Ernst Schering Research Foundation
Workshop 52

New Mechanisms for Tissue-Selective Estrogen-Free Contraception

H. B. Croxatto
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(Editors)

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Preface

Currently, combined oral contraceptives are the most convenient and accepted way of hormonal contraception. Nevertheless, there is a constant demand for innovation both from the medical community and from consumers. Despite the high safety profile of available products, consumers and physicians seek contraceptives with additional benefits and lower hormonal load. One option for addressing this need is tissue-specific progestins.

With the development of new molecules such as progestins with a tissue-specific mode of action, opportunities arise for new concepts in contraception.

Estrogen-free contraception – e.g., by progestin-only pills – is an established concept but with significant disadvantages which could be overcome by introducing potent locally active, tissue-specific progestins.

A major success factor for the realization of this concept is a deeper understanding of local pharmacological response to progestins in general and to new progestins in particular. The target organs in focus are the cervix, endometrium, ovary, tube and potentially the gonadotropic axis. To characterize specific effects on these targets is one of the major challenges in the development of contraceptives in general and new progestins in particular. There is a major interest from both molecular biologists and clinicians working in reproductive medicine to gain further insight into these mechanisms of action responsible for the contraceptive effect.

The aim of this workshop was to bring together experts in the field of molecular and pharmacodynamic actions of progestins with

clinicians and medical specialists to discuss potential clinical endpoints, physiological reactions and (bio)markers which would be useful to describe the tissue-selectivity and the contraceptive action of new progestins in different target organs.

The contributions of the experts in this field will lead to a better understanding of the mechanisms underlying the organ-specific action of progestins and will help to design future clinical studies addressing the tissue-specific action of those compounds using meaningful endpoints (preferably surrogate markers). These endpoints are needed for the proof of concept of new concepts for fertility control.

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U. Fuhrmann, I. Schellschmidt, R. Schürmann

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1 Progesterone Receptors and Opportunities for Contraception

H. B. Croxatto

1.1 Introduction

Progesterone is indispensable for reproduction as it regulates a number of processes which occur at various times throughout the menstrual cycle and pregnancy and at various levels of the reproductive axis from the brain to the genital tract. Progesterone is required for the production of a viable zygote, and it is essential for the establishment and maintenance of pregnancy. In order to accomplish these functions, progesterone production varies considerably from one phase of the ovarian cycle to the next and this is reflected in profound oscillations in its blood levels along the menstrual and conceptional cycles. Endogenous progestins intervene in the control of these processes, not only at the elevated serum levels of the luteal

phase and pregnancy, but also at the slightly rising levels around the onset of gonadotropin surge and at the very low levels of the follicular phase. Depending on the timing of progesterone administration, it can serve as a pro-fertility or an antifertility hormone. Producing an excess when its levels are normally low or simulating a progesterone deficit when its levels should be high, will interfere with normal fertility.

Since most progesterone actions are exerted through progesterone receptors (PR) belonging to the superfamily of nuclear steroid receptors, it follows that pharmacological manipulation of these PR with drugs other than progesterone itself has the potential for mimicking both the pro-fertility and antifertility actions of progesterone. Thus specific alterations in the action of this hormone on selected target cells, that lead to reduced fertility in a clinically useful manner, may be achieved by targeting PR. These are the basic concepts that led to review our knowledge about progesterone physiology in the female genital tract at this Ernst Schering Research Foundation Workshop. The aim of this workshop is to explore new avenues in contraception based upon direct pharmacological interventions on PR.

1.2 The Need for New Contraceptive Methods

Sexual and reproductive patterns have been evolving probably throughout the entire human history, but the contraceptive revolution unleashed in the last century accelerated this evolution far beyond any expectation Sigmund Freud or even the father of the pill, Gregory Pincus, anticipated. The current sexual and reproductive pattern dictates long-term use of contraception at the individual level. This is illustrated in Fig. 1. Sexual activity can extend from attainment of puberty until after women reach the post-menopausal age, a period that can exceed 40 years, most of which are fertile years for both men and women. Since increasing number of couples are choosing to limit their family size to one or two children, it follows that either abstinence or contraception need to be practiced for many years. Most couples, by far, choose to dissociate sexual relationships from reproduction rather than to practice abstinence. Therefore contraception is used by either member of the couple for many years.

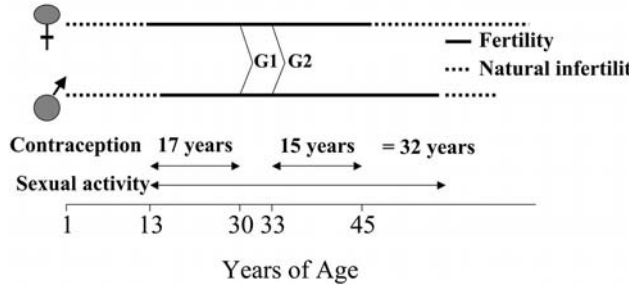

Fig. 1. Schematic representation of segments of the life span of a man and a woman, in which natural fertility and infertility, sexual activity and use of contraceptive methods may be expected to occur, when no more than two children (G1, G2) are desired. The reproductive pattern represented dictates long-term use of contraception at the individual level

Ideally, couples would like to have different contraceptive options in order to choose the optimal method for each stage and style of life as well as to be able to share the burden by alternating the responsibility between the male and female partner.

If contraceptive methods are going to be used for a long segment of the life span of a person they need to be very good. However, it is well documented that none of the existing methods will satisfy the vast majority of users throughout all of the stages of life and it is unlikely that in the future there will be such method. Therefore, for the time being, goodness identifies better in this case with variety than with panacea. Since the early 1960s, when the contraceptive pill and the interuterine device were launched, until now, there has been a constant proliferation of classes of methods as well as brands within each class (Table 1). In spite of that, many of the current contraceptive methods that offer either high efficacy or safety do it at the cost of noneasy use or menstrual disturbances or lack of reversibility or other inconvenience, and present barrier methods offer only modest or no protection against sexually transmitted diseases. Therefore there is ample room for improvement, and efforts in that direction are likely to persist for a large part of the current century. Male methods of contraception are practically nonexistent in comparison with female methods. Hormonal contraceptives have

Table 1. Growing diversity of contraceptives since introduction of modern methods

Method	First approval	Approved in USA
Intrauterine device inert	1950	1961
Pill	1960	1960
Intrauterine device Cu	1971	1971
Injectable	1975	1992
Intrauterine device hormonal	1976	1976
Implant	1983	1991
Emergency pill	1984	1998
Medical abortion	1988	2000
Vaginal ring	1998	2001
Patch	2001	2001
Microbicide/spermicide	*2006* [a]	*2006*
Male hormonal	*2006*	*2010*

[a] Years in italics are estimations.

now been on the market for some 40 years and by far, the most commonly used are combined oral contraceptives. The need for regular daily intake of a pill for highest efficacy is a burden to many women and the addition of ethynyl estradiol, even though it improves bleeding control, is also inconvenient to a fair number. Progestin-only methods are more amenable to long-term delivery from devices that relieve the user from permanent attention for maximum efficacy, but they are associated with annoying bleeding disturbances. Another class of estrogen-free method of contraception, not associated with bleeding disturbances, would be most welcome by many women and health care providers as well.

1.3 Some Reproductive Processes Regulated by Progesterone Receptors

Discrete steps of the reproductive process essential for the establishment of pregnancy are listed in Table 2. Further segregation of those taking place in the fallopian tube is presented in Table 3. PR participates directly or indirectly in the regulation of these processes, and

Table 2. Discrete steps of the reproductive process that are essential for the establishment of pregnancy

Sperm migration into the fallopian tube
Ovulation
Fertilization
Zygote development and transport to the uterus
Development of myometrial retentivity
Development of endometrial receptivity
Trophoblast adhesion and invasion
Corpus luteum rescue by human choriogonadotrophin

Table 3. Discrete processes taking place within the fallopian tube

Uptake of cumulus oocyte complex
Addition and removal of oocyte coverings
Adhesion of sperm to oviductal epithelium
Maintenance of sperm fertilizing capacity
Release of capacitated/hyperactivated sperm
Fertilization
Zygote initial development
Embryo transport to the uterus

the following paragraphs highlight direct effects of progesterone in some of them.

1.3.1 Sperm Migration

Sperm migration from the site of insemination to the site of fertilization is not just physical diffusion following a concentration gradient, but a hormonally regulated process. A number of elegant experiments have shown that the concentration gradient is discontinuous due to the formation of sperm reservoirs in discrete segments of the genital tract (Hunter 1988). In the human, semen containing millions of spermatozoa is deposited in the vagina, and only a small fraction of those may enter the cervical canal, depending on the rheologic properties of cervical mucus. Estrogens and progestins acting on mucus-secreting cells of the cervix have completely opposite effects.

Estradiol induces the secretion of abundant, clear mucus that allows spermatozoa to swim through and form a sperm reservoir in cervical crypts. This reservoir can supply fresh spermatozoa for several days to the upper segments of the genital tract until ovulation takes place. Although sperm cells can be found in the fallopian tube a few minutes after insemination, animal experiments have shown that fertilizing spermatozoa come from the sustained phase of transport and not from the rapid phase (Adams 1956; Overstreet and Cooper 1978). Progesterone, and progestins in general, acting through PR render the mucus hostile to spermatozoa by increasing its viscosity and probably through chemical mechanisms as well. This is a clear target for PR-mediated contraceptive effect since progestin-only methods prevent pregnancy to a major extent by impeding sperm entering into mucus, blocking their passage through the cervical canal and abrogating the formation of a sperm reservoir in the cervix (Kesseru et al. 1974; Croxatto 2002 a).

Whether or not sperm migration and survival beyond the cervix are also regulated by steroid hormones in the human has not been subjected to rigorous examination. A clear demonstration that sperm migration into the tube is under the influence of ovarian steroids comes from examining the number of spermatozoa that enter the oviduct in the rat following intrauterine insemination on different days of the estrous cycle (Orihuela et al. 1999). Differences up to two orders of magnitude were found between estrus and diestrus. Suppression of the ovarian cycle by treatment with a GnRH antagonist greatly decreased the number of spermatozoa entering the oviduct. This number was restored to levels observed in estrous rats, 6 h after a single subcutaneous injection of estradiol. Treatment with progesterone had no effect by itself, but antagonized the effect of estradiol (Fig. 2). Thus the number of sperm cells that migrate from the uterus into the oviduct is under steroid hormone regulation and progesterone inhibits this process.

