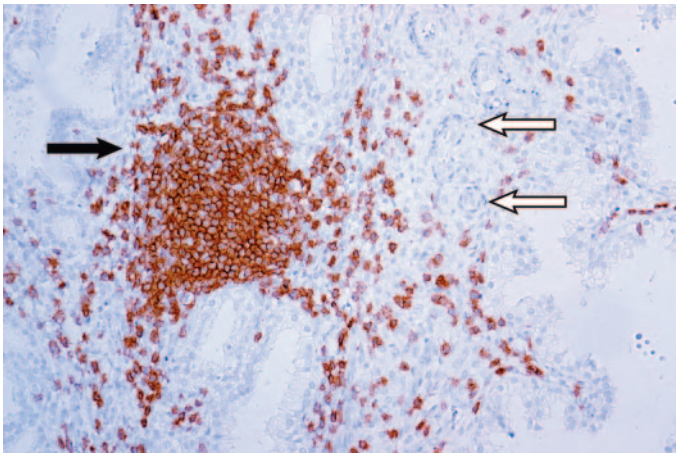

FIG. 2. Distribution of CD56+ve uNK cells in endometrium during the secretory phase of the cycle. This shows the close clustering of the uNK cells (*solid arrow*). The spiral vessels are indicated by *open arrows*.

It is notable that the exogenous administration of sex steroids produces a marked modulation of the classic histological features such as the glandular structure, mitotic status of glandular cells, and secretions in the lumen of the glands (7) when compared with accurately dated endometrium collected during a physiological cycle (11). Description of the morphological features of the endometrium may be related to the timing of ovulation. It has become widely accepted that when histological dating is in excess of 2 d ahead of classical anticipated histological features, the endometrium is advanced; if greater than 2 d delayed, then it is histologically described as delayed (12). Much controversy over endometrial dating exists (13, 14). It is likely that better methods for evaluating endometrial dating are now available and that a consistency across a set of parameters, such as date of last menstrual period in the context of regular cycles, histological dating, and serum endocrine profile on the day of biopsy, are important for accurate dating. Precision can also be obtained if chronological dating is based on determination of the LH surge (15) or timing of ovulation as defined with pelvic ultrasound (16). A morphometric analysis that provides a quantitative description of endometrial structure may also be used (12, 17). Li and Cooke (12) described five measurements, from a total of 17 morphometric parameters that are necessary to achieve a reliable correlation. These features are the volume fraction of the gland occupied by glandular cells, predecidual reaction, luminal secretion, pseudo stratification, and glandular mitoses.

Recently, the utility of histological dating of endometrium in the evaluation of infertile couples has been questioned. Coutifaris *et al.* (13) have reported on a prospective multicenter study where the out-of-phase biopsy results failed to provide good discrimination between fertile and infertile couples. Furthermore, a randomized observational study by Murray *et al.* (14) using histological features identified by both objective and systematic analyses failed to reliably distinguish a specific menstrual cycle day or narrow interval of days. This latter study concluded that histological dating had neither the accuracy nor the precision to provide a guide for clinical management. Additional criteria may emerge following studies such as endometrial stage prediction based on global gene expression (18). In this study, analysis was made of some 10,000 genes in endometrium, collected from across the cycle and histologically divided into seven stages: early, mid, and late proliferative; early, mid, and late secretory; and menstrual, according to criteria of Noyes *et al.* (7).

Classic histological criteria may consequently be inappropriate for the assessment of endometrium exposed to exogenous steroids, for example progestogens; or novel steroid receptor modulators. Indeed, it is likely that the endometrial features exhibited after exposure to steroid receptors modulators will require new descriptors to aid interpretation of possible unique and previously unreported effects upon endometrial cellular components. The endometrial features displayed in an endometrial biopsy will be indicative of the timing in the cycle of administration, route of steroid deliv-

ery and formulation, dose of compound administered, and the duration of therapy (19–21).

B. Structure of vessels

The blood vessels of the endometrium are critical to menstruation. The spiral form of the arterioles in the upper two thirds of the functional layer (2) are characteristic of menstruating species. Such vessels are involved in both leukocyte entry and vasoconstriction and clearly involved in menstruation. The strength of the small arterial blood vessels in endometrium is derived from a combination of endothelial cells, basement membrane, and cells with smooth muscle character that surround this membrane. By the late secretory phase, the spiral arterioles are surrounded by a characteristic cuff of cells that resemble the decidual cells of pregnancy (22). These cells possess smooth muscle character (*e.g.*, actin expression) as do all decidual cells (23). However, despite the similarities between the perivascular and decidual cell, a distinction can be clearly seen when progesterone is withdrawn from decidua. Immunohistochemical studies show the distinctive cuff, expressing inflammatory agents such as prostaglandins and cytokines, clearly defined against a background of decidual cells (24, 25). These perivascular cells appear to differ from the pericyte (the smooth-muscle cell that wraps around normal blood vessels) because they form a thicker layer, but they may still have processes that reach through the basement membrane and interact with endothelial cells.

The basement membrane, comprised of collagen type 4, fibronectin, and glycoseaminoglycans, varies from 50 to 350 nm in thickness, with an increase occurring during the luteal phase of the cycle. Certain components such as heparin sulfate proteoglycan show a decrease in the menstrual phase of the cycle (26) which may reflect destabilization of the vessels. The basement membrane can be broken down by various matrix metalloproteinases (MMPs) and it is the control of these MMPs by steroid hormones that is one conduit for progesterone action. The matrix components of the basement membrane are synthesized by neighboring (perivascular) stromal cells under the influence of progesterone. In particular, progesterone stimulates synthesis of fibronectin (27) and thrombospondin (28). Tenascin expression appears to reflect areas of proliferating cells (29) and thus, although global levels fall during the secretory phase of the cycle, they increase in cells immediately surrounding the blood vessels (30) where there is also an increase in proliferation markers such as Ki67 (31). Moreover, studies in the rat suggest that tenascin is clearly evident in the mesometrial gland region where it may interact with the uterine natural killer (uNK) cells in that area. These findings underline the potential for stromal cell–uNK cell interactions in women.

Some components of the membrane such as heparin sulfate proteoglycan decrease during the menstrual phase of the cycle (26). Because of the overall negative charge on many components of the extracellular matrix, these molecules can act as tethering points for growth factors. Thus, loss of molecules such as heparin sulfate proteoglycan during the menstrual process may lead to an increase in growth factor availability that will induce the regrowth of endometrial tissue (26).

C. Endometrial leukocyte populations

A dynamic leukocyte population exists within the endometrial stroma, the numbers and types of which vary across the menstrual cycle and throughout pregnancy. Endometrial leukocytes include T and B cells, mast cells, macrophages, and neutrophils. It is the phenotypically unique uNK cells that make up the majority of the leukocyte population in the late secretory phase and early pregnancy (32, 33). uNK cells are the major leukocyte population present in the endometrial stroma at the time when implantation, placentation, and decidualization occur. In the absence of pregnancy, uNK cells may be important in the initiation of menstruation. The observed cyclical increases in uNK cell numbers in the endometrium implicate direct or indirect regulation by endocrine signals, these being estrogen and/or progesterone. uNK cells have a unique phenotype (CD56 bright, CD16-, CD3-), which distinguishes them from peripheral blood NK cells (CD56 dim, CD16 bright, CD3-).

In the proliferative phase, few uNK cells are apparent but their numbers increase from day LH+3 and particularly so in the mid to late secretory phase (day LH+11–13) where they are located in close contact with endometrial glands and spiral blood vessels (32, 34). It remains to be established whether the increase in cell number is solely the result of *in situ* proliferation or whether there is also *de novo* migration from the peripheral circulation. A precursor cell type might be selectively recruited into the endometrium, where it differentiates to become the uterine-specific NK cell. In support of this theory is the existence of a subset of peripheral NK cells (around 1% of total circulating NK cells) that express a similar antigenic phenotype to uNK cells (35). On the other hand, proliferation of uNK cells in the endometrium has been described using the proliferation marker Ki67 (36, 37).

Quantitative real time RT-PCR studies have demonstrated an absence of ERα and progesterone receptor (PR) mRNA in purified uNK cells (33). In contrast, mRNA for ERβ isoforms (ERβcx/β2, ERβ1) and the glucocorticoid receptor (GR) have been localized in these cells (33). Colocalization using specific monoclonal antibodies has confirmed that uNK cells are immunonegative for ERα and PR protein (33, 38, 39). uNK cells are also immunonegative for ERβcx/β2 but do express ERβ1 and GR proteins. These recent data have raised the possibility that estrogens and glucocorticoids could be acting directly on uNK cells through ERβ and GR, respectively, to influence gene transcription in the endometrium and decidua (33).

Estrogen and progesterone may exert their effects on uNK cells indirectly via cytokines such as IL-15 and prolactin or other soluble factors (40–42). Because these factors are mainly secreted by the uterine stromal cell (USC) and because it is this cell that maintains PR, this is the likely conduit for progesterone action on uNK cells. Indeed, uNK cells and the endometrial stromal cell may have a special relationship as King (32) has commented that ectopic decidua is always associated with NK cells. Moreover, class I human leukocyte antigen (HLA; specifically HLA-B7) mRNA was increased on

the surface of endometrial stromal cells when these cells were decidualized (43). HLA would interact with the killer inhibitory receptors on the NK cells and prevent lysis of the stromal cell by the NK cell. Additional interactions between the uNK cell and the stromal cell may involve prolactin. Prolactin is a 23-kDa neuroendocrine pituitary hormone that is also secreted by other cells such as the decidualized stromal cell (44). Prolactin stimulates proliferation of lymphocytes by stimulating IL-2 (45) and may have growth-promoting effects on other cells (46). Prolactin secretion may be under the influence of cytokines produced by hematopoietic cells, which include the uNK cells (47). Because the uNK cells have the prolactin receptor (42), a two-way interaction between stromal and uNK cell may contribute to homeostasis with first-trimester decidua. Although the uNK cells have no conventional PRs, it is possible that they respond to progesterone via nongenomic membrane receptors. Membrane receptors for progesterone have been reported in fish, and human counterparts of the α, β, and δ forms have been identified (48). However, although mRNA for these proteins has been found in several cell types, including endometrial cells (O. Harding, H. O. D. Critchley, H. N. Jabbour, R. W. Kelly, and T. A. Bramley, unpublished observations), there have been no confirming reports that these molecules play any role in progesterone signaling.

The processes of endometrial differentiation, menstruation, and placentation involve the remodeling of the endometrial vasculature. The angiogenic factor, vascular endothelial growth factor (VEGF)-A plays an important role in regulation of vascular permeability and the establishment of new blood vessel formation and induces endothelial cell proliferation, migration, and differentiation in the endometrium (49). Of the VEGF family, VEGF-A has also been localized to individual cells, presumed to be leukocytes, distributed throughout the endometrial stroma. These cells have been identified as neutrophils through dual immunohistochemical staining by Mueller *et al.* (50). VEGF-A expression has also been reported in uterine macrophages in the secretory phase of the cycle (51). VEGF-C and other angiogenic factors, placental growth factor, and angiopoietin (Ang)-2 mRNA are expressed in uNK cells (52). VEGF-C was originally characterized as a growth factor for lymphatic vessels, but it can also stimulate endothelial cell proliferation and migration (53). These patterns of angiogenic growth factor expression and the intimate spatial association of uNK cells with spiral arterioles implicate a role for these cells in endometrial angiogenesis.

There are few data pertaining to mechanisms of neutrophil recruitment into the human uterus. Small numbers are present in endometrium during the majority of the menstrual cycle, except immediately premenstrually and during menses (54). The withdrawal of progesterone in the late secretory phase of the cycle may be the trigger for neutrophil influx because in the sheep, the withdrawal of progesterone results in a rapid influx of polymorphonuclear leukocytes into the uterus (55). Neutrophils synthesize and release a wide range of immunoregulatory cytokines and thereby initiate and augment cellular and humoral immune responses. One recent observation relating to the role for neutrophils in mucosal defense as well as in the mechanism of menstruation is the expression in human endometrial neutrophils of the antiproteinase and antimicrobial molecule elafin in a menstruation-dependent manner (56).

Macrophages contribute approximately 20% of the total leukocyte population in late secretory (premenstrual) endometrium (57, 58). These cells are present throughout the menstrual phase but increase in number in the mid to late secretory phase and in decidua (59). Although macrophages show a cyclical pattern of expression across the cycle, they do not express classic ERα or PR (38, 39). Control of their appearance by the ovarian sex steroids is, therefore, likely to be indirect. Endometrial macrophages display phenotypic differences, and subtypes have been described that express MMP-9 (60) and the membrane-bound MMP, MT1-MMP (61). Both these enzymes are involved in the breakdown of extracellular matrix and have been proposed to play a role in menstruation. Macrophages also produce a wide variety of regulatory molecules (58) that could stimulate the production of MMPs and proinflammatory cytokines from adjacent cells. Furthermore, macrophages are also a source of VEGF (62). Production of VEGF and the up-regulation of macrophage numbers premenstrually may implicate these cells in the menstrual process, where hypoxia and VEGF could lead to an induction of MMPs (63), and also in the revascularization of the endometrium after menstruation.