An additional effect of these hormones became evident when the number of spermatozoa adhering to the oviduct epithelium was segregated from the total number entering this organ. The absolute number and proportion of spermatozoa adhering to epithelial cells changed along the estrous cycle, and pharmacological experiments showed that progesterone is required to restore sperm adhesion to

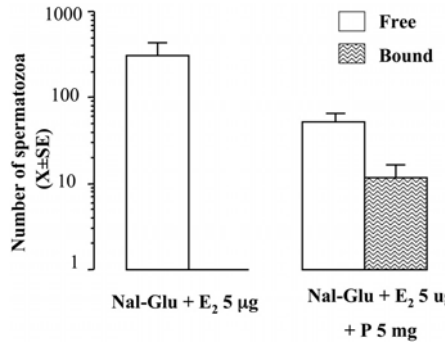

Fig. 2. Effect of estradiol (E_2) and progesterone (P) on the number of free and epithelium-bound spermatozoa recovered from the rat oviduct 3 h following intrauterine insemination. All animals were treated with the GnRH antagonist Nal-Glu for 7 days to suppress ovarian activity. On day 7 hormones were injected subcutaneously 6 h before insemination ($n=5$ per group). E_2 alone increased 100-fold the total number of spermatozoa entering the oviduct in comparison with Nal-Glu alone (not shown), but no sperm adhesion was observed. Addition of P partially reduced the total number of spermatozoa entering the oviduct, at the same time it restored sperm adhesion to the epithelium. (Redrawn from Orihuela et al. 1999)

oviductal epithelium when ovarian activity is suppressed (Fig. 2). Whether or not the same phenomena occur in the human is still to be seen. Sperm adhesion to human fallopian tube epithelium has been documented to occur at least in vitro (Gwathmey et al. 2003), but until now there is no evidence regarding its hormonal regulation. On the other hand there is a hint that the number of spermatozoa entering the oviduct may be under local influence of steroid hormones at least in a nonhuman primate. In the Cebus monkey, the number of spermatozoa recovered from the ampulla following mating increases significantly in the ovulatory side after ovulation (Ortiz et al. 1995).

Spermatozoa entering the tube can follow various pathways. Once they become capacitated, spermatozoa remain alive for only minutes or few hours, while noncapacitated sperm cells adhere to oviduct epithelium for many hours, retaining their vitality until they are released in a capacitated and hyperactivated state (Fazelli et al. 1999). This cell–cell interaction has been shown to be crucial for achieving

fertilization in experimental animals (Smith and Yanagimachi 1990, 1991). If these sperm cells released from the epithelium encounter the cumulus oophorus in the ampullary segment, fertilization may take place, otherwise they need to be replaced continuously with fresh sperm cells coming from the cervical reservoir (Croxatto 1996). This latter process may be critical in the human since the total number of sperm recovered from the entire fallopian tube of women between 8 h and 15 h after intercourse is well below 1000 in most cases and rarely exceeds that number (Croxatto et al. 1975). Even under optimal conditions, when single intercourse takes place in the fertile period, the rate of fertilization in the human appears to be around 50% (Alvarez et al. 1988; Wilcox et al. 1995), therefore the ability of human sperm to achieve fertilization is more vulnerable in vivo than in vitro and quite dependent on PR-mediated actions. In conclusion there are three links in the chain of events leading from insemination to fertilization that are regulated through PR. They are: sperm passage through the cervical canal, sperm entrance into the oviduct, and sperm adhesion to oviduct epithelium.

1.3.2 Oviductal Embryo Transport

Embryo transport to the uterus is also a regulated process. The time taken by embryos to traverse the entire length of the oviduct is not invariable in any given species and may change according to the physiologic condition of the female (Croxatto 2002b). The signals involved in the regulation of oviductal embryo transport vary across species. In the rat, mouse and rabbit, transport through the isthmic segment is regulated by ovarian hormones. In the rat it is the estradiol : progesterone ratio in serum that determines the time taken by eggs to reach the uterine cavity. The higher the ratio, the faster the transport through the isthmic segment (Croxatto and Villalón 1995). In other species such as the horse, some bats and hamsters, ovarian hormones have minimal influence on the rate of transport and the crucial signals are produced by the embryo. The equine embryo, at the morula stage, secretes prostaglandin E_2, whereas the hamster secretes platelet activating factor (PAF) beginning at the four-cell stage. In both cases, the embryonic signal acts on the oviduct to in-

duce the transfer of embryos to the uterus. In the human the single egg normally takes 3 days to traverse the tube and exogenous estradiol or progesterone administered shortly after ovulation do not seem to change this timing (Croxatto 1996). Since developing human zygotes secrete PAF in vitro and the endosalpinx expresses PAF receptor and PAF acetyl hydrolase, it is possible that this system operates in the human (Velasquez et al. 2001). The fact that egg transport in women is not under the influence of estradiol and progesterone represents an advantage for the development of contraceptive agents that act through PR. Otherwise they could raise the risk of tubal pregnancy and put women's health at stake. Nonetheless, the early low dose progestin-only methods that were less effective in inhibiting ovulation were associated with higher risk of tubal pregnancy, possibly due to loss of cilia from tubal epithelium or changes in the quality of tubal secretion. Therefore new agents for chronic use should select out those effects on the endosalpinx.

1.3.3 Endometrial Receptivity

Acting on the endometrium that has proliferated under sustained estrogenic stimulation, progesterone orchestrates two processes: one to nest the embryo, if it comes, and another to cause menstruation if it does not come. In addition, acting upon the myometrium it inhibits tonic and phasic contractions, insuring physical conditions needed to sustain uninterrupted blood flow and expansion of the tissues growing inside the uterus. Intracellular PR plays a central role in endometrial cyclicity. On the one hand, PR–ligand complexes promote the epithelial and stromal differentiation needed for the establishment and maintenance of pregnancy, and on the other hand, dissociation of these complexes at the time of corpus luteum demise leads to tissue breakdown and menstruation.

The study of human endometrial receptivity has moved swiftly from the morphologic to the molecular arena pari passu with technological advances in molecular biology and assisted reproduction. Protein expression in the endometrium exhibits neat and distinct changes in accordance with oscillations in estrogen and progesterone serum levels and with progress in the differentiation of various cell

phenotypes throughout the secretory phase. Current data indicate that at the onset of receptivity, expression of some proteins is temporarily turned on or increased while some others are temporarily turned off or decreased (Lessey 2000; Sunder and Lenton 2000; Martin et al. 2002). These changes are believed to be driven by PR as a consequence of sustained action of progesterone secreted by the corpus luteum and they are considered essential for implantation and maintenance of pregnancy. Several groups have recently disclosed concerted changes in gene expression in human endometrium chronologically associated with the onset of receptivity (Carson et al. 2002; Kao et al. 2002; Borthwick et al. 2003; Riesewijk et al. 2003). The major challenge is to determine cause–effect relationships between those changes and acquisition of receptivity, as progesterone drives not only the development of receptivity but the installation of catamenogenic mechanisms as well. A modest approach utilized at our center was to compare gene expression during the receptive period between endometria proven to have allowed implantation and endometria that had repeatedly nil implantation, both as recipients in oocyte donation cycles. As the success of implantation depends on embryo and endometrium quality, one way to isolate the endometrium as the study variable is to select cases in which oocyte donors became pregnant from the same oocyte pool. However, the source of the fertilizing sperm still remains an uncontrolled variable. This flaw can only be mitigated by selecting cases who exhibit normal semen parameters. Using these criteria, cases and controls were recruited among women who had been subjected to two or more oocyte donation cycles in the not too distant past. All were treated with exogenous hormones in order to induce an artificial endometrial cycle and a biopsy was taken on the seventh day of progesterone treatment in order to assess gene expression levels using microarray technology. A clear advantage of this model is that gene expression response to hormonal stimulation can be correlated with the outcome of oocyte donation attempted in previous cycles. More than 90 transcripts showed more than a twofold difference between the two groups. Twelve of these 90 transcripts encode proteins such as chemokine receptor, matrix metalloproteinase-7, progestagen-associated endometrial protein, the tissue level of which had been previously found to change at the time of the implantation. Differential expression of

these genes between cases and controls was confirmed by real time RT–PCR, and protein expression was assessed by immunocytochemistry. Of great interest is the finding that 11 of the genes that are upregulated in the transition from nonreceptive to receptive state, were found to be downregulated at this time in the endometrium of three women in whom nidation had failed repeatedly following embryo transfer. On the other hand, ankirin, which is normally downregulated at implantation, was upregulated in these women. Thus expression of some genes believed to play an important function in implantation was found to be altered in endometria which are inimical to implantation. This neither proves that normal expression of these genes is essential for successful implantation, nor proves that implantation failure in these women is due to altered expression of these particular genes, but the chances are high. This association between infertility and faulty expression of a dozen genes brings us closer to the possibility of engineering new pharmacological and diagnostic tools for fertility regulation based upon PR-mediated effects on the endometrium.

1.4 Versatility of Progesterone Receptors

Progesterone has multiple modes of action on cell function. The best known today is via binding to its nuclear receptors to form molecular complexes; these then interact with progesterone response elements in DNA to stimulate or repress transcription of progesterone-regulated genes. Another mode of action of progesterone, well documented in *Xenopus* oocytes and mammalian sperm, involves cell membrane PR. In both cases it is independent of genomic effects and does not require diffusion of progesterone into the cell. Another one is through gamma amino butyric acid receptor subtype A (GABA-R). This pathway is most important in nerve cells, where GABA-R are more abundant, and it is responsive mainly to 5*a* reduced progestins.

Two isoforms of PR nuclear receptors, known as A and B, have been found to be present in varying proportions across progesterone target cells. PR-A and PR-B isoforms are expressed from a single gene but differ in their transcription activating properties. Both func-

tion in a tissue-specific manner regulating distinct and overlapping subsets of progesterone responsive genes. Either isoform mediates antiprogestational effects. Isoform B mediates progestational effects, while PR-A mediates antiestrogenic and antiglucocorticoid effects. These isoforms can form homodimers and heterodimers and their actions as ligand-activated transcription factor differ between them. Besides the DNA and ligand-binding domain, PR has several other anchoring domains, which are used for dimerization and for association with other molecules such as chaperones, scaffolding, co-activators and co-repressors. Thus, PR associates with a number of molecules through these anchoring domains, and its ability to act as transcription factor depends not only upon ligand binding, but also upon these molecular associations. It is noteworthy that ligand-binding changes PR three-dimensional conformation and in doing so some anchoring domains become exposed while others are hidden. A simplified version of this concept is illustrated in Fig. 3. Furthermore, the conformational change is dependent on steric features of the ligand. It is possible then that different ligands confer distinct three-dimen-

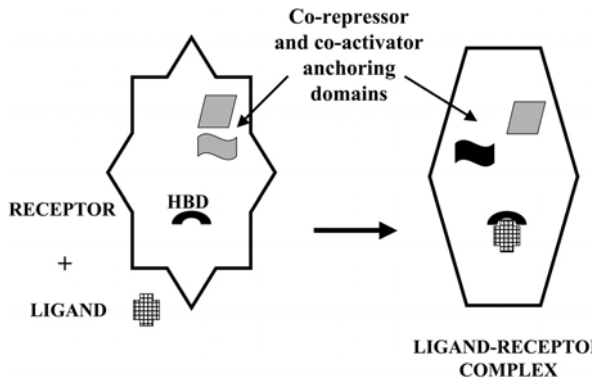

Fig. 3. Cartoon illustrating conformational changes in a progesterone receptor monomer as a result of ligand binding to hormone binding domain (*HBD*). For simplicity, only two-dimensional change and two anchoring domains, one for co-repressor and another for co-activator regulators, are represented. Upon ligand–receptor complex formation, the receptor conformation changes, exposing one of the regulator's anchoring domains (*dark*) leaving the other (*light*) unexposed

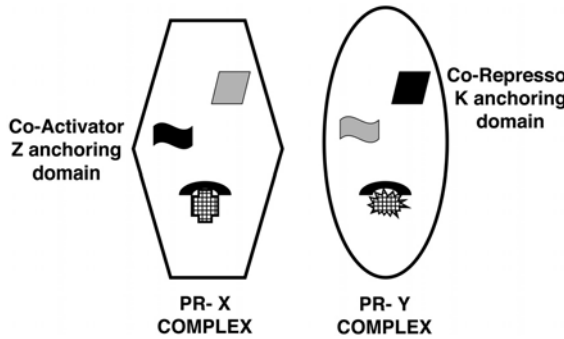

Fig. 4. Cartoon illustrating different conformational changes in a progesterone receptor monomer as a result of binding to ligand X or Y. The conformational change induced by ligand X exposes an anchoring domain for co-activator Z, whereas binding to ligand Y induces a conformational change that exposes an anchoring domain for co-repressor K. The interaction between the conformational effects of each ligand on PR-A and PR-B and the cell phenotype-specific expression of co-repressors and co-activators, confers great versatility to PR in its target cells

sional conformation and a different set of exposed anchoring domains on PR, which in turn determines the PR activity at the chromatin level. This concept is illustrated in Fig. 4. Since cell phenotype-specific expression of co-repressors and co-activators is gradually emerging (Rowan and O'Malley 2000; Shiozawa et al. 2003), it is conceivable that the plasticity of PR combined with the phenotype diversity of progesterone target cells, can generate a great diversity of tissue-selective effects. This selectivity can be enhanced by synthetic ligands exhibiting a wide range of steric features. In summary, PR presents great versatility in terms of adopting distinct three-dimensional conformations with different ligands and as a consequence establishing diverse molecular associations which depend on the interaction between each conformation with each cell phenotype. It is conceivable that progress in this area will lead to the discovery of cell phenotype-specific co-activators and co-repressors with defined functions. Once that information is on hand, the chemist will produce PR ligands able to modulate PR in a manner that affects only the function of a selected target cell in the desired direction.

1.5 Progesterone Receptor Modulators

Synthetic steroids that bind PR with clinically acceptable specificity and higher affinity than the natural ligand have recently increased in number and diversity leading to several classes according to the way they modulate PR. According to their effects on target cells, PR ligands can be classified in two groups, namely: (1) agonists, those which mimic the effect of progesterone; and (2) antagonists, those that prevent the action of agonists. With some exceptions, the presence of an antagonist may go unnoticed by cells, unless an agonist is present.

A novel class of PR ligands exhibiting progesterone agonistic and antagonistic activities in vivo has recently emerged. Recognizing that they represent a new class of compounds, they were designated progesterone receptor modulators. This denomination segregates them from antiprogestins with the advantage of avoiding political obstacles for their development. However, in as much as each PR ligand – regardless of being agonist, antagonist or having dual behav-

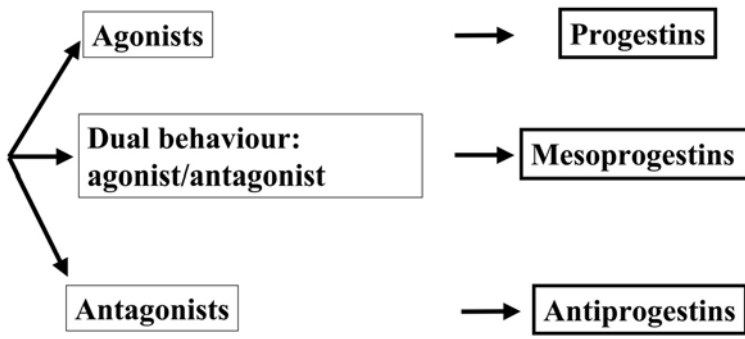

Fig. 5. Types of progesterone receptor ligands according to their pharmacological activity relative to that of progesterone. All of them modulate progesterone receptor by changing its three-dimensional conformation upon binding. Progestins mimic progesterone actions while antiprogestins prevent the access of progestins to the receptor. Another group of compounds behave as agonists in the absence of other agonists and as antagonists in their presence. As these fall between pure agonists and pure antagonists, they are designated as mesoprogestins

ior as agonist and antagonist – changes the three-dimensional conformation of the receptor molecule in ways that diverse anchoring domains are exposed/hidden, all of them are PR modulators. Therefore it seems inadequate to exclude progestins and antiprogestins from the progesterone receptor modulator designation. As the new compounds are endowed with agonistic and antagonistic properties in the same or different tissues depending on the presence or absence of other PR ligands, we propose to designate them as mesoprogestins, meaning between progestins and antiprogestins (Fig. 5). Mesoprogestins have the potential to alter in unforeseeable manners progesterone-dependent processes. Asoprisnil, one of the most studied compounds of this class, has the ability to suppress both endometrial growth and menstrual cyclicity in primates, independently of its effects on ovarian cyclicity (Chwalisz et al. 2000, 2002). This finding substantiates the possibility of designing PR ligands that behave as tissue-selective PR modulators. Use of such compounds for novel fertility regulation strategies is likely to become a reality within a decade or so.

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2 Reproductive Tissue-Selective Actions of Progesterone Receptors

B. Mulac-Jericevic, O. M. Conneely

2.1 Progesterone Receptors

The steroid hormone progesterone is a key modulator of normal reproductive functions. These include ovulation, uterine and mammary gland development and the neurobehavioral expression associated with sexual responsiveness (Clarke and Sutherland 1990; Lydon et al. 1995). The physiological effects of progesterone are mediated by interaction of the hormone with two specific intracellular progesterone receptors termed PR-A and PR-B. Progesterone receptors (PRs) are members of a large family of structurally related gene products known as the nuclear receptor (NR) superfamily (Evans 1988; Mangelsdorf et al. 1995; O'Malley and Conneely 1992; Tsai and O'Malley 1994a) of transcription factors. NRs regulate gene transcription by discriminative binding to DNA regulatory sequences as well as specific interactions with co-activator and/or co-repressor proteins to

regulate the activity of the RNA polymerase complex (McKenna and O'Malley 2001).

PRs are expressed from a single gene as a result of transcription from two alternative promoters (Kastner et al. 1990; Kraus and Katzenellenbogen 1993) and translation initiation at two alternative AUG initiation codons (Conneely et al. 1989). The production of these two isoforms is conserved in a number of vertebrate species including humans and rodents (Conneely et al. 1989; Giangrande and McDonnell 1999; Lessey et al. 1983; Shyamala et al. 1990) and the ratios of the individual isoforms vary in reproductive tissues as a consequence of developmental (Shyamala et al. 1990) and hormonal status (Duffy et al. 1997) and during carcinogenesis (Graham et al. 1996).