Another uterine leukocyte, the mast cell, has been identified in the endometrium in small numbers, mainly in the stromal compartment (64, 65). Human mast cells are hemopoietic cells that are characterized by their content of neutral protease and contain either tryptase alone or tryptase and chymase (66). Mast cell distribution and numbers are not altered during the menstrual cycle, but the cells are activated before menstruation when a diffuse pattern of immunoreactivity is observed. *In vitro* data have implicated a role for mast cells in the up-regulation of MMPs before the onset of menstruation (67, 68). Endometrial mast cells do coexpress the enzyme mast cell tryptase and MMP-1 in the same granules (69). Human endometrial mast cells do not express the genomic PR (H. Critchley, S. Milne, and S. Brechin, unpublished observation) and so progesterone is unlikely to be acting directly to regulate mast cell traffic in the endometrium across the cycle. Progesterone does, however, regulate MMP expression (including MMP-1) by other cell types that in turn may influence mast cell activation (67, 70).

III. Steroid Control in Endometrium

A. Endometrial steroid receptor expression

Steroids interact with their target organs via specific nuclear receptors. Members of the nuclear receptor superfamily include PR, ER, GR, and androgen receptors (ARs). At least two isoforms of the human PR have been described (71, 72). PRA and PRB derive from a single gene and function as interactive transcriptional regulators of progestin-responsive genes. Two structurally related subtypes of ER, known as alpha (ERα) and beta (ERβ) have now been identified in the human and are derived from separate genes (73–75). The actions of steroids may also involve membrane as well as nuclear receptors. Some actions of estrogens are mediated via

intracellular second messengers or other signal transduction pathways through nongenomic action (76). Recently, nongenomic (membrane bound) PRs have been characterized with no sequence homology to the nuclear receptor (48, 77).

The nuclear steroid receptors share common structure and functional domains, denoted A/B, C, D, E, and F (78). The A/B region is located at the N-terminal end and is not well conserved. This region (A/B) contains a transactivation domain (AF1). The C domain contains a highly-conserved DNA-binding domain consisting of two "zinc" fingers. Sequences within the C domain determine the specificity of the different receptors for specific hormone response elements. Aberrations in DNA and mutations in this region can result in receptor dysfunction. Next to the DNA-binding domain is the variable hinge region (D). The ligand-binding domain (LBD), region E, has a dimerization region and two transactivation domains (AF-2 and AF-2a). The LBD determines whether or not the receptor is activated. There are also accessory proteins involved in the stabilization or destabilization of the transcriptional complex necessary for steroid hormone action (79, 80). Further detailed discussion of the mechanisms of steroid receptor function lies beyond the scope of this review. However, it is worth noting that although most genes that respond to progesterone can be identified by progesterone response elements in their promoter region, this is not invariably so. A recent review highlights mechanisms by which progesterone sensitizes key kinase cascades to growth factors such as epidermal growth factor (EGF) and deals with synergistic up-regulation of growth genes such cyclin-D1 (81). Thus, although progesterone may restrict estradiol-driven endometrial growth in general, there are areas of growth in the progesterone-dominated uterus: for example, in cells surrounding the spiral arterioles (31). Moreover, the growth rate of endometrial stromal cells in culture is enhanced by a combination of progesterone and growth factors such as basic fibroblast growth factor (FGF) (82).

Estrogen is the steroid responsible for endometrial proliferation. Progesterone (and progestogens) will only result in differentiation if PRs are present in endometrial cells. PR expression requires previous exposure to estrogen. Progestogens exert an antiestrogenic effect with inhibition of endometrial growth and induction of maturation and differentiation of the glandular and stromal cells.

The expression of endometrial sex steroid receptors (PR, ERα and β, AR) varies temporally and spatially across the menstrual cycle (1, 9, 83–85). The expression of ERα and PR is under dual control by estradiol and progesterone. Both endometrial ERα and PR are up-regulated during the proliferative phase by ovarian estradiol and subsequently down-regulated in the secretory phase by progesterone acting at both the transcriptional and posttranscriptional level (86). The presence of PR is considered evidence of a functional ER-mediated pathway. The administration of a PR antagonist, mifepristone (RU486), in the early secretory phase (LH+2) has been demonstrated to block the progesterone-induced down-regulation of PR (and ERα) in nonpregnant human endometrium (87, 88).

B. Endometrial paracrinology

Progesterone is at a maximum concentration in peripheral blood at the mid secretory phase of the cycle when PR in the epithelial cells is waning. PR is absent in leukocytes throughout the menstrual cycle, and therefore the action of progesterone, both prolonged, heralding pregnancy, or falling, in the absence of pregnancy, will be mediated by the USC. The distinct relationship between the USC and the uNK cell has been described above, and the interactions between the USC and the epithelial cell that are necessary to maintain tissue integrity are detailed below. Critically, it is clear that many epithelial functions are controlled by progesterone in a paracrine manner. Not least among these functions is the ability to synthesize and release natural antimicrobial agents that contribute to the normally sterile nature of the uterus (89, 90). Sterility is partly maintained in the uterus by the many innate defenses of the cervix (91), but also by the ability of the endometrium to express a range of natural antimicrobial agents, specific to different phases of the menstrual cycle (89).

A series of experiments by Cunha's group (92–94), using knockout mice and tissue recombination, have elegantly shown that both growth and PR expression in epithelial cells is dependent on stromal-epithelial interaction. Estrogen action on the ERα receptor in the stromal cells is responsible for the down-regulation of the PR in the epithelial cells (92–94). Moreover, many of the implantation-permissive changes in murine endometrium have been attributed to optimum estrogen levels (95). Translation of these findings into women needs caution, and evidence from *in vitro* fertilization programs suggests that estrogen levels in women may not be so critical (95).

C. Endometrial intracrinology

Modification or catabolism of steroids at the level of the target tissue has been described as intracrinology (96). In human endometrium, steroidal regulation of receptor action is dependent on ligand availability. Hence, in reproductive tissues the local actions of sex steroids, estrogens, progestogens, and androgens, is modulated by hydroxysteroid dehydrogenase (HSD) enzymes. The various dehydrogenases are multigene families. The human 17β HSD family has at least six known members, each being a separate gene product from a different chromosome with distinct properties in terms of substrates and redox direction (96, 97). The type 2 enzyme (17β HSD-2) plays a major role in inactivation of estradiol to estrone (98). The enzyme 17β HSD-2 also inactivates testosterone to androstenedione and converts inactive 20α-dihydroprogesterone to active progesterone (96, 97). 17β HSD-2 is expressed in the endometrial glandular epithelium and is up-regulated by progesterone (87). Its activity decreases when progesterone concentrations decrease (as with luteal regression) or after antiprogestogen administration (87, 98).

D. Progesterone and progesterone receptors

Progesterone is essential for the transformation of an estrogen-primed endometrium in preparation for implantation. The molecular and cellular mechanisms by which the

sex steroid hormones promote uterine receptivity remain poorly understood. It is, however, recognized that sex steroids, acting via their cognate receptors, initiate a pattern of gene expression essential for implantation and the early stages of pregnancy.

There are two main isoforms of the human PR (71): PRA (Mr 94,000) and PRB (Mr 120,000) arising from a single gene with specific promoters for the two isoforms (99). These function as specific transcriptional regulators of progestin-responsive genes. A third, truncated form (PR-C) (Mr 60,000) can also migrate to the nucleus after steroid activation and may act as a repressor of PRA and PRB (100). PR-C was identified in the breast cancer cell line T47D but has also been reported in the uterus (100). Another molecule (PR-M) with homology to the nuclear receptors mentioned above, lacks a DNA binding domain and therefore may function as a membrane-associated receptor (101).

PRA is the shorter subtype, devoid of 164 amino acids present at the N terminus of the B subtype. It is otherwise identical to the B subtype (102). A significant decline in PR expression in the glands of the functional layer of the endometrium (the upper two thirds of endometrium region that is shed at menstruation) with the transition from the proliferative to the secretory phase of the cycle is well described (9, 84). In contrast, PR expression persists in the stroma in the upper functional region, being particularly highly expressed in stromal cells in close proximity to the uterine vasculature. The basal layer is differentially regulated in that the glands and stroma of the deeper zones express PR throughout the cycle (9). These differences between the superficial and basal layers of the endometrium are likely to be functionally important because only the upper functional zone is shed at menstruation. Localization studies utilizing antibodies that recognize both PR subtypes have described differential regulation of PR in the endometrial epithelium and stromal cells (103–105). In the secretory phase, the PRB subtype appears to decline in both the stroma and glands, and there is agreement that PRA is the predominant isoform in stroma throughout the cycle (103–106).

Study of PRA- and PRB-null mice (72) has provided insight into the roles of PR isoforms. In the PRA knockout mouse, estrogen treatment induces uterine epithelial hyperplasia that progesterone treatment cannot suppress. This indicates that the progesterone-mediated suppression of epithelial growth stimulated by estrogen depends on PRA, not PRB. Furthermore, in the PRA+PRB-null mouse, there is a dramatic traffic of inflammatory leukocytes into the uterus, which cannot be prevented by progesterone (107). This implicates a role for progesterone in the suppression of the influx of inflammatory cells into the uterus in wild-type animals. Furthermore, by selective ablation of PRA in mice, it has also been shown that the PRB isoform modulates a subset of reproductive functions of progesterone, by regulation of a subset of progesterone-responsive target genes (108). PRA and PRB are therefore functionally distinct mediators of progesterone action *in vivo*. It is still not known if these observations in mice can be extrapolated to reproductive function in the human.

A valuable insight about progesterone (and exogenous progestogen) action in human endometrial function may be derived from the observations of pharmacological withdrawal of progesterone from the endometrium (109). Consequently, studies that address the actions of PR antagonists have informed the knowledge base about the local mechanisms that may be targeted to maximize the contragestive and abortifacient properties of these compounds. For example, the antiprogestogen, mifepristone (RU486), is known to exert its inhibitory effects by impairing the gene regulatory activity of the PR (110). Administration of the antiprogestogen, mifepristone, has become a useful model to study local events in both nonpregnant endometrium and early pregnancy decidua *in vivo*. Examples include studies on the antagonism of progesterone action at the level of its receptor that result in an up-regulation of key local inflammatory mediators (chemokines and prostaglandins) and an influx of leukocytes (24, 111).

Evidence for those functions in the nonpregnant endometrium regulated by progesterone may be derived from *in vivo* studies where antiprogestogens have been administered acutely in the secretory phase of the cycle or chronically at a low dose. Administration of an antiprogestogen in the early secretory phase will adversely affect local factors of potential importance to implantation, whereas administration in the mid secretory phase will influence factors implicated in endometrial bleeding (109). An increase in steroid receptors (ERα, PR, and AR) in both the glandular and stromal compartments in mid secretory phase endometrium after early secretory phase (on day LH+2) administration of antiprogestogens has been described by several authors (85, 87, 88, 112); whether this is due to a failure to down-regulate these receptors has yet to be determined. The endometrial changes (including marked alterations in the endometrial vasculature, Ref. 113) associated with withdrawal of progesterone and menstrual bleeding supports the involvement of vasoactive local mediators in this process.

The chronic administration of low-dose oral mifepristone inhibits ovulation and induces amenorrhea or a marked reduction in endometrial bleeding (114), revealing the sensitivity of endometrial morphology to antiprogestogen exposure. Chronic antiprogestogen administration inhibits both endometrial secretion and proliferation and has some intriguing "endometrial antiproliferative effects" (115) that likely involve the AR.

E. Estrogen and estrogen receptors

The precise molecular mechanisms regulated by estrogen in the uterus have not yet been fully defined. Two structurally related subtypes of ER, commonly known as ERα and ERβ, have been identified in the human, as well as in other mammals (73, 74), and reviewed by Saunders and Critchley (116). The ERβ gene, like ERα, is encoded by eight exons with maximum levels of homology between ERα and ERβ present in the DNA and LBDs (75). The function of ERβ in the uterus is still not fully elucidated. In both the human and nonhuman primate endometrium, ERβ, like ERα, is expressed in the nuclei of glandular epithelial and stromal cells and has been reported to decline in the late secretory phase in the functionalis layer (1). However, unlike ERα, ERβ has been detected with both polyclonal and monoclonal anti-ERβ anti-

bodies in the nuclei of the vascular endothelial cells. The presence of ERβ in endometrial endothelial cells indicates that estrogen may act directly on endometrial blood vessels (117, 118). Estrogen may therefore have direct effects on endometrial angiogenesis and vascular permeability changes during the cycle. Thus far, PR is reportedly absent from the vascular endothelium (117, 119) of the spiral arteries. The effect of progesterone withdrawal on these vessels, which plays a key role in menstrual induction, is likely to be indirectly mediated by the PR-positive perivascular stromal cells.