Binding of progesterone to PRs induces a significant conformational change on the receptor proteins (Allan et al. 1992a, 1992b) that results in dimerization of two ligand receptor complexes (Tsai and O'Malley 1991; Tsai et al. 1988), increased receptor phosphorylation (Weigel et al. 1995), binding of receptor dimers to specific hormone responsive DNA elements located in the promoter regions of target genes (Gronemeyer 1991; Tsai and O'Malley 1994b), and interaction of the receptor complex with specific coactivator proteins and general transcription factors (Kamei et al. 1996; Onate et al. 1995) to form a productive transcription initiation complex on specific target gene promoters. The overall structural features of the PRs that are responsible for these activities are well defined (Fig. 1). The amino-terminal or A/B region of PRs is the most hypervariable region in terms of both size and amino acid sequence among members of the superfamily. This region contains transactivation domains (AF1 and AF3) that that recruit coactivator proteins to the receptor to modulate the level and promoter specificity of target gene activation (Dobson et al. 1989; Meyer et al. 1992; Sartorius et al. 1994; Tora et al. 1988) as well as an inhibitory domain responsible for recruitment of transcriptional inhibitory corepressor proteins (Giangrande et al. 1997). The most conserved region (C) is the DNA binding domain that in the case of PRs is centrally located. This domain consists of approximately 66–68 amino acids and is composed of two type II zinc finger structures that facilitate binding of the receptor to specific *cis*-acting DNA sequences and are the hallmark of

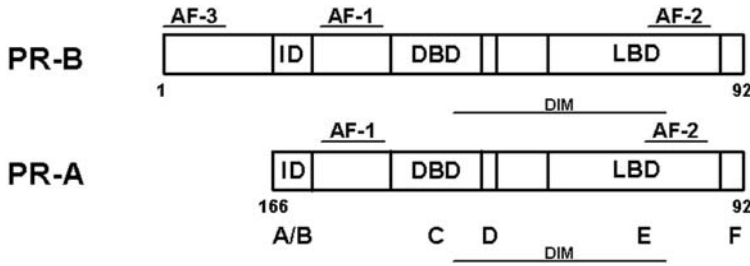

Fig. 1. Structural organization of the human PR-A and PR-B isoforms. *Numbers* denote the amino acid position in each protein. *AF-1*, *-2* and *-3* are activation domains; *DBD*, DNA binding domain; *LBD*, ligand binding domain; *ID*, inhibitory domain; *DIM*, sequences important for receptor dimerization

the nuclear receptor superfamily (Freedman 1992; Luisi et al. 1991). A highly conserved ligand binding domain is located on the carboxy-terminal side of the DNA binding domain. In addition to its progesterone binding function, this region contains an additional transactivation domain (AF-2) required for hormone dependent coactivator recruitment, sequences important for interaction of inactive receptors with heat shock proteins and for receptor dimerization (Fawell et al. 1990; Pratt et al. 1988; Vegeto et al. 1992; Webster et al. 1988).

In the early 1990s, in vitro biochemical studies revealed that the two PR isoforms displayed quite distinct transactivational properties that are specific to both cell type and target gene promoter used (Hovland et al. 1998; Meyer et al. 1992; Tora et al. 1988; Vegeto et al. 1993). Specifically, PR-B has been shown to function as a strong activator of transcription of several PR dependent promoters and in a variety of cell types in which PR-A is inactive. In addition, when the PR-A and PR-B proteins are co-expressed in cultured cells, the PR-A can repress the activity of PR-B as well as the activity of other nuclear receptors (Giangrande and McDonnell 1999). Further, the PR-A and PR-B proteins also respond differently to progesterone antagonists (reviewed in Giangrande and McDonnell 1999). While antagonist bound PR-A is inactive, antagonist bound PR-B can be converted to a strongly active transcription factor. When expressed in equimolar ratios in cells, the PR-A and PR-B proteins can dimer-

ize and bind DNA as three distinct species: A:A or B:B homodimers or A:B heterodimers. The differential transactivation properties contributed to these complexes by the presence or absence of the PR-B-specific AF3 domain may contribute to the complete repertoire of physiological responses to progesterone.

While it is well established that PRs mediate the transcription regulatory effects of progesterone, the signal transduction properties of PRs are not restricted to respond to their steroidal ligand. Studies using tissue culture systems have revealed that PRs, as well as some other nuclear receptors, can be activated in a ligand independent manner by stimuli that modulate intracellular kinase activity (Aronica and Katzenellenbogen 1991, 1993; Beck et al. 1993; Denner et al. 1990; Sartorius et al. 1993). The physiological relevance of a progesterone independent mechanism of receptor activation has been substantiated in vivo: PR-mediated lordosis behavior exhibited by rats (Mani et al. 2001) and mice (Mani et al. 1996) can be stimulated either in response to progesterone or in the absence of progesterone by dopamine activated intracellular signaling pathways.

In addition to its PR mediated genomic actions, progesterone also stimulates cellular responses that are independent of the transcription regulatory function of PRs by at least two distinct mechanisms: The first is PR dependent and involves PR mediated activation of intracellular phosphorylation cascades, at least one of which is the Src/Ras/MAPK pathway, to modulate cellular responses to the hormone (Ballare et al. 2003; Boonyaratanakornkit et al. 2001). The second mechanism is independent of PRs and appears to be mediated by interactions with specific membrane receptors for progesterone. Plasma membrane associated progesterone specific receptors have been isolated and cloned from a range of tissues and from a number of species (Bramley 2003) and are identified as G protein-coupled receptors distinct from classical intracellular PRs (Zhu et al. 2003). Rapid non-genomic effects of progesterone have been implicated in modulation of the sperm acrosome reaction (Bronson et al. 1999; Patrat et al. 2000), modulation of neuronal excitability (Genazzani et al. 2000; Lambert et al. 2003), prevention of preterm labor (Grazzini et al. 1998) and *Xenopus* oocyte maturation (Bayaa et al. 2000). While several of these responses appear to be regulated in a PR independent manner, the contribution of PR mediated non-genomic

signaling to the physiological activities of progesterone remains poorly understood.

2.2 PR Knockout Models Demonstrate Tissue-Specific Role of PRs in Reproductive Tissues

Phenotypic studies of the PRKO mouse model in which the expression of both isoforms was inhibited by null mutation of the PR gene provided compelling evidence of an essential role of PRs in all aspects of female reproduction. Specifically, female mice lacking both PRs exhibit impaired sexual behavior and neuroendocrine gonadotrophin regulation, anovulation, uterine dysfunction and impaired pregnancy associated mammary gland morphogenesis (Chappell et al. 1999; Lydon et al. 1995; Mani et al. 1996; Tibbetts et al. 1999). Furthermore, studies of the PRKO mouse reveal that PRs also play an essential role in regulation of thymic involution during pregnancy (Tibbetts et al. 1999) and in the cardiovascular system through regulation of endothelial and vascular smooth muscle cell proliferation and response to vascular injury (Vazquez et al. 1999).

The differences in transcriptional activities and coregulator interactions between the PR-A and PR-B observed in vitro predicted that these proteins may mediate different physiological responses to progesterone. In addition, the selective ability of PR-A to inhibit transcriptional responses induced by both PR-B and the estrogen receptors suggested that PR-A has the capacity to diminish overall progesterone responsiveness in certain tissues as well as contribute to the antiestrogenic activities of progesterone previously observed in the uterus (Lydon et al. 1995). However, physiological validation of the functional differences between the PR-A and PR-B isoforms has been hampered due to a lack of information on the specific cell types that express each isoform in vivo and a lack of appropriate animal models with which to dissect their selective functions.

The observation that PR-A and PR-B are produced by translation at two distinct AUG signals encoded by a single gene predicted that mutation of either ATG codon in the PR gene would result in selective ablation of expression of a single isoform in vivo (Conneely et al. 1989; Kastner et al. 1990). Thus, the CRE-loxP gene targeting

approach in embryonic stem cells was used to introduce a point mutation into the PR gene at the ATG codon encoding Met 1 (M1L) in order to specifically ablate expression of the PR-B protein and at the ATG encoding Met 166 (M166A) in order to ablate expression of PR-A (Mulac-Jericevic et al. 2000, 2003). This strategy has provided a powerful model system with which to examine the selective expression of each isoform in situ as well as to assess the selective contributions of PR-A and PR-B in their normal cellular context to the physiological functions of progesterone.

2.3 PRs and Ovarian Function

Luteinizing hormone (LH), the primary signal for rupture of preovulatory ovarian follicles leading to ovulation, stimulates transient expression of PR mRNA and proteins in granulosa cells (Natraj and Richards 1993; Park-Sarge and Mayo 1994) and the antiprogestin, RU486, inhibits ovulation (Loutradis et al. 1991). Definitive proof that PRs are essential mediators of ovulation has been provided by analysis of the ovarian phenotype of the PRKO mouse. Analysis of this model revealed that PRs are required specifically for LH-dependent follicular rupture leading to ovulation but not for differentiation of granulosa cells to form a corpus luteum (luteinization) (Lydon et al. 1995). Follicular rupture requires induction of a prostaglandin-mediated inflammatory response to LH as well as tissue degradation at the apex of the preovulatory follicle, an event that is mediated by matrix proteinases (Espey 1994). Analysis of the expression of potential mediators of ovulation in PRKO mice has demonstrated that LH-induced regulation of Cox-2, an enzyme that catalyzes the production of prostaglandins and is essential for ovulation, is unaffected (Robker et al. 2000). In contrast, the expression of two metalloproteinases, ADAMTS-1 (a desintegrin and metalloproteinase with thrombospondin motifs) and cathepsin-l (a lysosomal cysteine protease) is inhibited in mural granulosa cells of the mature follicle (Robker et al. 2000; Tetel et al. 1999) in PRKO mice. One of these proteases, ADAMTS-1 is essential for ovulation (Robker et al. 2000) suggesting that this protein may represent a critical mediator of the progesterone-induced ovulatory event.

Both the PR-A and PR-B proteins are induced in preovulatory follicles in response to LH stimulation (Natraj and Richards 1993). Analysis of PRAKO mice, in which PR-A is ablated, showed that ovulation is severely impaired but unlike in PRKO mice, is not completely absent (Mulac-Jericevic et al. 2000). Histological analysis of the ovaries of PRAKO mice showed numerous mature anovulatory follicles that contained an intact oocyte and were arrested at a similar stage to that previously observed in PRKO mice. In contrast, ovulation is unaffected in PRBKO mice indicating that PR-A expression is both necessary and sufficient to mediate the ovulatory response to progesterone (Mulac-Jericevic et al. 2003). The ovulatory defects in PRAKO mice must therefore be due to an inability of PR-B to regulate signaling pathways necessary for follicular rupture that are as yet unidentified. The observation that the PR-A and PR-B proteins are not functionally redundant in the ovary provides physiological validation of previous studies in tissue culture demonstrating that these transcription factors have different functional activities. From a mechanistic standpoint, the observation that PR-A alone is sufficient to support normal ovulation indicates that heterodimeric interactions between the PR-A and PR-B proteins are not required for regulation of essential progestin-responsive target genes associated with ovulation. With the exception of the above-mentioned proteases, the PR-dependent signaling pathways that mediate follicular rupture have not yet been elucidated. Because the PRKO mouse has a specific defect in follicular rupture while luteinization is maintained, differential array analysis using this model provides an excellent system with which to delineate the signaling pathways regulated by PRs that are specific to follicular rupture. The identification of these genes should facilitate the identification of PR isoform selective target genes that are essential for ovulation in addition to providing important new information on the molecular mechanisms of progesterone-induced follicular rupture.