In vitro studies have recently demonstrated that homodimers (ERα-ERα or ERβ-ERβ) or heterodimers (ERα-ERβ) may be formed when both isoforms are expressed in the same cell (120, 121). The amount and pattern of expression of each ER subtype is likely to influence gene transcription within that cell. It has been reported that mRNAs encoding isoforms of human ERβ formed by alternative splicing of the last (eighth coding) exon are expressed in human tissues (122, 123). Both the mRNA and protein corresponding to one of these splice variants (ERβcx/β2) are expressed in human endometrium (124). This splice variant lacks the ligand binding site and may act as a negative inhibitor of ERβ action (122).

A detailed and thorough inclusive investigation of the nuclear receptor and cofactor mRNA levels in human endometrium and myometrium has recently been conducted by Vienonen *et al*. (125) and accompanying editorial (126). This study utilizing real-time quantitative PCR has confirmed previous reports describing the menstrual cycle-dependent regulation of expression of endometrial sex steroid receptors. The expression of coactivators was not observed to be regulated.

Retinoic acid receptor (RAR) isoforms may be implicated in the action of progesterone during the secretory phase of the cycle (127). RARα mRNA expression has been reported as more abundant in the proliferative phase of the menstrual cycle, and this follows the cycle-dependent regulation pattern of other sex steroid receptors (ERα, ERβ, and PR). At the protein level, all isoforms of the RAR, α, β, and γ, have been described as maximal in the late proliferative phase and to decline in the secretory phase (128). The role of the RAR in human endometrium is yet to be determined.

F. Androgens and the androgen receptor

The AR is expressed in human endometrium (85, 129). This reproductive tissue is a target for androgen action either directly via the AR or indirectly via the ER after aromatization to estrogen (130). Circulating concentrations of testosterone have been reported to show little if any changes throughout the menstrual cycle (in contrast to the cyclical variations in estradiol and progesterone). Testosterone levels are, however, approximately 10 times greater than those of estradiol (131, 132). During the menstrual cycle, the AR is expressed predominantly in the endometrial stroma, and there is considerably higher intensity of AR immunostaining during the proliferative compared with the secretory phase (85). Treatment with androgen will suppress estrogen action in the endometrium, and this effect is most likely mediated by endometrial AR. The physiological role, if any, for AR in the menstrual process is yet to be ascertained. Furthermore, the regulation of AR expression is unknown.

In a clinical situation of chronic hyperandrogenism associated with poor reproductive outcome, polycystic ovarian syndrome (PCOS), there is an elevation of expression of endometrial AR (133). The increase in AR expression was observed in the glandular and luminal epithelium. It is of note that the endometrium from women with PCOS also displays aberrant expression of a proposed biomarker for uterine receptivity, αvβ3. The expression of this integrin is modified by estrogen and androgens (133, 134). Endometrial epithelial AR is up-regulated by estrogens and androgens *in vitro*. Expression *in vitro* is inhibited by progestins and EGF (133).

There is also an intriguing up-regulation of the AR in both glandular and stromal cells after administration of antiprogestogen in both human and nonhuman primate endometrium (85). Androgens do suppress estrogen-dependent endometrial proliferation, and Brenner *et al*. (115, 135) hypothesized that the endometrial AR is involved with the antiproliferative effects induced by antiprogestogens. Indeed, Brenner's group (136) has demonstrated in the rhesus macaque that the administration of an antiandrogen, flutamide, will counteract the suppressive effects produced by antiprogestogens on endometrial thickness, stromal compaction, and mitotic index. The endometrial AR may be a critical component of the mechanism by which antiprogestogens suppress endometrial proliferation in the presence of circulating estrogens. Chronic antiprogestogen administration inhibits both endometrial secretion and proliferation (antiestrogen effects). This effect has been described as a "functional noncompetitive antiestrogenic action" of an antiprogestin (137). Because only the endometrial epithelium demonstrates this phenomenon, it has been termed an "endometrial antiproliferative effect" of antiprogestogens (115, 135).

G. Glucocorticoids and the glucocorticoid receptor

Glucocorticoids have been shown to exert specific effects on endometrial cells (138–141), but their role in endometrial physiology is not defined. Bamberger *et al.* (142) have briefly described the localization of GR across the menstrual cycle. The GR is almost exclusively expressed in the stromal compartment including endothelial and lymphoid cells (33, 142). Confirmation for an absence of cycle-dependent expression of GR mRNA expression is provided in the recent data on endometrial mRNA levels across the cycle (125). It has been demonstrated both at the mRNA and protein levels that uNK cells express GR (33). The role of glucocorticoids in endometrial immune function remains to be extensively studied. Elsewhere in the body, the immunosuppressive effects of glucocorticoids have led to their wide application in the treatment of inflammatory states. Suggested roles in the uterus for glucocorticoids include effects on implantation (138), endometrial cellular proliferation (139), apoptosis (140), and endometrial remodeling (70). Glucocorticoids have also been shown to repress the decidual prolactin promoter (143) and corticotropin-releasing hormone promoter (144), both of which are markers of decidualization. This and

the expression of GR in the endometrium (142) may implicate glucocorticoids in the process of decidualization.

Glucocorticoid function is regulated not only by GR expression but also by the expression of steroid-metabolizing enzymes (which determine the availability of the ligand). The 11β HSD family modulates the action of glucocorticoids by converting either cortisone (inactive) to cortisol (11β HSD-1) or cortisol (active) to cortisone (11β HSD-2). Smith (62) reported that levels of the 11β HSD-2 are higher across the nonpregnant menstrual cycle than 11β HSD-1 and that 11β HSD-2 was present in the luminal and glandular epithelium with raised levels in the secretory phase. Hence, it was suggested that the balance of expression of 11β HSD isoforms could facilitate trophoblast invasion by removing the glucocorticoid-mediated inhibition of MMPs. In this context, data have been reported on the expression of 11β-HSDs during *in vitro* decidualization of human endometrial stromal cells (145). Decidualization was observed to involve an enhancement of the corticosteroid-metabolizing capacity of stromal cells, thereby implicating a mechanism by which stromal cells might influence the health and invasiveness of the implanting trophoblast.

Detailed *in vivo* studies of 11β HSD-1 mRNA and protein levels in very early pregnancy tissues have not to our knowledge been reported. To date, there is no commercially available antibody for the immunolocalization of the 11β HSD-1 enzyme. It is therefore of particular interest that uNK cells that express GR mRNA and protein are found aggregated close to the glandular epithelium and also have proposed roles in the control of early trophoblast invasion (146).

IV. Progesterone Withdrawal and the Mechanisms of Menstrual Bleeding

A. Progesterone action in the endometrium

Several array-based studies have examined the changes in gene expression associated with the mid secretory phase of the menstrual cycle (5, 6, 147), and these have recently been reviewed by Dey *et al.* (148). The mid secretory phase of the cycle is a critical time because it represents the height of progesterone priming, providing both the implantation window and heightened sensitivity to progesterone withdrawal. Thus, changes induced at this time will be critical to effective implantation of the blastocyst and, in the absence of pregnancy, to menstruation. These studies have shown good agreement in reporting important gene changes around this time. These investigations of the implantation window have been supported by studies on the induction of the decidualized phenotype in endometrial stromal cells (149, 150). Because this system is more readily manipulable, time courses of gene expression have been possible (149), and these show very rapid increases in well-identified markers of decidualization such as IGF binding protein (IGFBP)-1.

Some changes seen in the mid secretory phase of the cycle such as the expression of Dickkopf-1 (Dkk-I) (5, 147, 151) are novel and revealing. Dkk-I has been established as an inhibitor of the WNT signaling pathway, which is active in human endometrium (152). Although most WNT gene expression does not change through the cycle, the inhibitor Dkk-I in stromal tissue is progesterone dependent (152). WNT-3 was the one gene modulated, with elevated expression during the proliferative phase of the cycle, and it is WNT-3 that is implicated in increased cyclooxygenase (COX)-2 expression and prostaglandin E_2 (PGE_2) synthesis in mammary epithelial cells (153).

Dkk-I inhibits a coreceptor for the Frizzled receptor low-density lipoprotein associated receptor-6, and thus a potential new pathway of progesterone control of endometrial differentiation is apparent (152). Frizzled in turn is implicated in HOX gene control. HOX genes are best characterized as developmental signals, with HOX-10 playing an important role in uterine differentiation (154), but HOX-10 in particular is essential for implantation (155, 156), and pathologies such as endometriosis, polycystic ovarian disease, and the presence of an hydrosalpinx are all associated with aberrant HOX-10 expression (157–159). One role for HOX-10 has been identified as the mediator of progesterone-controlled expression of the prostaglandin receptors E-series prostanoid (EP)3 and EP4 (160).

Additional examples of endometrial genes that are regulated by progesterone include glandular secretion of glycodelin (otherwise known as pregnancy protein 14, progestagen-dependent endometrial protein, or α2 pregnancy-associated endometrial globulin) (161), 15-hydroxyprostaglandin dehydrogenase (PGDH) (162–164), 17β HSD-2 (87), prolactin (46), and calcitonin. The secretion of glycodelin is of interest because endometrial and blood levels are maximal 10 to 15 d after the LH surge (165). Thus, levels remain high (or even increase) after the levels of progesterone begin to fall; explanations for this are made more difficult because of the pleiotropic nature of glycodelin. However, the most likely role for glycodelin in endometrium is as an epithelial morphogen (166, 167), and as such the effect of progesterone is likely to be indirect through the influence of stromal cells. It is thus possible that further differentiation of glandular epithelium is a prerequisite to menstruation.

Expression of calcitonin mRNA has been demonstrated to be temporally restricted to the mid secretory phase of the cycle, a period that coincides with the putative window of implantation (168). The site of postovulatory synthesis of calcitonin mRNA and protein is the glandular epithelium. Evidence for regulation of this gene by progesterone has been derived from examination of endometrium collected from women treated with an antiprogestogen, mifepristone. Calcitonin expression was dramatically reduced in women exposed to acute administration of mifepristone in the early secretory phase (administered day LH+2).

The cellular interactions and progesterone target genes involved in the decidualization process are complex. Multiple growth factors, cytokines, and protein hormones have been recognized as important signals for initiation and maintenance of decidualization (reviewed in Refs. 46 and 169). Gene array techniques have helped our understanding of the uterine changes in the implantation window and their progesterone dependence. Most importantly, the decidualized stromal cell, by virtue of its retained PR, is likely to be a cell that is critically affected by falling progesterone, signaling the onset of menstruation.

The disturbed bleeding patterns reported by women with

the use of progestogen-only contraceptives are likely to reflect modifications in the endometrial vessels from which bleeding arises, including changes in vessel integrity and/or hemostasis. Furthermore, the aberrant bleeding may arise from a different vascular source than normal menstrual bleeding (170). Breakthrough bleeding arises mainly from capillaries and veins adjacent to the uterine lumen and considered to be related to increased vessel fragility (171–173).

The levonorgestrel (LNG)-releasing intrauterine system (LNG-IUS) is now widely used for the management of heavy uterine bleeding, although its principal indication for use is contraception (174). The intrauterine delivery of LNG induces a rapid and dramatic transformation of the endometrium, characterized by extensive decidualization (175, 176). The observed morphological changes are consistent with progesterone-mediated differentiation as observed during the progesterone-dominated secretory phase and during pregnancy (32). With local intrauterine LNG administration, there is no longer cyclical activity within the endometrium and there is a general thinning of the functional layer of the endometrium. The features of atrophy and decidualization are evident within 1 month of insertion of the LNG-IUS. Importantly, the morphology of the endometrium returns to normal within 1–3 months of the removal of the device, and there is a complete return of previous fertility (177). Initially, after LNG-IUS insertion, sex steroid receptor content is decreased with consequent altered expression of local mediators that may play a role in aberrant bleeding episodes. It is disappointing that no single factor has as yet been identified to explain the mechanism(s) of abnormal bleeding patterns associated with the use of this or indeed any other progestogen-only contraceptive. The endometrial responses to local LNG exposure have been documented and in summary include (reviewed in Ref. 178): down-regulation of ER, PR, and AR; expression of prolactin (stroma) and prolactin receptors (epithelium and isolated leukocytes in stroma) and IGFBP-1; elevation of leukocyte infiltrate after insertion (uNK cells, macrophages); enhanced expression of local inflammatory mediators (cytokines and prostaglandins); evidence for aberrant angiogenesis; changes in vessel integrity and/or hemostasis; and abnormally fragile superficial endometrial vessels.