2.4 PRs and Uterine Function

Progesterone plays a critical role during early pregnancy in the preparation of the uterine epithelium for receptivity, and differentiation of endometrial stromal cells to a decidual phenotype that supports

the development of the implanting embryo. Furthermore, progesterone is a potent antagonist of estrogen induced proliferation in the uterine epithelium.

The uterus of the PRKO mouse fails to support implantation after embryo transfer and is unresponsive to a decidual stimulus (Lydon et al. 1995). In PRKO mice, epithelial cells in the uterus become hyperplastic as a result of unopposed proliferative estrogen action. PRs are expressed in the epithelial, stromal and myometrial compartments of the uterus and their spatio-temporal expression within these compartments is regulated by both estrogen and progesterone (Tibbetts et al. 1998) and undergoes dynamic changes during the estrus cycle and early pregnancy (Tan et al. 1999). This intercompartmental regulation of PR expression is essential for the appropriate regulation of uterine gene expression (Kurita et al. 1998). Recent analysis of the effects of PR ablation on the expression of several genes previously implicated in progesterone dependent uterine implantation has shown that the defects observed in PRKO mice are associated with inhibition of expression of several epithelial markers of uterine receptivity (Mulac-Jericevic et al. 2000) and at least one essential stromal mediator of decidualization, hoxa-10 (Lim et al. 1999).

Analysis of progesterone dependent uterine function in PRAKO and PRBKO mice have revealed that PR-A and PR-B have distinct functions in the uterus (Mulac-Jericevic et al. 2000, 2003). In PRAKO mice, progesterone induced differentiation of endometrial stromal cells to a decidual phenotype is inhibited, suggesting that PR-A plays a crucial role in decidualization of the stroma prior to implantation (Mulac-Jericevic et al. 2000). In contrast, analysis of uterine function in PRBKO mice has shown that expression of PR-A is both necessary and sufficient to mediate both the antiproliferative and implantation associated responses to progesterone (Mulac-Jericevic et al. 2003). The uterine defects observed in PRAKO mice are due to an inability of PR-B to regulate a subset of PR dependent target genes rather than to differences in the spatio-temporal expression of PR-A relative to PR-B in the uterus (Mulac-Jericevic et al. 2000).

Surprisingly, selective activation of PR-B in the uterus of PRAKO mice resulted in an abnormal progesterone dependent induction of epithelial cell proliferation in contrast to its ability to inhibit estrogen induced proliferation in the wild-type uterus (Mulac-Jericevic et

al. 2000). This gain of PR-B dependent proliferative activity upon removal of PR-A indicates that PR-A is required not only to inhibit estrogen induced hyperplasia of the uterus but also to limit potentially adverse proliferative effects of the PR-B protein.

In the human endometrium, the levels of PR-A and PR-B are differentially regulated during the reproductive cycle (Mangal et al. 1997; Mote et al. 2000). The physiological importance of maintaining the correct relative expression levels of PR isoforms in the uterus is indicated by the identification of aberrant ratios of PR isoforms in human endometrial cancers, and the recent identification of a functional polymorphism in the human PR promoter that results in increased expression of the human PR-B isoform and is associated with increased risk of endometrial cancer (Arnett-Mansfield et al. 2001; De Vivo et al. 2002). Given the opposing effects of the PR isoforms in this uterus, one could predict that aberrant changes in the relative spatio-temporal expression patterns of PR isoforms in the uterus could play an important role in determining appropriate responsiveness to progestin therapy in the treatment of uterine epithelial hyperplasias.

2.5 PRs and Mammary Gland Morphogenesis

Mammary gland development is predominantly postnatal and is controlled by a complex interplay of endocrine hormones, in particular estrogen, progesterone and prolactin, acting together with locally acting growth factors (Anderson 2002; Soyal et al. 2002). Postnatal mammary gland development involves two distinct growth phases that are initiated at the onset of puberty and pregnancy respectively. At puberty, estrogen promotes ductal elongation and dichotomous branching to the limits of the mammary fat pad. At adulthood, the virgin gland becomes relatively quiescent with the exception of minimal side branching and alveolar budding that occur over time as a result of the cyclic rise of ovarian steroids during the estrus cycle. At pregnancy, exposure to progesterone and prolactin results in extensive epithelial proliferation, increased dichotomous side branching and differentiation of milk filled alveolar lobules. At weaning, removal of the suckling stimulus elicits involution of the lobular

alveolar system through apoptosis and matrix degrading proteinase mediated remodeling. At the end of the involution process, the postnatal developmental cycle of mammary gland development is completed and the mammary gland resembles the general architecture of the pre-pregnant mammary gland (Anderson 2002; Soyal et al. 2002).

Null mutation of both PR isoforms in PRKO mice has demonstrated that PRs are specifically required for pregnancy associated ductal proliferation and lobuloalveolar differentiation of the mammary epithelium. Mammary glands of PRKO mice failed to develop the pregnancy associated side branching of the ductal epithelium with attendant lobular alveolar differentiation despite normal postpubertal mammary gland morphogenesis of the virgin mice (Lydon et al. 1995; Seagroves et al. 2000). Thus, in contrast to its antiproliferative role in the uterus, progesterone is an essential pregnancy associated proliferative stimulus in the mammary gland.

PRs are expressed exclusively in the mammary epithelium (Ismail et al. 2002; Seagroves et al. 2000; Sivaraman et al. 2001) and development of the mammary gland from juvenile to adult state is associated with a change in pattern of expression of PRs from a uniform to a scattered pattern of expression in a subset of epithelial cells (Grimm et al. 2002; Seagroves et al. 2000). Recent studies have shown that PR expressing cells are segregated from proliferating cells in the normal mammary glands of both rodents and humans (Clarke et al. 1997; Ismail et al. 2002; Seagroves et al. 2000). Consistent with this finding, the proliferative responses of the ductal and alveolar epithelium to progesterone are associated local induction of PR dependent growth factors that act in a paracrine manner on PR negative cells to control their proliferation (Brisken et al. 1998, 2000). In contrast to the normal mammary gland, segregation of the steroid receptor expressing cells from proliferating cells is lost in mammary epithelial cells that have been exposed to carcinogen (Sivaraman et al. 2001) and in cells of breast tumors (Graham and Clarke 2002). This aberrant change in pattern of receptor expression is likely to contribute to abnormal growth of breast cancer cells.

Both isoforms of PR are expressed in the mammary gland of the virgin mouse (Shyamala et al. 1990) and during pregnancy (Fantl et al. 1999) and the levels of PR-A protein exceed those of the PR-B

isoform. Progesterone activated PR-B in PRAKO mice elicits side branching and lobular alveolar development in the mammary gland comparable to that in the wild-type mammary gland (Mulac-Jericevic et al. 2000). Thus, PR-B is sufficient to elicit normal proliferation and differentiation of the mammary epithelium in response to progesterone and neither process appears to require functional expression of the PR-A protein. In contrast, recent analysis of the mammary glands of PRBKO mice has shown reduced pregnancy associated ductal side branching and lobuloalveolar development as a consequence of decreased ductal and alveolar epithelial cell proliferation and increased apoptosis of alveolar epithelium (Mulac-Jericevic et al. 2003). Despite these defects, PR-A retains its normal segregated spatio-temporal pattern relative to proliferating cells in PRBKO mice and is expressed at a higher level than that observed for PR-B in PRAKO mice. Examination of the molecular genetic signaling pathways that are differentially regulated by PRs in the mammary gland, showed that the defects observed in PRBKO mice are associated with a PR-B isoform selective regulation of the *r*eceptor *a*ctivator of *n*uclear factor *k*appa B (NFκB) *l*igand (RANKL) signaling pathway (Mulac-Jericevic et al. 2003) which is essential for alveologenesis (Fata et al. 2000). In contrast, progesterone dependent activation of secreted growth factor, wnt4, a key mediator of branching morphogenesis (Brisken et al. 2000) is normally regulated by either PR-A or PR-B (Mulac-Jericevic et al. 2003). Thus PR-A and PR-B appear to regulate both overlapping and distinct subsets of progesterone dependent signaling pathways required for mammary gland development.

2.6 Conclusion

Analysis of the phenotypic consequences of selective ablation of the PR-A or PR-B isoforms in mice has facilitated a comprehensive analysis of the selective spatio-temporal expression of these receptors in the reproductive tract and mammary gland, as well as elucidation of their individual contributions to the established reproductive activities of progesterone. These studies have revealed that PR-A and PR-B exhibit mostly distinct but partially overlapping hor-

Table 1. Components of female reproductive system and phenotype identified in progesterone receptor knockout mouse models

Component	Genotype	Phenotypes
Ovaries	PRKO	Inability to ovulate
	PRAKO	Severely impaired ovulation
	PRBKO	Normal ovulation
Uterus	PRKO	Impaired implantation/decidualization/ infertility
	PRAKO	Impaired implantation/decidualization/ infertility
	PRBKO	Normal implantation/decidualization
Mammary gland	PRKO	Impaired mammary gland development
	PRAKO	Normal pregnancy induced mammary gland morphogenesis
	PRBKO	Reduced pregnancy associated side-branching and lobuloalveolar development

monal responses (Table 1). PR-A activation is both necessary and sufficient for establishment and maintenance of pregnancy but elicits reduced mammary gland morphogenic responses to hormonal stimulation relative to PR-B. In contrast, PR-B activation is insufficient to support female fertility but is a potent proliferative mediator in the mammary gland and most surprisingly, in the uterus. The distinct tissues selective activities of PR-A and PR-B observed in vivo in these studies support the conclusion that modulation of PR activity using isoform selective progestins may contribute toward elimination of adverse tissue-specific side effects of progestins.