The sc delivery of LNG (Norplant) also has a marked effect on the endometrial vasculature. A decreased expression of a number of components of the endothelial cell basement membrane is evident with Norplant administration (172). These architectural changes are highly likely to play a role in endometrial vessel integrity and fragility. The processes that lead to increased vessel fragility and changes in vessel density are, however, yet to be determined. Indeed, modulation of the vascular basement membrane is likely to be part of a cascade of events that results in aberrant angiogenesis.

B. Decidual changes preceding menstruation

The primary purpose of decidualization is to prepare the endometrium for the implanting blastocyst, but at the same time preparation has to be made for a failed implantation. The depth of the implantation in humans is a characteristic that may necessitate sloughing off a large proportion of the endometrial surface. The changes seen in USCs during the secretory phase will be relevant to the onset of menstruation, particularly as these cells retain the PR. Decidualization of stromal cells can be viewed as differentiation with a concomitant reduction in growth factors induced by an early appearance of agents such as IGFBP-1 and -3 and spermidine/spermine N-acetyl transferase (149, 150, 179). Although IGFBP-1 may have other, non-IGF-related functions (180), it is likely that this binding protein restricts IGF levels in the immediate environment of the secreting cell. IGF is an estrogen-induced growth factor prominent in the proliferative phase (180), and polyamines are also growth factors for endometrial stromal cells (181) whose action is restricted through acetylation by acetyl transferase.

Decidualization is also accompanied by an increase in the secretion of matrix components, particularly such as collagen, fibronectin, and laminin (169). Moreover, agents that degrade matrix such as MMP-3 have to be maintained at low levels by a progesterone and IL-1-mediated mechanism to allow decidualization (182). Cytokine changes that suggest paracrine actions of the decidual cell also occur during decidualization; the decidual cell secretes IL-15, which is an essential growth and differentiation factor for the uNK cell (40, 41, 183) (also see *Section II.C*).

C. Physiological withdrawal of progesterone

The withdrawal of progesterone prevents implantation and converts the refractory pregnant uterus once again into a spontaneously steroid responsive organ (184, 185). The physiological withdrawal of progesterone from an estrogen-progesterone primed endometrium (that occurs with demise of the corpus luteum due to the absence of pregnancy) is also the triggering event for the cascade of molecular and cellular interactions that result in menstrual bleeding. A current hypothesis for menstruation (described below) is based on lines of evidence derived from studies on local endometrial response to progesterone withdrawal (1).

The withdrawal of progesterone up-regulates key inflammatory mediators, many of which have a key perivascular location (25, 186, 187), underlining the role of the stromal cell. Among the agents stimulated are chemokines: the α-chemokine CXCL8 (neutrophil chemotactic factor, IL-8) and the β-chemokine CCL-2 (monocyte chemotactic peptide-1, MCP-1), as well as the inducible enzyme responsible for synthesis of prostaglandins, COX-2 (111, 188).

Early studies of menstruation implicated prostaglandins (189) and accord with both increases in prostaglandin synthesis and decreases in metabolism in response to falling progesterone levels (190). Prostaglandin synthesis via COX-2 is particularly relevant in the vascular compartment because this provides an explanation for the action of nonsteroidal antiinflammatory drugs (which inhibit COX enzyme activity) in menstrual pathology. Moreover, the actions of prostaglandins on blood vessels and surrounding cells is underlined by the significant distribution of prostaglandin receptors in this locus (Fig. 3) (191, 192). PGDH, the enzyme responsible for conversion of prostaglandins to inactive metabolites, is a progesterone-dependent enzyme (163, 164). Antagonism of progesterone action results in an inhibition of

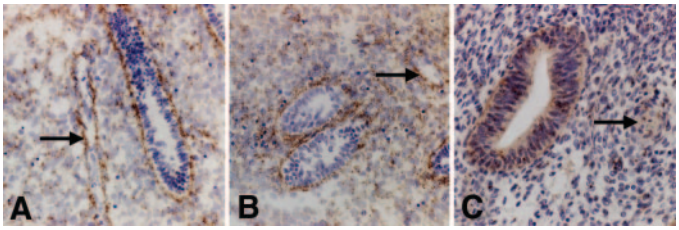

FIG. 3. Localization of EP2 (A), EP4 (B), and FP (C) receptors in the vascular compartment (*arrowhead*) of human endometrium.

PGDH expression, and progesterone withdrawal from decidua clearly demonstrates a perivascular locus for this enzyme (193).

Taken together, these early local responses to progesterone withdrawal result in an elevation of prostaglandin concentrations (PGE_2) and prostaglandin F ($PGF_{2\alpha}$) and potential synergism with the chemokine, CXCL-8 (194, 195). This synergism has been supported by studies in the prostaglandin E synthase knockout mouse, which shows reduced influx of macrophages in mice with ablated microsomal prostaglandin E synthase when compared with the wild type (196). There is a marked perimenstrual influx of leukocytes consisting of neutrophils, macrophages, and other hematopoietic cells, and before menstruation the neutrophils constitute 15% of the USC population (58). Whether CXCL8, which is widely expressed in human endometrium (25, 186, 197), is the primary chemotactic signal for neutrophil entry in endometrium is uncertain because many other CXC (α-chemokine) ligands such as CXCL1 [GROα (198], CXCL2 [GROβ (199)], CXCL5 [ENA-78 (200)], CXCL6 [GCP-2 (199)], and CXCL10 [IP10 (201)] have been reported.

It has long been accepted that myometrial contractions and vasoconstriction are a consequence of an increased production of $PGF_{2\alpha}$, consequent upon progesterone withdrawal (190). Coincident vascoconstriction of the endometrial spiral arteries takes place (2), and so the uppermost endometrial zones are presumed to become hypoxic. Hypoxia is a potent inducer of angiogenic and vascular permeability factors such as VEGF (202), and a hypoxia-dependent mechanism to initiate menstruation is attractive, but there is however controversy concerning the role for hypoxia (if any) in the menstrual process (203).

The angiogenic factor, VEGF is a local mediator stimulated by hypoxia in endometrial stromal cells (63). Progesterone withdrawal has been reported to up-regulate the endometrial stromal expression of the VEGF type 2 receptor, kinase domain receptor (KDR), in women and nonhuman primates (204). This stromal but not vascular endothelial expression of KDR is blocked by adding back progesterone 24 h after progesterone withdrawal. Pro-MMP-1 is also up-regulated in a coordinate manner in the same stromal cell population by withdrawal of progesterone. Furthermore, Nayak and Brenner (205) have described the up-regulation of VEGF-A mRNA in the glands and stroma of the same superficial endometrial zones. Hence, given that VEGF-A, KDR, and MMPs are coordinately expressed by stromal cells of the upper zones of premenstrual stage endometrium at the time of progesterone withdrawal, the conclusion is that a VEGF-KDR-MMP link is an important component of the premenstrual/menstrual process (115, 117, 204).

D. Menstruation, the sequence

Thus, early events occurring in PR-positive cells herald the onset of menstruation but may be inhibited by "add back" of progesterone. In the rhesus macaque monkey, the adding back of progesterone before 36 h following progesterone withdrawal prevented menstrual bleeding (206). However, add back of progesterone after 36 h was ineffective in preventing the onset of menstruation. Thus, the withdrawal of progesterone will initially affect cells expressing the PR in a reversible manner. These early, progesterone withdrawal events in menstruation involve vasoconstriction and cytokine changes (207). Subsequent events are likely to be irreversible and include the activation of lytic mechanisms in a cascade of activation of pro-MMPs and accentuation by hypoxia. Hence, the latter phase of menstruation is progesterone independent and will involve cells that may not express the PR. These changes will involve the disruption of the progesterone-dominated epithelial-stromal interaction that suppresses key mediators such as IL-1, MMP-1 (208), and MMP-7 (209). IL-1 may be particularly important because this cytokine has far-reaching effects needing tight control by the multiple pathways that include posttranslational modifications, decoy receptors, and the receptor antagonist (IL-1 receptor antagonist) (Fig. 4).

E. Origin and control of MMPs—the final effectors

Currently, there is no certainty as to the origin of the MMPs, which starts the process of menstruation. These could arise from the stromal cells, particularly those surrounding the blood vessels, or they could arise from invading leukocytes, prominent among which are neutrophils. Progesterone suppresses several stromal cell-derived, lytic enzymes such as urokinase-type plasminogen activator (uPA), MMP-1, and MMP-3 (210). Moreover, as described above, progesterone inhibits neutrophil entry into endometrium, a process controlled by the stromal cells which, during the critical secretory phase, is the main cell type retaining the nuclear PRs in the functional layer of the endometrium. Immediately before the onset of menstruation, the phenotypical change characteristic of decidualization has already occurred in many stromal cells. Thus, the cell that would have facilitated implantation in the event of pregnancy will be the cell that responds to continuously falling progesterone levels and initiates menstruation. This will result in either activation of MMPs or the release of chemotactic agents that recruit neutrophils.

The roles of the leukocyte and the resident stromal cell scenarios are not mutually exclusive because early progesterone-related events are dependent on the PR, which is absent from leukocytes. Moreover, local release of agents such as MMP-1 and MMP-3 from the stromal cell (210) may activate other proteases released from the invading neutrophils. This is supported by the presence of substantially more latent MMP-9 than active MMP-9 before menstruation (211).

1. MMPs from stromal cells. In general, MMPs in uterine cells are repressed by progesterone, but this is not always direct

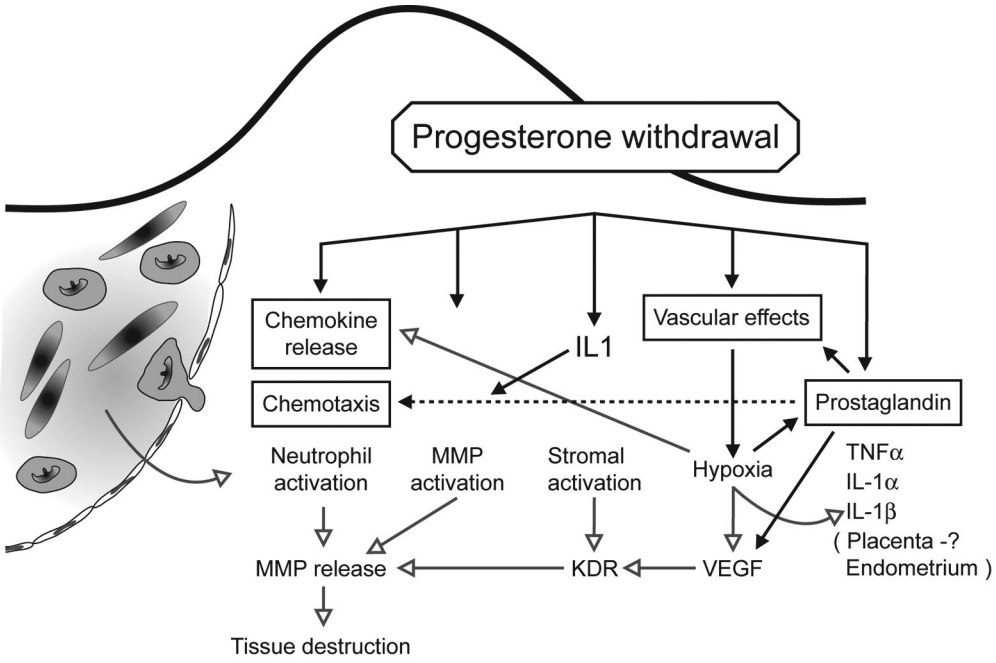

FIG. 4. Progesterone withdrawal activates many pathways; prominent among these are those releasing vasoactive agents. Chemotactic agents, which synergize with vasoactive agents, are also expressed. There is no doubt that MMPs, which degrade the interstitial matrix of the endometrium, play a major role in the menstruation process, but their origin is uncertain. MMPs can be released from both resident endometrium stromal cells and invading leukocytes.

and may involve interaction with the stromal cell. Indirect action is likely to affect MMPs from both epithelial and endothelial cells. In epithelial cells, MMP-7 is suppressed at a time of high progesterone concentrations because of the release of TGF-β from stromal cells in response to progesterone (209). The endothelial cell may also respond to TGFβ-1 signaling because it does not possess the nuclear PR at any stage in the cycle. These interactions underline the critical role of the stromal cell in the initiation of menstruation. By the late secretory phase, the spiral arterioles are surrounded by a characteristic cuff of distinct cells expressing smooth muscle characteristics (*e.g.*, smooth muscle actin expression) in common with decidual cells (23). Withdrawal of progesterone from decidua shows that these distinctive cells release proinflammatory agents in a manner that is clearly different from surrounding decidual cells (24, 25).

Observation of changes *in vivo* suggests progesterone-dependent suppression of collagenase and other lytic enzymes in the uterus (212) and in particular the suppression of MMP-1 and MMP-3 (213) and MMP-9 (214). The effects of progesterone on the lytic enzymes may be enhanced by progesterone-stimulating synthesis of tissue inhibitors of MMPs (TIMPs), such as TIMP-3 (215). Although mRNA for MMP-1 and MMP-3 can be detected in proliferative phase endometrium by *in situ* analysis, this disappears during the secretory phase. Moreover, when progesterone levels decline in the late secretory phase, expression of MMP-1 and MMP-3 is reinitiated. These findings are supported by evidence from culture studies showing that MMP-1 expression and protein release were inhibited in the presence of progesterone (216).

Initial steps in the degradation of collagen are ascribed to MMP-1, and thus this enzyme is critical to the stability of the basement membrane of the blood vessels. After initial cleavage by MMP-1, other MMPs will contribute including MMP-2, which, although expressed throughout the menstrual cycle in stromal cells (217), rises in response to the withdrawal of progesterone (218).

Because MMP-1 plays a key role in extracellular matrix breakdown, tight control of the protein is necessary. This is achieved by transcriptional control as well as control of the stability of the mRNA. The coding region for MMP-1 contains both activator protein-1 and nuclear factor κB (NFκB) response elements (219) that allow stimulation not only by inflammatory agents such as IL-1 and TNFα but also by progesterone.

MMP-1 and MMP-3 have similar promoter regions and in this respect differ from MMP-2 (220). NFκB is important in control of MMP-1, MMP-3, and MMP-9 and, when activated along with other transcription factors, can stimulate many other proinflammatory genes such as inducible nitric oxide synthase, TNFα, IL-1β, toll-like receptor-4, and COX-2 (221, 222). NFκB may be controlled by progesterone in several ways: progesterone may stimulate inhibitor of κBα, the protein that retains NFκB outside the nucleus (223), or the PR may compete with binding sites for NFκB on promoter regions of the gene (224). However, the real key to the importance of NFκB is the finding that TGFβ-1 and progesterone may have a coordinate suppressive effect (225) that may be through NFκB because of the interactions of both with this pathway.

Suppression of MMP-1 production is affected by TGFβ-1 through a SMAD3/4-dependent mechanism (226) acting on NFκB. This inhibition occurs by competition between SMAD3 and the NFκB complex for P300, which is a transcriptional coactivator for both (226). This results in an inhibition of the acetylation of key lysine residues that would

normally render the NFκB complex immune to inhibition by inhibitor of κBα (226).

In addition, apart from suppressing MMP activity, TGFβ-1 is also responsible for the stimulation of expression of TIMPs (227) and increasing the synthesis of major matrix proteins such as collagen and fibronectin (228). The other function of TGFβ is the control of cell growth, and epithelial cell growth is inhibited by TGFβ (229). Thus, TGFβ-1 stabilizes tissue by limiting MMP activity, which accords with the cyclical expression of TGFβ with the highest levels in the secretory phase stromal cell (230, 231). Moreover, TGFβ-1 has to be activated in a proteolytic step, although little is known about the physiological agents involved. One candidate lytic enzyme is uPA, which is reduced in the mid secretory phase (231) but is expressed when progesterone levels fall before menstruation. uPA activity in turn is inhibited by tissue factor (TF), which is progesterone dependent (232) and appears in stromal cells in the secretory phase of the cycle and in decidua (233). Expression of TF, which in decidualized endometrial stromal cells is both delayed and chronic (234), is largely controlled by the specificity protein (SP)1 transcription factor (235). *In vitro* studies show that progesterone stimulates SP1 and inhibits SP3, which antagonizes SP1, and that SP1 is enhanced and SP3 ablated in perivascular cells in the secretory phase of the menstrual cycle (235). Because TF is a major hemostatic agent (236), it is beneficial in perivascular cells in that potential bleeding will be limited around the time of implantation and later when extravillous trophoblast cells invade the maternal arteries. However, TF levels fall before menstruation to allow menstrual-associated hemorrhage (237).

Another relevant gene with an SP1 response element is plasminogen activator inhibitor-1 (PAI-1) which inhibits the fibrinolytic pathway. Thus, progesterone stimulates PAI-1 expression in endometrial stromal cells, possibly moderating decidual cell migration within tissue. Trophoblast certainly expresses plasminogen activator that is inhibited by a PAI-1-vitronectin complex, and thus the expression of PAI-1 by decidua may be a mechanism for the restriction of trophoblast invasion. With the decline of progesterone, PAI-1 expression will be restricted, and thus fibrinolytic activity will be present at the time of menstruation, which will account for the reduced clotting in menstrual blood.

2. MMPs from leukocytes. Invasion of the endometrium by leukocytes is an integral part of the process of menstruation, both contributing to tissue breakdown and repair (see *Section I.C*). Leukocytes that are attracted into the uterus before menstruation are a major source of lytic enzymes. The neutrophil in particular represents an almost unlimited source of MMP-8 (neutrophil collagenase) and MMP-9 (gelatinase B). The neutrophil characteristically has a high turnover rate and a high production rate of 10^{11} cells per day with any deficiency quickly replenished by the bone marrow (238). Although neutrophils usually have a lifetime of only a few hours, their lifetime is increased in inflammatory situations. The role of the neutrophil may have been underestimated because current views maintain that an excess of MMPs over TIMPs is necessary for lytic activity. However, a surface-bound form of MMP-8 has been identified on the neutrophil membrane, and this is not affected by TIMPs (239). The other major cell type to invade the endometrium before menstruation is the monocyte, which is a source of MMP-1, MMP-3, and MMP-9 as well as TIMPs. Macrophages and neutrophils represent the two major groups of phagocytic cells, and clearing debris that is not discharged into the lumen may be an important function of these cells.

F. Animal models of menstruation

A mouse model of menstruation was first reported by Finn and Pope (240). Mouse uteri, in an animal treated with progesterone, were induced to decidualize with mineral oil. In this model, progesterone withdrawal results in an influx of leukocyte and tissue shedding. To this extent, the mechanism of menstruation is reproduced. This study found that "menstrual" shedding induced by progesterone withdrawal only occurred if the uterus had been decidualized with the oil. This was a key finding implicating the decidual cell in the initiation of menstruation. Thus, in women, the predecidualization of cells in the normal secretory phase is sufficient to prime these cells to progesterone withdrawal, which in turn suggests that defects in decidualization may lead to menstrual pathology. This study has recently been reevaluated (241) and refined using silastic implants which, on removal, will give a more rapid and predictable decline in progesterone levels. This new technique allows a better assessment of leukocyte dynamics and the role of apoptosis in menstrual shedding and thus allows the delineation of very early events after progesterone withdrawal. The limitation is that mouse leukocytes respond differently from human leukocytes, and chemotactic agents such as IL-8 have no direct equivalent in the mouse. However, such a model may well answer the critical question: what is the initial site of MMP expression upon progesterone withdrawal?

V. Repair and Vessel Regrowth

After menstruation, the endometrium is programmed to regrow under the influence of estradiol. The restructuring of the functional layer is critical to the development of a tissue ready for implantation or for menstruation. Vessel growth is particularly important in the endometrium of menstruating species where the spiral arterioles are a characteristic feature. Indeed, early studies in a primate model show that straight arteries growing into the functional zone acquire a coiled structure (2). The growth of these vessels is clearly important but poorly understood. Early studies of intraocular endometrial explants in the rhesus monkey (2) showed that the degree of bleeding was related to the vascular growth within the preceding cycle.

A. Epithelial growth

After separation of the functional layer of the endometrium, the regeneration of all cell types, epithelial, endothelial, and stromal, occurs rapidly. The remaining basal layer acts as a germinal compartment from which the different cell types grow and differentiate (242). Slow-growing pluripotent stem cells have been reported in primate endometrium

(243) where they are resident in the basal layer nearest to the myometrium. These cells differentiate into committed progenitor cells with increased growth in the adluminal half of the basal layer. These progenitor cells are responsive to appropriate growth factors that ensure a very rapid growth of tissue.

Regrowth is estrogen dominated, and for epithelial cells EGF, TGFα, and EGF receptor are all likely to be involved. Both TGFα and EGF compete for the EGF receptor, and both, along with platelet-derived growth factor (PDGF), are mitogens for epithelial cells from the basal layer (242). Although endothelins are found in endometrial epithelial cells (244) and are potent mitogens for these cells, their receptors are maximally present in the secretory phase of the cycle (245). However, the contribution of endothelins to early epithelial growth should not be fully discounted because endothelin receptor subtypes termed ETA and ETB are present (246) and the critical question is whether receptors will be sufficiently expressed in the residual epithelial cells after tissue shedding.

The best evidence of early endometrial growth is presented in a scanning electron microscopic study of human endometrium by Ludwig and Spornitz (247). After the shedding of the functional layer, the exposed surface is covered by fibronectin and leukocytes. This fibronectin is rapidly removed once epithelialization occurs. Regrowth of the epithelium occurs soon after shedding: growth from the stumps of the glands starting on menstrual d 2, and surface epithelium grows out of cone-shaped edges to the glands, rapidly covering the luminal surface (248), two thirds of which is covered by d 4 and which is fully covered by epithelium at least by d 6 of the cycle (249).

B. Pattern of angiogenesis during the cycle

The uterus and the ovary are the principal sites of active cyclical angiogenesis in the adult. Changes in angiogenesis and the process of vascular development in the endometrium have been quantified in the human, nonhuman primate, and rodent models. During the last few years, many of the factors regulating angiogenesis have been identified, but their role in regulation of endometrial angiogenesis remains to be elucidated. The field is being aided by the development of specific antagonists to angiogenic factors, providing powerful tools by which the functional role of a factor in tissues such as the endometrium can be established by *in vivo* experimentation in suitable animal models.

It is generally agreed that angiogenesis occurs in at least three different stages during the menstrual cycle. The first stage is at menstruation to repair ruptured blood vessels; the second, during the proliferative phase during the period of rapid growth of the endometrium; and the third, during the secretory phase with development of the spiral arterioles and growth of the subepithelial capillary plexus (250, 251). Patterns of angiogenesis throughout the cycle in the human endometrium have been studied using proliferating cell nuclear antigen (PCNA) or Ki67 to identify proliferating cells, together with dual staining by an endothelial cell marker. During the last 20 yr, numerous determinations of changes in angiogenesis throughout the menstrual cycle been reported. Recent reviews of these studies have led to the conclusion that although there was a tendency for angiogenesis to be highest during the proliferative phase, no significant peaks of endothelial cell proliferation at the various stages of the human cycle were apparent (252). However, it is pointed out that quantification is made particularly challenging because endometrial cell proliferation rates can be extremely variable at the same stage of the cycle.

Nayak and Brenner (205) suggested that lack of clarity in the pattern of angiogenesis in the human endometrium may in part be the result of variation in hormone levels at the time of sampling or to variations in the region from which biopsy was obtained. To obtain a precise relationship between steroid exposure and changes in endometrial angiogenesis, these workers studied ovariectomized monkeys treated sequentially with estradiol and progesterone implants to create artificial menstrual cycles. The progesterone implant was withdrawn to induce menstruation, with the estradiol implant either left in place to mimic the proliferative phase or also withdrawn to create a hormone-deprived state that was studied between 2 and 14 d. Additional groups were manipulated to represent the mid- and late secretory phases of the cycle. This detailed and elegant study appears to have provided the most precise model required to elucidate some of the important questions that could not be addressed in the human. Using Ki67 or bromodeoxyuridine to identify proliferating endothelial cells, a 6-fold increase in angiogenesis was observed 8–10 d after progesterone withdrawal (mid-proliferative phase). Apart from a nadir at menses, there were no significant changes in angiogenesis at any other stage of the artificial cycle. This increase did not occur in the hormone-deprived macaques, showing it to be estrogen-dependent. The authors suggest that a steady level of angiogenesis may be sufficient to explain the increased vascularity occurring during the secretory phase.