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3 The Progesterone Receptor/Estradiol Receptor Association and the Progestin-Triggered S-Phase Entry

A. Migliaccio, G. Castoria, M. Di Domenico, C. Ballaré, M. Beato, F. Auricchio

3.1 Introduction

The mechanism by which steroids stimulate cell proliferation is still debated. Regulation of growth factor production or stimulation by steroids of expression of genes required for cell division has been proposed (Dickson and Lippman 1995). In addition to the early genes, cell cycle controlling genes and cyclin-dependent kinases are also regulated by steroid hormones (Musgrove et al. 1993). Ovarian hormones stimulate cell proliferation in several female tissues (Clarke and Sutherland 1990). Estrogens and progesterone increase alveolar formation as well as ductal branching in mammary gland (Warner et al. 1978). In addition, progestins stimulate mammary gland cell proliferation in a variety of systems, including the mouse mammary gland (Bresciani 1968), mammary tumors of rodents (Kiss et al. 1986; Manni et al. 1987) and primary culture of human mammary epithelial cells (Longman and Buehring 1987; Hisson and Moore 1987). Targeted disruption of the estrogen receptor α (ERα) gene demonstrates a role for this receptor in the postnatal development of the uterus and the mammary gland, as homozygous ER$\alpha^{-/-}$ female mice exhibit hypoplastic uteri and undeveloped glands, with only vestigial ducts present at the nipples (Lubahn et al. 1993; Korach 1994). On the contrary, PR$^{-/-}$ mice have normally developed uteri at puberty but respond to repeated administration of estrogens with a dramatic hyperplasia and inflammation of the uterus, suggesting that progesterone receptor (PR) is involved in controlling the proliferative response to estrogens and in preventing uterine inflammation (Lydon et al. 1995). In contrast, progesterone is the actual proliferative hormone in the postpuberty growth of the mammary gland (Lydon et al. 1995). Although both stromal and epithelial cells participate in mammary gland development, the PR of epithelial cells seems to be essential for lobulo-alveolar development (Humphreys et al. 1997). Such a requirement is probably mediated by cyclin D1 expression, as a disruption of this gene yields a very similar mammary phenotype, namely lack of postpuberty development (Sicinski et al. 1995).

Steroid hormones exert their effect through regulation of gene transcription (Beato et al. 1995). Nevertheless, they produce rapid, nontranscriptional events mediated by activation of multiple signal-

ing pathways. Such activation has been described by our and other groups under different experimental conditions and/or in different cell types. It triggers various effects, including cell cycle progression, neuroprotection, vasorelaxation or bone protection (Migliaccio et al. 1998, 2000; Castoria et al. 1999, 2001, 2003; Honda et al. 2000; Simoncini et al. 2000; Kousteni et al. 2001). It has been reported that progesterone activates calcium influx in sperm (Blackmore et al. 1990) and induces protein tyrosine phosphorylation (Mendoza et al. 1995). Progestins stimulate oocyte maturation in *Xenopus* by a mechanism involving protein kinase activation (Sagata et al. 1989; Muslin et al. 1993). Furthermore, involvement of the Src/ERK pathway in progesterone-induced oocyte maturation in *Xenopus* has also been described recently (Boonyaratanakornkit et al. 2001).

Analysis of the mechanisms responsible for the hormone-dependent and steroid receptor-mediated pathway activation in reproductive cells reveals association between steroid receptors [ERα or β, progesterone receptor (PR) and androgen receptor (AR)]. These complexes recruit several cytoplasmic-coupled effectors, which trigger DNA synthesis of responsive cells (Migliaccio et al. 2000; Castoria et al. 1999, 2001). Our results highlight the central role of the hormone-regulated protein–protein interactions in the steroid action. The mechanism of signaling pathway activation by progestins in human mammary cancer-derived T47D cells is now reviewed.

3.2 Progestin Stimulates the Src/RAS/ERK Signaling Pathway in Human Cancer-Derived T47D Cells

3.2.1 Progestin Stimulates the Src/Ras/Erk Pathway in T47D Cells, Antiprogestins and Antiestrogens Inhibit This Action

The Erk-2 activity was evaluated in quiescent T47D cells challenged for different times with 10 nM synthetic progestin R5020. Progestin treatment resulted in a rapid stimulation of Erk-2 activity, which was already detectable after 2 min, reached the maximal values after 5 min and returned to basal levels after 60 min (Migliaccio et al. 1998). The antiprogestin RU486 (at 1 μM) inhibited the 5-min stimu-

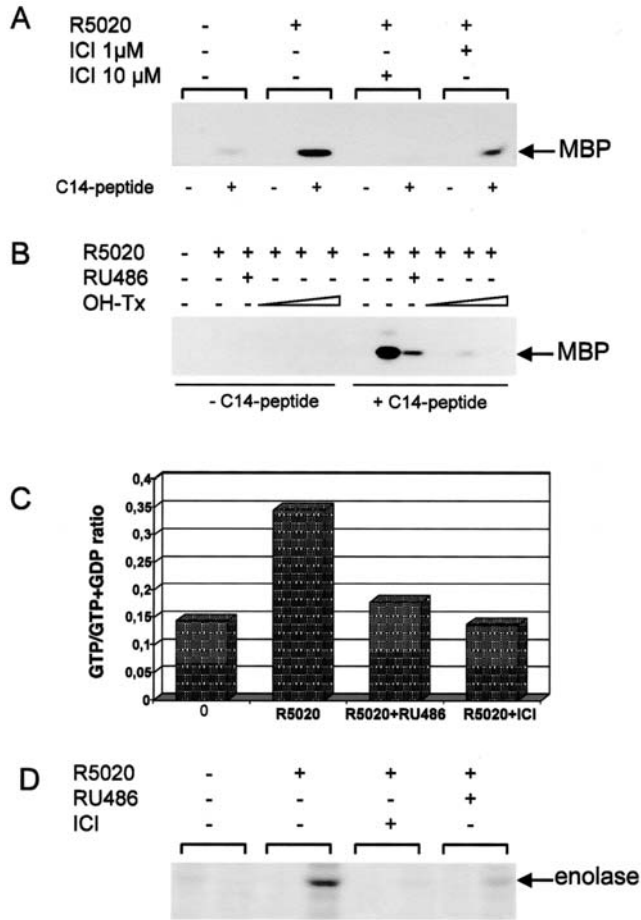

Fig. 1 A–D. Activation of Erk-2, Ras and Src in progestin-treated T47D cells. Effects of antiprogestins and antiestrogens. In **A** and **B**, 10 nM R5020, in the absence or presence of either 1 or 10 μM ICI 182 780 or increasing concentrations of OH-Tamoxifen (0.1, 1 and 10 μM) were added to the cell medium for 5 min. Lysates were immunoprecipitated with anti-Erk-2 antibody in the absence or presence of the C-14 peptide, against which the antibody had been raised. Immunoprecipitates were assayed for Erk activity using myelin basic protein (*MBP*), as a substrate. In **C**, cells were radiolabeled with [^{32}P] orthophosphate and incubated for 4 min in the absence or presence of 10 nM R5020, alone or together with 1 μM ICI 182 780.

latory effect of the agonist treatment, indicating that binding to the PR is required for the progestin effect (Fig. 1 B). Surprisingly, the stimulatory effect of the progestin on Erk-2 activity was inhibited by two different antiestrogens, the pure steroidal antiestrogen ICI 182 780 and the nonsteroidal antiestrogen OH-Tamoxifen (Fig. 1 A, B).

Activation of signaling effectors upstream of the MAP kinases was analyzed next. Ras activity was measured in [^{32}P]ortho-phosphate-labeled T47D cells treated for 4 min with 10 nM R5020, in the absence or presence of antiprogestins or antiestrogens. The GTP : GTP + GDP ratio was calculated using a PhosphoImager. Figure 1C shows that the R5020 treatment increased the GTP : GTP + GDP ratio 2.6-fold. Such an increase was strongly reduced or abolished by the addition of either the antiprogestin RU486 or the antiestrogen ICI 182 780. These findings show that progestin activates Ras by a mechanism which is likely to require both PR and ER.

Treatment of T47D cells with 10 nM R5020 activated Src kinase. Such activation was detected 2 and 5 min after hormone addition and decreased to the basal levels thereafter (Migliaccio et al. 1998). T47D cells were also treated with 10 nM R5020 for 2 min in the presence of either 1 μM of the antiprogestin RU486 or 10 μM of the antiestrogen ICI 182 780 (Fig. 1 D). The stimulatory effect of R5020 on Src activity was almost completely abolished by the antiprogestin, indicating a requirement for PR occupancy by the agonist. Src activation by R5020 was strongly reduced by ICI 182 780, suggesting the ER requirement for progestin activation of the Src/Ras/ERK pathway.

Nucleotides bound to Ras were analyzed. The ratio GTP : GTP + GDP averaged from two experiments is shown. In **D** cells were untreated or treated for 2 min with R5020 in the absence or presence of either RU486 or ICI 182 780. Lysates were incubated with either control or anti-Src 327 antibody. The immunoprecipitates were assayed for Src kinase activity using acid-treated enolase as a substrate

3.3 Progestin Stimulation of the Src/RAS/ERK Signaling Pathway Requires Estrogen Receptor

3.3.1 Progestin Stimulation of Erk-2 and c-Src Activities in Cos-7 Cells After Transient Expression of Progesterone and Estradiol Receptors

Because of the inhibitory effect of antiprogestins and antiestrogens on progestin activation of the Src/Ras/ERK pathway, we hypothesized that progestin action is mediated by PR, but also depends on antagonist-free ER. To address this issue, Cos-7 cells lacking ER and PR were transfected with either pSG5-HEGO plasmid encoding the human ERa (Tora et al. 1989), or pSG5-hPR-B encoding the hu-

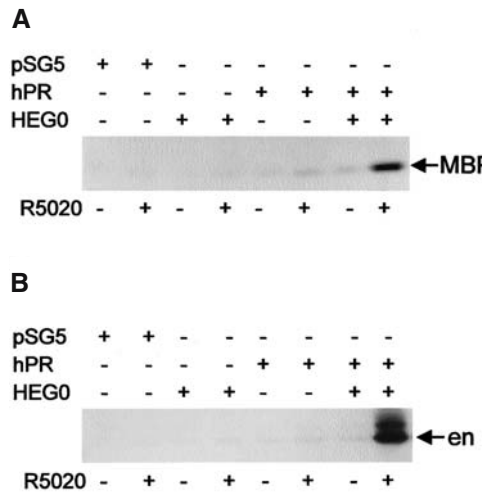

Fig. 2 A, B. Effect of progestin on Erk-2 and c-Src in Cos-7 cells co-transfected with hPR-B and hER*α* cDNA. Cos-7 cells were transfected with either empty pSG5 vector, or pSG5-hPR-B, or pSG5-hER*α* (HEG0) or both the pSG5-hPRB and -hER*α* vectors. Cells were then left unstimulated or stimulated with 10 nM R5020. Lysates from cells stimulated for 5 min were incubated with anti-Erk-2 antibody (**A**), whereas lysates from cells stimulated for 2 min were incubated with anti Src antibodies (**B**). Immunoprecipitates were assayed for Erk-2 (**A**) or Src (**B**) activity, respectively

man PR-B (Kastner et al. 1990) or with both plasmids. The effect of progestins on Erk-2 activity of transfected cells was assayed. Both receptors were co-expressed to a similar extent (Migliaccio et al. 1998). Fig. 2 shows that in ER-expressing cells, progestin treatment did not affect the basal level of Erk-2 and Src activities. While in PR-B expressing Cos cells the progestin-induced pathway activation was undetectable or very weak, a strong stimulation of the Src/Erk-2 pathway by R5020 was observed in Cos cells expressing both ER and PR-B.