The finding that most vascular proliferation occurs during the mid-proliferative phase in the macaque concurs with an early study in the human (253), although these authors demonstrated a second wave of endothelial cell proliferation during the mid secretory phase. The explanation of tight synchrony of sampling in relation to progesterone and estradiol levels in the macaque study seems to be the most logical to account for the differences in results to the detailed data from the normal human cycle (252). However, the peak in angiogenesis is so marked that it would be surprising that it was not revealed to some degree by the observations in the human. Thus, other explanations need to be considered, of which the most likely are subtle differences in the steroidal profile induced during the artificial cycles, the influence of ovarian products in addition to estradiol and progesterone, or species variation. If, as it seems, measuring changes in endothelial cell proliferation fails to reveal changes in angiogenesis at different stages of the cycle in human specimens, the question arises as to whether other measures of blood vessel growth would be more informative. It is generally agreed that most of the vascular proliferation occurs in the upper zones of the human and nonhuman primate endometrium (254). In the endometrium, proliferating endothelial cells are found within existing vessels, rather than in association with vascular sprouts as is common in ovarian

and tumor angiogenesis (250). This suggested that growth of blood vessels in the endometrium may involve more complex mechanisms. Consequently, Gambiono *et al.* (251) compared the three mechanisms of angiogenesis, sprouting, intussusception, and elongation (255), in full thickness endometrial sections from women. Sprouting is the most common mechanism and involves breakdown of the basement membrane, endothelial proliferation and migration, sprout formation from existing vessels, and lumen formation. Nonsprouting angiogenesis, or intussusception, occurs from proliferation of endothelial cells inside a vessel and results in remodeling of a vessel internally into two discrete capillaries as dividing cells migrate inward (256, 257). Elongation occurs by restructuring of existing vessels by longitudinal growth. Gambiono *et al.* (251) performed stereological analysis of blood vessel length density, branch point density, and mean vessel length per branch point for three endometrial zones during five phases of the human menstrual cycle. This study distinguished between determination of peaks of endothelial cell proliferation and measuring new vessel formation. They concluded that vessel elongation is the major mechanism by which endometrial angiogenesis occurs between the early and the mid to late proliferative phase, whereas intussusception is the main angiogenic mechanism in the early-mid secretory phases. Blood vessel length density was highest at the mid-late proliferative and early to mid secretory phases of the cycle, demonstrating that between the early proliferative and mid to late proliferative phases, new vessel growth on a length per unit volume basis occurs more rapidly than surrounding tissue growth. These patterns were similar in each area of the endometrium. The results imply that proliferative phase estrogen may drive vessel elongation, whereas vascular remodeling occurs during the secretory phase. Evidence for elongation of endometrial vessels during the early proliferative phase has also been provided by Maas *et al.* (257). Thus, angiogenesis in the endometrium is among the more complex in the body and supports the concept of organ-specific angiogenesis.

Because it appears that endometrial angiogenesis in the human during the normal cycle is not subject to detectable changes in response to cyclical hormonal variations, increasing emphasis has been placed on animal models in which the roles of specific factors such as the ovarian steroids can be elucidated by precise manipulation of steroid exposure. The rhesus monkey model described above is a powerful model but has restricted availability (205). Use of this model showed an estrogen-dependent peak in angiogenesis and lowest endothelial cell proliferation at menses and also showed that withdrawal of both estrogen and progesterone resulted in a fall in proliferation within days (205).

Most detailed studies have been carried out in the mouse. In agreement with the primate data, ovariectomized adult animals, 7 d after castration, had extremely low endothelial cell proliferation that was markedly increased by 1 d after commencement of estradiol administration (258). Angiogenesis was maintained at a lower rate on d 2 and 3 of the study. Progesterone replacement appeared to have an initial inhibitory effect, but continued treatment was associated with a second peak in angiogenesis 2–3 d after treatment with estradiol and progesterone. The rapid effect of estrogen raised the question of direct effects of the steroid on the endometrial endothelial cells. It should be noted that in this model, vascular density was paradoxically highest in the endometrium of ovariectomized animals and was lower in all the steroid-treated animals. This was the result, at least in part, of increased edema after replacement (258). The concept that estrogen induces angiogenesis in the mouse was challenged by the findings of Ma *et al.* (259) who proposed, using reporter and mutant mice, that although estrogen stimulates vascular permeability, it has an inhibitory effect on uterine angiogenesis, whereas it is progesterone that provides the angiogenic stimulus. The importance of these models is that by defining peaks of angiogenesis after stimulation by ovarian steroids, the role of angiogenic factors and their receptors in mediating these changes can be determined by specific inhibitors as is discussed further below.

As the endometrium grows and matures with accompanying angiogenesis during the cycle, it might be expected that quantification of blood vessel density would demonstrate a progressive increase as the cycle progressed. However, measuring blood vessel density shows that it does not appear to be influenced by cycle stage in human (260) or baboon (261). Paradoxically, in some instances where steroidal stimulation is ablated and angiogenesis is low, blood vessel density may increase. It is apparent that simply measuring blood vessel density in a given area fails to take into account changes in endometrial thickness. In addition, results will be influenced by changes in the volume of other cellular components and by changes in edema.

Another marker of vessel maturation into specialized arterioles is reflected by the acquisition of vascular smooth muscle cells (262); however, detecting these cells with the markers smooth muscle actin and myosin heavy chains did not reveal significant cyclic changes (263, 264).

C. Angiogenic factors and their receptors in the uterus

The cyclic endometrium has constant angiogenic potential. Human endometrial fragments implanted onto the chick embryo chorioallantoic membrane *in vitro* assay demonstrated angiogenic activity for all stages of the cycle, with lowest activity at the late proliferative phase (257). This angiogenic potential constitutes the activity of a large number of pro- and antiangiogenic factors that have been reported in the uterus and have been the subject of a recent review (265). Here, we will focus on the most actively studied factors, especially where they have been the subject of manipulative experiments in animal models. Of the known angiogenic factors, VEGF, also known as vascular permeability factor, is a major specific stimulator of endothelial cell proliferation and vascular permeability and has attracted most attention with respect to the endometrium. The most significant of the VEGF family is VEGF-A (hereafter VEGF), which is produced in five isoforms (266). Other members are -B, -C, and -D, together with placental growth factor. VEGF acts through two tyrosine kinase receptors, VEGFR-1 (flt-1) and VEGFR-2 (KDR), the latter being considered the most important in regulation of angiogenesis. In addition, VEGF-C acts via a third receptor, VEGFR-3, which is present on lymphatic cells, as well as on VEGFR-2. VEGF is generally considered to be

the critical factor for the development of the vascular system because inactivation of a single VEGF allele results in embryonic lethality (266). In addition, VEGF acts as a survival factor for immature vessels (267).

Coordination of blood vessel formation, maintenance, stabilization, and regression also involves other factors. These include the angiopoeitins, Ang-1 and Ang-2, which are of particular interest with respect to regulation of endometrial vasculature because Ang-2 causes destabilization of vessels to help initiate angiogenesis, whereas Ang-1 confers stability to new blood vessels (268), divergent processes that occur over a short time frame in the endometrium. Ang-2, acting through its tyrosine kinase receptor Tie-2, is proposed to enhance the action of VEGF by reducing endothelial contact with the extracellular matrix, and hence with adjacent endothelial cell interactions. In contrast, Ang-1 acting via Tie-2 as a competitive antagonist enhances the stability of the newly formed blood vessels by recruiting pericytes (268). It was proposed that during periods of vascular regression, VEGF would be down-regulated, whereas Ang-2 would be increased, leading to destabilization and regression of newly formed vessels.

FGF and PDGF are also known to stimulate angiogenesis and have been demonstrated in the endometrium of a number of species (265). A novel angiogenic factor, endocrine gland VEGF (EG-VEGF) and its receptors have recently been reported to be expressed in the uterus (269). EG-VEGF is unrelated structurally to VEGF and was originally described as being preferentially expressed in steroidogenic tissues (270); however, the same sequence was reported independently in the small intestine and named prokineticin-1 (271).

Naturally occurring angiogenesis inhibitors may also play a role in the regulation of the endometrial vasculature. Thrombospondin 1, a multifunctional extracellular protein with inhibitory effects on endothelial cell proliferation, has been shown to be stimulated by progesterone, is detected at highest levels during the secretory phase in human endometrium, and has been proposed to act as an inhibitory factor at this stage of the cycle (28, 272).

D. Localization and changes in angiogenic factors throughout the cycle

It had been anticipated that determination of the localization and quantification of these angiogenic factors and their receptors in the endometrium during different stages of the cycle would help clarify their roles in regulation of the endometrial vasculature. Although considerable progress has been made, a definitive picture has yet to emerge from the data generated from studies on the human endometrium. This in part has been the result of a lack of uniformity in findings between groups coupled with the difficulties presented in correlating changes in expression patterns to angiogenesis when distinct peaks of endothelial proliferation are absent. Techniques employed have commonly been quantitative PCR, *in situ* hybridization, and immunohistochemistry. Sometimes, apparent differences in results between groups must be ascribed to technical reasons, such as differences in precision of quantitative PCR or differences in the sensitivity of *in situ* hybridization, whereas immunohistochemical studies may be influenced by specificity of different antibodies.

In the human and nonhuman primate endometrium, it is established that VEGF mRNA and protein are localized in both the stroma and glandular epithelium. In addition, VEGF protein is also found in neutrophils (50, 256). There is general agreement that VEGF produced in the epithelial cells is largely secreted apically and may not be involved in angiogenesis (156). However, the possibility of significant amounts diffusing across the epithelial cell basement membrane to induce a gradient affecting the subepithelial complex of capillaries is also favored by some (271, 273). Because most studies report a higher level of expression of VEGF in the glands than the stroma, this makes for potential difficulties when trying to correlate changes in endometrial VEGF with angiogenesis at different stages of the cycle.

Although a number of early studies seemed to provide evidence for cyclic changes in VEGF mRNA and protein during specific stages of the cycle, subsequent work fails to confirm these observations (274). Detailed studies in the human (252) and baboon (261) uterus did not detect a significant change in stromal VEGF mRNA at different stages of the cycle. However, the presence of neutrophils, in blood or near blood vessels, containing VEGF protein correlated with endothelial cell proliferation (256). These authors propose that correlating changes in focal, rather than bulk, VEGF is crucial for understanding the regulation of endometrial angiogenesis.

In the rhesus macaque with synchronized artificial cycles described above, changes in VEGF and its receptors were determined by *in situ* hybridization. VEGF mRNA production in the superficial stromal zone correlated with the peak in angiogenesis at the mid-proliferative phase, whereas changes in VEGF mRNA in the epithelium did not relate to angiogenesis (205). Localization of VEGF receptors by *in situ* hybridization has proven challenging, but clear expression has been described in this study, especially in blood vessels immediately below the surface epithelium (205). Highest levels were found during the postmenstrual repair phase. A unique rise in VEGFR-2 mRNA occurs during the menstrual premenstrual/menstrual phase in the macaque and human endometrium (204). Here, expression is increased in the upper endometrial zone stroma, rather than endothelial cells, and it has been proposed that this change mediates a VEGF-induced up-regulation of MMP-1 (see *Section IV.C*).

Expression patterns of the angiopoietin family in the human endometrium have been examined, although results have been conflicting (264), perhaps reflecting a relatively low expression of these factors. Ang-2 expression is consistently greater than that of Ang-1 (52, 275). Li *et al.* (52) demonstrated that uNK cells are a major source of Ang-2 mRNA, reaching a peak during the late secretory phase, whereas Ang-1 was absent from these cells and was not detectable by *in situ* hybridization in the same study. Using nonisotopic *in situ* hybridization combined with immunocytochemistry, a wider distribution was reported with Ang-1 and Ang-2 in the stromal and glandular epithelium (275). A subsequent study employing RT-PCR found an increase in Ang-1 mRNA in the human endometrium during the secretory phase, whereas Ang-2 and Tie-2 showed only minor variations (276). The latter results pointed to a stabilizing role for Ang-1 during the

secretory phase. Li *et al.* (52) suggest that the production of Ang-2 by uNK cells may serve to antagonize Ang-1 during the premenstrual period to destabilize blood vessels and induce vascular regression before menstruation.

Expression of EG-VEGF/prokineticin-1 has been shown to be highest during the secretory phase of the human menstrual cycle (269), and it has been proposed that its role may be in vascular differentiation and spiral artery formation during the secretory phase. In addition, its presence in myometrial smooth muscle, as well as intestinal smooth muscle, suggests that it may also play a role in myometrial contraction (269).

E. Regulation of endometrial angiogenic factors

In many tissues, the stimulus for increasing VEGF and other angiogenic factors is hypoxia, mediated via hypoxia-inducible factor activation, which acts rapidly to increase translational efficiency, stabilize the protein or enhancement of transactivation (277). However, in endocrine glands such as the ovary, the gonadotropins exert an additional regulatory mechanism that seems to fluctuate in importance according to the stage of follicular or luteal development (278). Because ovarian steroids dominate endometrial growth, it is not surprising that they will impact on the regulation of angiogenesis and vascular development (279). However, as discussed above, there is no clear relationship between the marked fluctuations in ovarian steroid secretion seen during the normal cycle to changes in angiogenesis in the human, whereas there is evidence that hypoxia plays an additional role in stimulating synthesis of VEGF (63, 280).

Despite there being little clear evidence for a close relationship between estrogen peaks and angiogenesis, there is no doubt that estrogen stimulates VEGF mRNA in the rodent, primate, and human uterus when administered exogenously. Overall, there is good evidence from experimental models that exposure to estradiol during the proliferative phase drives VEGF production and angiogenesis, suggesting a crucial maintenance role throughout the cycle (251, 261, 273, 279). In the rhesus monkey model of Nayak and Brenner (205), withdrawal of progesterone resulted in a marked transitory rise in VEGF mRNA in the stroma and luminal epithelium. This was not estrogen-dependent because it occurred at the same time after withdrawal of both steroids. An additional rise occurred 8–10 d after progesterone withdrawal (mid-proliferative phase); this was estrogen dependent because total steroid withdrawal resulted in suppression of VEGF mRNA over the same time period (205). Also, without estrogen replacement, mRNA for VEGF receptors declined. Because receptor synthesis is predominantly dependent on VEGF, the authors suggest that estradiol maintains expression of the receptors through stimulation of VEGF. Alternatively, the discovery of ERβ on the vascular endothelium provides a pathway by which a direct effect could be mediated (117). The acute response of VEGF mRNA to estradiol has been studied in the baboon 60 d after ovariectomy. VEGF synthesis was markedly reduced compared with intact animals, but within 2 h of estradiol treatment VEGF synthesis was stimulated in stroma and epithelium, dissected by laser capture, whereas no effect was observed after progesterone administration (261, 273). Thus, despite the absence of a clear relationship between fluctuations in estradiol and VEGF during the cycle, there is convincing evidence from ovariectomized, estrogen-replaced animals that estrogen stimulates VEGF synthesis. Thus, it is likely that estradiol has a role in maintaining VEGF expression throughout the menstrual cycle.

VEGF also has potent permeability properties, and the secretory phase is accompanied by increased vascular permeability and stromal edema. The growth-stimulating effects of estrogen are in part the result of increased vascular permeability and water retention. It has been proposed that this is mediated via estrogen-induced VEGF production (273).

F. Effects of manipulation of angiogenic factors: in vivo models

Because defining the relationship between endogenous angiogenic factors and changes in angiogenesis has proven elusive in many studies, it is important to take advantage of the increasing availability of specific antagonists to putative angiogenic factors to conduct experiments on appropriate *in vivo* models. Detailed evaluation of the effects of manipulation of angiogenic factors on normal endometrial angiogenesis must also be determined before the concept of pro- or antiangiogenic therapy for treatment of menstrual/endometrial pathologies can be evaluated.

However, the absence of a clear cyclical pattern of endothelial cell proliferation in the human in most studies published to date (252, 255) and in some animal models means that targeting a specific period where angiogenesis is known to be intense may be difficult. To overcome this problem, studies have focused on use of animal models with steroid manipulations known to induce changes in angiogenesis.

In the ovariectomized mouse treated with estrogen for 24 h to stimulate angiogenesis, inhibition of VEGF by the tyrosine kinase VEGFR-2 inhibitor, SU5416, or a VEGF antibody has been shown to almost completely inhibit endothelial cell proliferation (281). A less dramatic effect was observed in mice receiving longer-term estrogen and progesterone replacement. In another study, inhibition of VEGF had no effect on angiogenesis but inhibited edema and epithelial cell proliferation (282). The proliferation rates recorded in control mice differed markedly between the two studies (28 and 1%, respectively), probably attributable to technical differences such as the fact that perfusion fixation was employed by Heryanto *et al.* (281) in an effort to retain the size of the lumen of capillaries to allow more effective identification of proliferating endothelial cells. When FGF and PDGF were also inhibited by specific antagonists, SU5402 and SU11685, respectively, angiogenesis was inhibited in ovariectomized mice receiving estrogen and progesterone replacement (281). In the immature estrogen-treated rat, VEGF immunoneutralization was also shown to block estrogen-induced edema (283). In the adult rat, anti-VEGF treatment blocked implantation (284).

Further developments in the synthesis of specific antiangiogenic agents will help to elucidate the role of these factors

in endometrial angiogenesis. Use of these tools in *in vivo* nonhuman primate models is eagerly awaited.

VI. Disorders of Menstruation

A. Parameters of normal menstruation

The menstrual cycle is generally defined in the context of its length, regularity, frequency, and pattern of menstrual blood loss. These parameters have been well defined in population (285) and long-term observational studies (286) and have been recently reviewed by Fraser and Inceboz (287). These studies have indicated that the mean menstrual cycle length in the mid-reproductive years is between 28 and 30 d. The duration of the period of menstruation is commonly 4–5 d, with the period of heaviest bleeding reported in the first 2 d of menses, whereas the volume of blood loss varies between 25 and 35 ml (for review, see Ref. 287).

B. Menstrual dysfunction

Disorders of the menstrual cycle place a considerable burden on general practice and specialist health service resources (288) and are the most common indication for hysterectomy (289). It has been postulated that the increasing incidence in menstrual disorders is due to changes in life style in the second half of the twentieth century. The development of the contraceptive pill, reduction in family size, and /or the incidence of lactational amenorrhea has meant that today a woman would experience a much greater number of menstrual cycles (approximately 400) compared with her ancestors [30–40 cycles (288)]. Menstrual dysfunction encompasses benign pathologies such as menorrhagia (excessive menstrual blood loss); dysmenorrhea (painful periods); irregular, frequent, and prolonged periods; oligomenorrhea (infrequent or scanty periods); and amenorrhea [absent menstrual periods (288, 290)]. A number of these disorders are closely associated with disturbances in the hypothalamic-pituitary-ovarian axis and morphological changes within the uterus that are not precipitated by local disturbances within the endometrial environment (for example, the complaints of oligomenorrhea and amenorrhea). The causes of aberrations in regular menstruation have been functionally attributed to ovarian failure (primary or secondary), disordered regulation of gonadotrophin secretion, and PCOS (291). Amenorrhea may also be due, albeit rarely, to the formation of intrauterine adhesions or synechiae (Asherman's syndrome), which is an occasional consequence of surgical intervention in the uterus (such as dilatation and curettage of the uterine cavity at the time of surgical termination or evacuation of retained products of conception) or infection. In keeping with the focus of this review, the remainder of this section will focus on menstrual complaints that may be exacerbated by disturbances in expression and signaling of local mediators within the endometrium, with special emphasis on heavy menstrual bleeding and dysmenorrhea.

C. Menorrhagia

Menorrhagia is the complaint of unacceptable and excessive menstrual blood loss and has been defined as an objective measured blood loss in excess of 80 ml of blood lost per menstrual cycle. This definition was derived from data obtained in a Swedish study in which the incidence of anemia increased in women with blood losses over 60 ml per menstrual cycle and 80 ml of blood loss represented the 90th centile of the distribution. In light of these observations, the authors recommended that a blood loss in excess of 80 ml be regarded as "pathological" (292, 293). Unacceptable heavy menstrual blood loss affects 10–30% of women of reproductive age and up to 50% of perimenopausal women (294, 295). It is also estimated that 5% of women aged 30–49 yr will consult their general practitioner for excessive blood loss each year (296). Epidemiological studies suggest that the incidence of excessive menstrual blood loss may have a genetic link. A correlation has been described in the menstrual blood loss of monozygotic but not dizygotic twins (297).

There are clear instances where aberrations in clotting mechanisms, such as Von Willebrand's disease (298, 299) or deficiencies in PAI (300), can contribute to excessive bleeding, but these represent a small proportion of women presenting with abnormally high blood loss. More importantly, reduced clotting is a feature of normal menstruation, and there are likely to be other factors or a combination of factors contributing to excessive blood loss that are still poorly understood.

Progesterone, partly in conjunction with the growth factor EGF (301), plays a major role in the control of clotting mechanisms in endometrium through inhibition of proteases (which in normal circumstances limit thrombus formation, Ref. 302) and through stimulation of protease inhibitors such as PAI-1 (210). Thus, the removal of progesterone before menstruation would allow release of active tissue plasminogen activator, which would effectively reduce clot formation accompanied by reduced secretion of PAI-1 to augment the effect.

Benign disorders of the uterus may present with the complaint of excessive menstrual blood loss and/or an associated irregularity in the pattern of menstrual bleeding. Such benign disorders include endometrial polyps, fibroids, and adenomyosis. However, the vast majority of women complaining of excessive menstrual blood loss have normal endometrium. In a study of 1033 women who were investigated for excessive menstrual bleeding, approximately 90% of the subjects had a normal endometrium with no known pathology (303). Analysis of menstrual blood loss has shown that although women with menorrhagia menstruate for a longer period, the main difference between women with excessive and normal menstrual blood loss is in the rate of menstrual blood loss, with an approximately 3-fold difference in blood loss between the two groups of women. However, more recent studies suggest that the volume of blood loss is only one concern (and not necessarily the main concern) of women with heavy periods. The problem of heavy bleeding also reflects acute unmanageable blood flow in the first few days of menstruation (304, 305). These studies do suggest that in the majority of women the incidence of menorrhagia is precipitated by disturbances in the local endometrial environment (263, 264). This could be mediated by alterations in the expression and signaling of local endometrial factors that are

involved in the establishment and maintenance of vascular homeostasis.

D. Dysmenorrhea

Dysmenorrhea is a clinical term used for excessive pain experienced during menstruation. Dysmenorrhea is a significant clinical problem and results in considerable public health burden (306, 307). Dysmenorrhea is classically distinguished from an etiological perspective into primary or secondary. Primary dysmenorrhea is not associated with any underlying anatomical cause. It is a frequent occurrence in ovulating women and is often preceded by premenstrual tension and intense painful menstrual cramps occurring in the absence of a pelvic abnormality (308, 309). Primary dysmenorrhea is associated with uterine hypercontractility. During contractions, endometrial blood flow decreases, and there is now good correlation between minimal blood flow and maximal pain, suggesting that uterine ischemia due to hypercontractility maybe a major contributor to the symptom (308, 309). Secondary dysmenorrhea is differentiated from primary dysmenorrhea, because it is associated with uterine abnormalities or adnexal diseases such as endometriosis, pelvic inflammatory disease, submucous leiomyomas, adhesions, or the presence of an intrauterine contraceptive device. The reported prevalence rate for dysmenorrhea in women of reproductive age ranges from 43 to 90%. This wide range in the reported prevalence rate is primarily due to absence of standardized tests for diagnosis and measurement of dysmenorrhea. The precise mechanisms responsible for the symptoms of primary dysmenorrhea are yet to be elucidated.

VII. Local Mediators Associated with Menstrual Dysfunction

A. Prostanoids

The precise local mediators that may result in excessive menstrual blood loss remain largely unexplained. Histological studies have failed to reveal differences in endometrial or myometrial histology in women with normal and excessive blood loss (310). Moreover, no discernible differences in expression of sex steroid receptors are detected in the endometrium of women with normal and excessive blood loss (311). However, substantial evidence suggests that excessive menstrual blood loss is associated with disturbances in the process of angiogenesis and the expression of a number of local vasoregulatory factors that may affect either vascular development or permeability. A comparative study, which investigated endometrial tissue from women with heavy and normal blood loss, has demonstrated increased endothelial cell proliferation in women with excessive menstrual blood loss. After endometrial resection and reestablishment of normal blood loss, the endothelial cell proliferation index was comparable to that observed in women with normal blood loss (312). Additionally, the proliferation and differentiation pattern of the vascular smooth muscle cells around spiral arterioles of the endometrium of women with menorrhagia is significantly reduced compared with that of women with normal blood loss (262, 313). Together, these data outline a fundamental alteration in the composition and structural integrity of blood vessels in women with heavy menstrual blood loss. These may result in altered vascular fenestrations/permeability and integrity that may alter the accessibility and availability of endocrine and paracrine factors that precipitate menstruation and regulate blood loss within the endometrial microenvironment.