These findings demonstrate that a strong progestin effect on the Src/ERK signaling activation in Cos cells requires not only binding to the PR, but also ER expression. They also support the interpretation that antiestrogen inhibition of progestin-stimulated Src/Ras/ERK pathway in T47D cells (see Fig. 1) is a consequence of their binding to ER, with the consequent inhibition of the ER function in T47D cells.

3.3.2 Progestin Does Not Stimulate Src Activity in Cos Cells in the Absence of ER/Src Association

Experiments with T47D cells showed that association of ERα with Src is parallel to progestin-induced Src activation (Migliaccio et al. 1998). In addition, our in vitro findings demonstrate a lack of this interaction when HEG537F was used in place of the wild-type receptor (Migliaccio et al. 2000). On this basis, it is expected that HEG537F should be unable to interact with Src and activate Src in whole cells stimulated with either steroid, according to our view that ER/Src association is necessary for Src activation by estradiol or progestin. Cos cells were transfected with the control plasmid pSG5 or plasmids expressing either HEG0 or its mutant HEG537F (Migliaccio et al. 2000). Stimulation of transfected cells with 10 nM estradiol induced association between ER and Src, with the consequent activation of tyrosine kinase. A weak, and possibly nonspecific, association was observed in Cos cells expressing the ER mutant, HEG537F. In agreement with this result, no Src activation by estradiol was detected in cells transfected with HEG537F (Fig. 3 A, B). Expectedly, in Cos cells co-expressing PR-B and ER, a strong Src

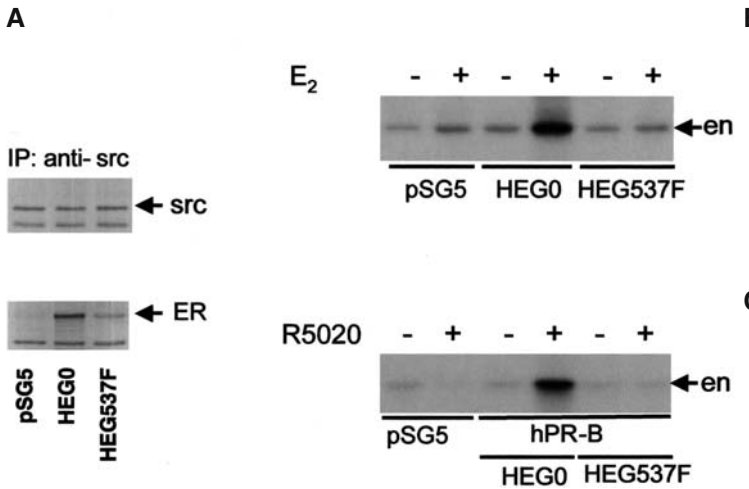

Fig. 3 A–C. Association of ER*α* with Src is required for estradiol or progestin activation of Src. Cos cells were transfected with either empty pSG5 vector or vector pSG5-HEG0 or pSG5-HEG537F and challenged with 10 nM estradiol for 2 min. Lysates were immunoprecipitated with anti-Src antibody. **A** Immunoprecipitates were blotted with either anti-Src (*upper*) or H222 anti-ER (*lower*) antibodies. **B** Immunoprecipitates were assayed for Src activity using enolase (*en*) as substrate. **C** Cos cells were transfected with the empty pSG5 vector or PSG5-PR-B together with either PSG5-HEG0 or pSG5-HEG537F. Cells were stimulated for 2 min with 10 nM R5020 progestin and Src activity of the immunoprecipitates from cell lysates was assayed

activation was detected upon progestin stimulation. In contrast, it was undetectable in the same cells co-expressing PR-B together with the HEG537F mutant (Fig. 3C).

From these findings we conclude that phosphotyrosine 537 is crucial for association between ER and Src with the consequent Src activation by estradiol. Phosphotyrosine 537 is also required for the progestin-induced cross-talk between PR-B and ER*α* leading to Src activation by progestin. This is in excellent agreement with the view that such a cross-talk leading to Src-dependent pathway activation requires ER*α*–Src association.

3.3.3 Association of PR-B with ER Is Required for Signaling Activation by Progestin

In T47D cells, 6% of the total PR-B is associated with 5% of the total ER (Ballaré et al. 2003). Far Western blotting analysis revealed that this association is direct and involves the hormone binding domain of ERα (Migliaccio et al. 1998). A detailed analysis of this interaction by yeast two-hybrid experiments (Ballaré et al. 2003) identified two regions in the N-terminal half of PR-B, ERID-I and ERID-II, as necessary for the PR-B–ERα association observed in transfected Cos cells (Fig. 4 A). Panel B shows stimulation of Erk-2 activity in Cos cells expressing both the wild-type PR-B and ERα. Interestingly, no Erk-2 activation was detected in Cos cells expressing the PR-B deletion mutants lacking either one or two association domains. These data further confirm that progestin stimulation of the signaling pathway requires PR-B/ER association.

In analogy with our findings of a direct association between AR and Src (Migliaccio et al. 2000), a direct association between PR-B and Src has also been described recently (Boonyaratanakornkit et al. 2001). In each case, requirement of a proline stretch for these associations has been described (Migliaccio et al. 2000; Boonyaratanakornkit et al. 2001). Indeed, mutation of three prolines in the cluster abolishes the PR-B/Src association (Boonyaratanakornkit et al. 2001). Nevertheless, this mutant, when expressed instead of the wild-type PR-B, does not affect the progestin-induced Src and Erk-2 activation in Cos cells (Ballaré et al. 2003), implying that direct association of PR-B with Src does not play a major role in signaling activation by progestin in the analyzed cells.

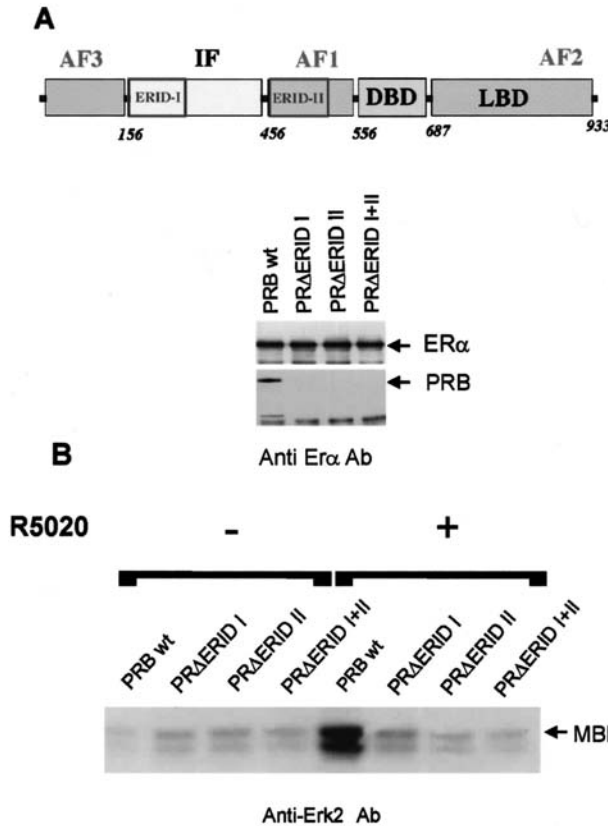

Fig. 4 A, B. Interaction between ER*α* and PR-B in Cos-7 cells is required for Erk activation by progestin. Cos-7 cells were transiently co-transfected with ER*α* along with the indicated PR expression vectors (PRB wild-type, or mutants lacking one or two domains interacting with ER*α*: PR ΔERID-I, PR DERID-II, or PR DERID-I+II). Expression of ER*α* and the different PR constructs was verified by immunoblotting with appropriate antibodies. **A** Scheme of the location of ERID-I and ERID-II in PRB and the co-immunoprecipitation of ER*α* and PR from lysates incubated with anti-ER*α* antibody. Each immunoprecipitate was analyzed with anti-ER*α* (*upper*) or anti-PR (*lower*) antibodies. ER*α* and PRB bands are indicated by *arrows*. **B** Transfected cells were treated for 5 min with or without 10 nM R5020 and lysates assayed for Erk-2 activity using MBP as a substrate

3.4 Signaling Activation Is Required for Progestin-Induced DNA Synthesis of Mammary Cancer Cells

3.4.1 Microinjection of a Dominant Negative Form of Src or Anti-Pan Ras Antibody Inhibits the Steroid-Induced S-Phase Entry of MCF-7 and T47D Cells

The findings that estradiol and progestins activate the Src/Ras/Erk_s signaling pathway raised the question of the role of this stimulation (Castoria et al. 1999). Microinjection experiments of human mammary cancer-derived cells (MCF-7 and T47D) with cDNA of catalytically inactive Src or anti-Ras antibody prove that Src and Ras are required for estradiol and progestin-dependent progression of cells through the cell cycle (Fig. 5A,B). In panels A and B, the mutant form of Src, with Lys295 changed to methionine (Src K^-; Barone and Courtneidge 1996), was microinjected into nuclei of target cells and the DNA synthesis was analyzed in cells stimulated with either estradiol (MCF-7 cells, panel A) or progestin R5020 (T47D cells, panel B). The results expressed in Fig. 5A and B show that microinjection of Src K^- inhibits the steroid-stimulated entry into S-phase of both MCF-7 and T47D target cells by 70%. In contrast, microinjection of a plasmid expressing the wild-type form of Src (Src wt) did not affect the steroid-induced S-phase entry (Fig. 5A,B). Requirement of Ras signaling for steroid-elicited DNA synthesis was then verified by injection of blocking anti-Ras monoclonal antibody (Y259 MAb) into the cytoplasm of quiescent MCF-7 and T47D cells (Fig. 5C,D). Data show that inhibition of steroid-induced BrdU incorporation by neutralizing anti-Ras antibody was almost total for both MCF-7 (C) and T47D (D) cells. The S-phase entry of cells was unaffected by injection of purified IgGs from nonimmunized rabbits (Fig. 5C,D).