Heavy menstrual blood loss has been associated with aberrations in the synthesis and production of vasodilatory prostanoids from the uterus (314–316). In women diagnosed with menorrhagia, PGE_2 synthesis and prostaglandin E binding sites are greater in uterine tissues compared with normal women and correlate directly with menstrual blood loss (315, 317–320). Prostaglandin I_2 (prostacyclin) and nitric oxide synthesis are also elevated in menstrual blood collected from women with menorrhagia (316, 321). This suggests that the degree or duration of menstrual bleeding in women diagnosed with menorrhagia may be augmented after elevation of vasodilatory factors. Elevation of these vasodilatory factors may further enhance menstrual bleeding and vascular dysfunction by up-regulating the COX/prostaglandin biosynthetic pathway via a positive feedback loop and promoting an autocrine/paracrine up-regulation of growth factors specific for vascular function (such as VEGF) (322, 323). Prostaglandins are known to elevate COX-2 enzyme expression, arachidonic acid metabolism and prostanoid biosynthesis in several model systems (324, 325). The elevated expression of prostanoids present in the endometrium of women with excessive blood loss has led to the administration of COX enzyme inhibitors as a means of therapy (326). COX enzyme inhibitors have been shown to reduce menstrual blood loss by approximately 30% in women with menorrhagia (321, 327–331). More recently, a dual mode of action has been demonstrated for fenamates such as sodium meclofenamate and mefenamic acid. As well as reducing prostaglandin synthesis, they also inhibit binding of PGE_2 to its receptor (332). Little is known about the expression of prostanoid receptors in the endometrium of women with menorrhagia. Because the binding sites for prostanoids are elevated in these women, it would be anticipated that this is a reflection of higher expression levels of receptors at the cell surface. Elevated expression of these receptors in various cell types of the endometrium, including the epithelial and vascular cells (Fig. 3), may be associated with enhanced signaling and regulation of expression for angiogenic factors that may affect vascular function and permeability (Fig. 5). Interestingly, overexpression of COX enzymes and prostaglandin receptors has been shown to promote the production of angiogenic factors and down-regulate the production of antiangiogenic factors. Several angiogenic factors are up-regulated in reproductive epithelial cells in response to induced expression of COX enzyme such as basic FGF, VEGF, and angiopoeitins. Moreover, COX enzymes down-regulate the generation of antiangiogenic factors such as thrombospondin, angiostatin, and endostatin that are known to have a vasoconstrictive effect, thus promoting vasodilation (333–335). Ultimately, vascular permeability and leakage at the time of menstruation will be determined by a milieu of pro- and antiangiogenic factors. The presumed overexpression of COX enzymes in menorrhagia and the established role of COX enzymes in regulation of pro- and antiangiogenic factors would suggest a disturbance in the balance of these factors

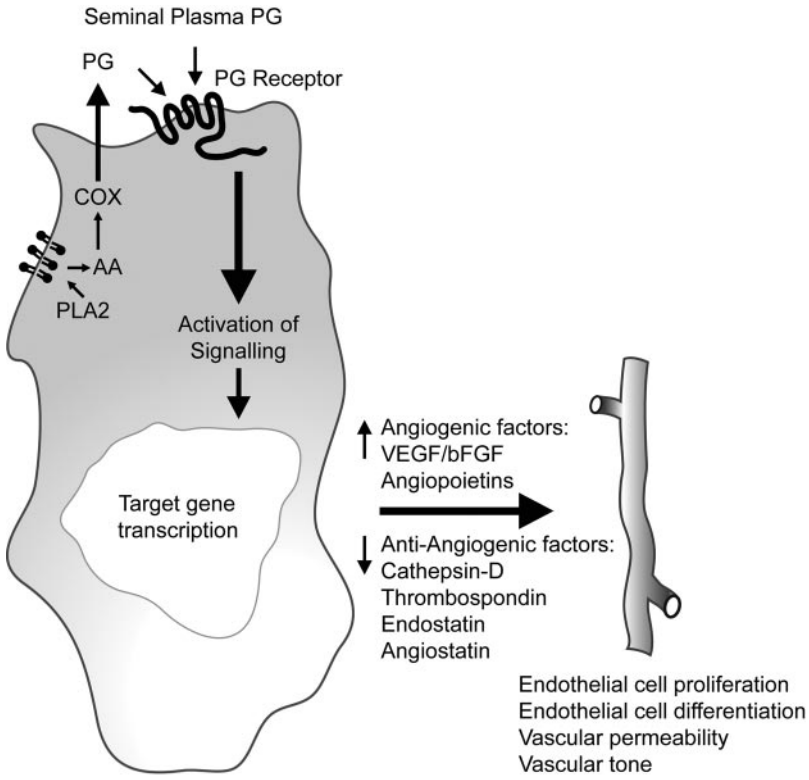

FIG. 5. Autocrine/paracrine regulation of prostanoid receptor signaling and the downstream effects on biological function. This diagram illustrates that prostaglandins are synthesized locally and subsequently released to activate specific prostanoid receptors and initiate intracellular signaling pathways. Activation of intracellular signaling results in regulation of gene transcription. These include up-regulation in expression of various angiogenic factors and down-regulation of anti-angiogenic factors. The alteration in gene expression can in turn affect vascular function. AA, Arachidonic acid.

in heavy compared with normal menstruation. Identification of the prostanoids responsible for these effects and the specific receptors/signaling pathways that are associated with their function may result in the development of novel therapeutic targets for menstrual pathology.

Little is known about the altered response to endocrine and local factors that may result in primary dysmenorrhea. However, many researchers have suggested that dysmenorrhea is associated with local disturbances in expression and synthesis of inflammatory mediators. Primary candidates that have been described to date are COX enzymes and their prostanoid products. Prostanoids are generated by most cells in response to inflammatory insults, and this results in activation of nearby sensory nerve endings, which express the respective prostanoid receptors (336, 337). The hyperalgesic effects of PGE_2 and $PGF_{2\alpha}$ have been described in several inflammatory models of nociception (for review, see Ref. 338). Elevated synthesis of prostanoids such as PGE_2 and $PGF_{2\alpha}$ has been demonstrated in the menstrual fluid of women with dysmenorrhea compared with menstrual fluid of women with painless periods (309, 320). *In vitro* studies have demonstrated that endometrial explants from women with dysmenorrhea produce more prostanoids in response to arachidonic acid compared with endometrium from pain-free women, thus suggesting a higher level of expression of COX enzymes and specific prostanoid synthase enzymes (339). This has prompted the use of COX enzyme inhibitors such as mefenamic acid, ibuprofen, and naproxen as therapeutic regimens for management of this disorder, with treatment being administered during menstruation or before the onset of menses (309). More recently, selective COX-2 inhibitors have proven to be even more efficacious in the treatment of dysmenorrhea (340), making it a preferential choice for treatment of women with primary dysmenorrhea.

Although no experimental data are currently available on the signaling of prostanoid receptors in endometrium of women with menstrual dysfunction, it is highly plausible that primary dysmenorrhea may be associated with an aberration in the expression and downstream intracellular effects of one or more prostanoid receptors. It is generally thought that PGE_2 is the principal inflammatory prostanoid, and several lines of evidence indicate that sensory neurons express EP receptors (338). Studies investigating pain perception in mice lacking individual prostaglandin receptors strongly suggest that EP3 is the major receptor mediating pain perception (341, 342). Interestingly, these studies also outlined the importance of the I-series prostanoid (IP) receptor in pain perception. Treating the knockout mice with lipopolysaccharide to induce COX enzyme expression and prostanoid synthesis, only the IP and EP3 receptor-deficient mice demonstrated significantly reduced pain perception, whereas the response in EP1-, EP2-, and EP4-deficient mice was similar to wild-type animals (341). These observations together with the significantly elevated expression and signaling of the IP receptor during the menstruation (343) strongly suggest a role for this receptor in dysmenorrhea. Future research is warranted to assess the pattern of expression and signaling of various prostanoid receptors in endometrium of women with dysmenorrhea.

B. Other angiogenic/permeability factors

The expression pattern of other angiogenic factors and their receptors may also contribute to the pathogenesis of

heavy menses. Candidates that have been investigated in the pathogenesis of excessive uterine bleeding include endothelin, endometrial bleeding associated factor (EBAF), and the angiopoietins. Endothelin is a 21-amino acid peptide of which there are three different isoforms that are encoded by distinct genes (246, 344). Endothelins act through interaction with two receptor subtypes termed ETA and ETB (345). Endothelins and their receptors have been detected in the human endometrium and localized to various cellular components including the endothelium (244, 346–348). In addition, the expression of neutral endopeptidase (an exoenzyme that inactivates endothelin) is localized in the endometrium, and its activity is reported to decrease premenstrually, thus increasing the local availability of endothelin during menstruation (349). Endothelins have potent vasoconstrictor properties on vascular smooth muscle, and the increase in their concentrations/function at the time of menstruation results in contraction of spiral arterioles, initiating local ischemia and resulting in menstrual bleeding. Endothelin expression is reduced in women with excessive menstrual blood loss possibly through enhanced metabolism by neutral endopeptidase. This may result in a fragile endometrium that is predisposed to bleed through alteration in constrictive potential of blood vessels (350).

Another gene whose expression has been investigated in normal and excessive menstrual bleeding is EBAF, also known as LEFTY-A (351). This gene was isolated by differential display technology to isolate genes whose expression is confined to the perimenstrual phase (352). EBAF is a member of the TGF-β superfamily, it is expressed in the stromal compartment of the human endometrium, and its expression is dramatically up-regulated (approximately 100-fold) at the perimenstrual phase. Moreover, expression of EBAF is increased and dysregulated in the endometrium of women with abnormal endometrial bleeding (352). *In vitro* studies suggest that EBAF stimulates the expression of a number of MMPs that initiate tissue breakdown at menstruation. Interestingly, expression of EBAF and its effects on metalloproteinases is down-regulated by progesterone, thus suggesting regulation of EBAF by inhibitory signals (351). The exact molecular mechanism by which EBAF mediates its cellular effects during menstruation remains to be elucidated.

Further evidence to support the hypothesis that excessive menstrual blood loss is associated with aberrant build up of the vascular smooth muscle cells can be deduced from recent data demonstrating disrupted expression of the angiopoietins and their Tie-2 receptor. In women with menorrhagia, expression of Ang-1 is significantly reduced, and that of Tie-2 is elevated in the endometrium. This results in a reduced ratio of Ang-1 to Ang-2, with enhanced signaling of the latter via the elevated Tie-2 receptor expression. This may result in reduced vascular stability thereby contributing to excessive blood loss at menstruation in women with menorrhagia (264, 275). Interestingly, the expression of angiopoietins is regulated by COX enzymes (353) that are presumed to be elevated in the endometrium of women with menorrhagia. It is tempting to speculate that the disrupted balance in angiopoietin expression and signaling in the endometrium of women with menorrhagia is mediated via the COX/prostanoid pathway.

Collectively, these data highlight the complexity and diversity of autocrine/paracrine factors that may be dysregulated within the endometrial environment, thereby resulting in an altered pattern of blood vessel development and function. Undoubtedly, when other angiogenic systems are investigated in detail, the list of dysregulated gene expression and function in the endometrium of women with complaints of heavy menses will expand.

VIII. Perspective and Future Direction

The advent of global mining of genes, using approaches such as genomics and proteomics, will greatly enhance our knowledge on the aberrations in gene expression in menstrual disorders. Several researchers have applied such global approaches to human endometrial function (5, 10, 147, 354, 355). These have included comparative studies to assess the gene signatures at various stages of the menstrual cycle such as proliferative *vs.* secretory phases. These studies were initiated to investigate the genes that may be crucial in preparation of the endometrium for the process of implantation. It is anticipated that elucidation of these signatures will greatly assist in the future in the development of therapeutic strategies for pathologies of implantation failure (such as recurrent miscarriage) or improper implantation/placentation (such as preeclampsia or intrauterine growth restriction). Other studies have assessed the gene signatures in aspects of endometrial pathologies such as endometriosis (10, 355, 356). These global approaches when applied in context of specific pathologies can give a great insight into the various genetic and molecular pathways that may be associated with the pathology and the cellular and phenotypic changes that they may mediate. These in turn may outline novel therapeutic intervention strategies.

The application of such approaches to menstrual disorders will greatly advance our knowledge of the library of genes that may be dysregulated in these complaints. Moreover, they will outline the molecular signatures that are characteristic of each of the menstrual complaints being investigated. The challenge in the future is to unravel the exact nature of the molecules, the cross-talk that may exist between them, and the signaling pathways they activate that precipitate menstrual disorders. It is important to accurately assess whether these pathways function independently or converge to amplify specific signals that are crucial for the progression of the dysfunctional state. Only with this knowledge will we be able to apply novel therapeutic intervention strategies. It is also important to bear in mind that menstrual disorders may result from multiple etiologies. Hence, the selection criteria for endometrial samples in the profiling studies have to be carefully selected to accurately discriminate between individual patient variability and the varying etiological states that may contribute to a common pathology.

Research into menstrual disorders is hindered by the lack of availability of rodent models to assess the role of various factors in regulating menstrual function. Our knowledge of the factors regulating menstrual function/dysfunction has relied historically on candidate gene approaches with a requirement for knowledge of the gene structure and sequence.

This approach has been applied to investigate the variation in expression of the candidate genes in the human endometrium across the menstrual cycle. The absence of rodent models to study menstrual problems has also necessitated that the research community rely predominantly on the use of human clinical material to identify the candidate genes whose expression may be dysregulated in the endometrium of women with menstrual disorder. To this end, researchers in the field have designed rigorous criteria for selection of clinical material that fit the disorder being investigated. In addition, this has paved the way for the development of intricate *in vitro* cell model systems that allow for the culture of primary cells isolated from human endometrium. These cell model systems have greatly enhanced our knowledge of the cell-cell interaction/communication that exists in the human endometrium. Hence, a further challenge in the future will be the development of experimental strategies that will allow us to assess the exact role of the various factors deduced from gene mining studies in menstrual function/dysfunction and which of these genes/pathways constitute a sensible target for novel therapeutic application in the clinic.

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