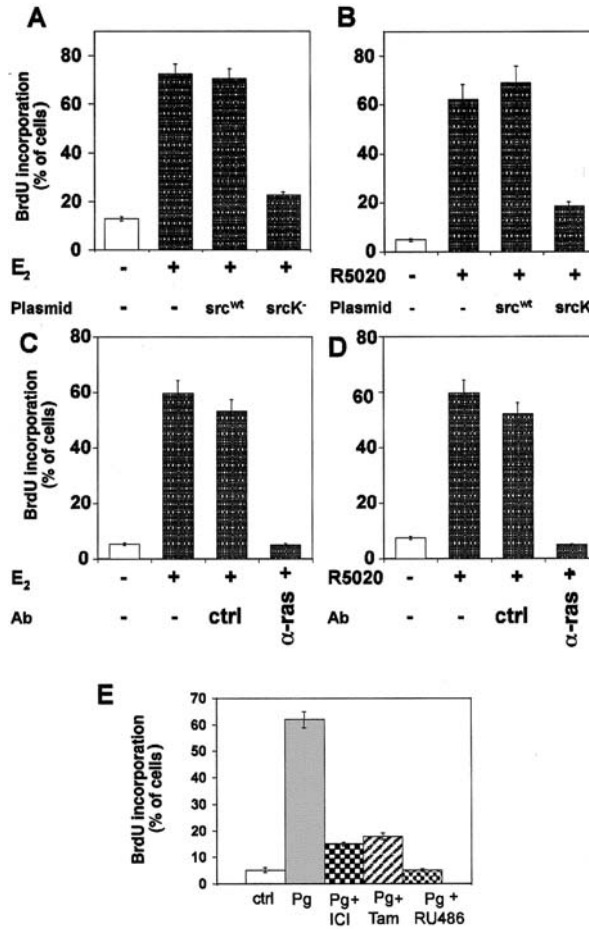

Fig. 5 A–E. Signaling activation is required for steroid-induced S-phase entry in T47D cells. PR-B/ERα cross-talk is involved in DNA synthesis stimulated by progestins. Quiescent MCF-7 (**A** and **C**) or T47D (**B** and **D**) cells on coverslips were microinjected, in **A** and **B** with the indicated plasmids (Src K^- or Src wt), in **C** and **D** with either anti-Pan Ras or purified rabbit antibodies. Either 10 nM estradiol (**A** and **C**) or 10 nM progestin R5020 (**B** and **D**) was added to the cells together with BrdU, and 24 h later they were fixed and stained. Coverslips were analyzed for DNA synthesis. It was calculated in injected cells by the formula: percentage of BrdU-positive

3.4.2 The Cross-Talk Between PR-B and ER*α* Regulates Progestin-Induced DNA Synthesis of T47D Cells

The synthetic progestin, R5020, triggers Src/Ras/Erk activation as well as Src-dependent proliferation and each of these steps is prevented not only by antiprogestins but also by the antiestrogens (Fig. 1). In addition, progestin stimulation of the Src/Ras/Erk pathway triggers DNA synthesis. On the basis of present evidences it was also expected that progestin stimulation of the DNA synthesis in T47D cells should be inhibited by antiprogestin and antiestrogen. Therefore, the effect of these antagonists on progestin-induced S-phase entry of T47D cells was analyzed (Migliaccio et al. 2002). Panel E in Fig. 5 shows that BrdU incorporation of TD47D cells is strongly stimulated by the progestin R5020. This effect is abolished by the antiprogestin RU486 and drastically reduced by two antiestrogens, ICI 182,780 and OH-Tamoxifen.

Taken together, data in Fig. 5 demonstrate that targeting of Src and Ras interferes with steroid-induced progression of target cells into S-phase. They also show that cross-talk between PR-B and ER*α* regulates cell cycle progression of T47D cells.

Present data show that cross-talks between steroid receptors are regulated by direct association between receptors (PR-B and ER*α*) as well as their interaction with important effectors of the signaling pathways. The hormone-triggered assembly of these multimolecular complexes has multiple effects and implications. It makes possible or reinforces the hormone action, generating a strong activation of the signaling pathways. It also offers the opportunity to specifically interfere with cell growth disrupting protein–protein interactions involved in signaling activation, without directly affecting the transcriptional activity of steroid receptors.

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cells = (number of injected BrdU-positive cells/number of injected cells) $\times 100$. In **E** quiescent T47D cells on coverslips were left untreated or treated for 24 h with the indicated compound. After BrdU pulse coverslips were fixed, stained and analyzed for DNA synthesis, which was calculated by the formula: percentage of BrdU-positive cells = (number of BrdU-positive cells/number of total cells) $\times 100$

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4 Endometrial Morphology and Progestogens

H. O. D. Critchley

4.1 Introduction

Crucial reproductive events for continuation of the human species are implantation, and in the absence of pregnancy, menstruation and endometrial repair. The human endometrium is a dynamic tissue that, in response to the prevailing steroid environment of sequential oestrogen and progesterone exposure, undergoes a well-characterized cycle of proliferation, differentiation and breakdown each month. Pivotal to reproductive events is the ability of the uterine endometrial lining to shed and regenerate if pregnancy does not occur. Menstruation is the reproductive process whereby the upper two-thirds of the endometrial (functional layer) is shed and regenerated on a repetitive basis. The endometrium is consequently a site of physiological injury and repair.

Studies undertaken by Markee (1940), and Corner and Allen (1929) established the role for ovarian steroids, oestradiol and progesterone, in regulating the changes in endometrial structure across the menstrual cycle. Menstruation is the response of the endometrium to the withdrawal of progesterone (and oestrogen) that occurs with demise of the corpus luteum in the absence of pregnancy. 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 and have been reviewed (Critchley et al. 2001 a).

Progesterone is essential for the establishment and maintenance of pregnancy consequent upon the transformation of an oestrogen-primed endometrium. Much remains to be established about the molecular and cellular mechanisms orchestrated by sex steroid hormones and the determinants of uterine receptivity. Sex steroids, acting via their cognate receptors, initiate a cascade of gene expression important for implantation and the early stages of pregnancy. It is therefore necessary to delineate the local mechanisms regulating local endometrial events involved in implantation and menstruation if we are to understand early pregnancy failure, aberrations of menstrual bleeding and the requirements for endometrial contraception. Indeed, armed with a better understanding of the local mechanisms involved in endometrial function, there will be the potential to modulate sex steroid interactions with far reaching applications for the management of female reproductive health.

4.2 Endometrial Morphology

The endometrium is a target tissue for steroid hormones and is composed of two layers. The upper functional layer is shed at the time of menstruation. The endometrium regenerates after menstrual shedding from an underlying basal layer. Oestrogen is the steroid responsible for proliferative changes during the follicular phase of the ovarian cycle and exposure of the endometrium to progesterone results in differentiation during the luteal phase. Thus in response to cyclical ovarian activity the pre-menopausal endometrium undergoes a series of classic morphological changes (described and reviewed in detail by Noyes et al. 1950; Buckley and Fox 1989). The exogenous administration of sex steroids produces a marked variability in classic histological criteria (Noyes et al. 1950) when compared with carefully dated endometrium collected during the corresponding phase of the physiological cycle (Habiba et al. 1998). Classic histological criteria may therefore be inappropriate in the assessment of endometrium exposed to exogenous steroids (for example progestogens), or steroid receptor modulators. Indeed the endometrial features observed will reflect the time in the cycle of administration, route of delivery and formulation and dose of steroid administered and the duration of therapy (Pekonen et al. 1992; Carranza-Lira et al. 1998; Buckley and Fox 1989). Progestogens will result in a morphological response only if progesterone receptors (PRs) are present in endometrial cells. PR expression requires prior exposure to oestrogen (see Sect. 4.3). Overall progestogens exert an anti-oestrogenic effect with inhibition of endometrial growth and induction of maturation and differentiation of the glandular and stromal cells. If hormone delivery is in the early proliferative phase then minimal stromal decidualization and glandular secretory development will occur. Spiral artery differentiation will be absent. Progestogens administered in the later proliferative phase result in marked pseudodecidualization of the upper endometrial zones. Prolonged administration of exogenous progestogens produces an atrophic endometrium with few inactive glands distributed in a shallow compact stroma. Thin-walled blood vessels are a noticeable feature and the endometrium has a marked leukocytic infiltrate [particularly of natural killer (NK) cells; see Sect. 4.9]. Again spiral artery growth is absent with prolonged

exogenous progestogen administration. For a detailed description of the endometrial morphological response of the endometrium to exogeous sex hormones see Buckley and Fox (1989). In order to appreciate the morphological and functional changes induced in the endometrium after exposure to progestogens it is necessary to review some of the critical parameters that regulate normal endometrial morphology and function.

4.3 Overview of Endometrial Sex Steroid Receptor Expression

Steroids interact with their target organs via specific nuclear receptors. Recently non-genomic (membrane bound) steroid receptors have been characterized, including a membrane bound PR (Losel et al. 2003; Zhu et al. 2003). Members of the nuclear receptor superfamily include progesterone, oestrogen, androgen and glucocorticoid receptors. The expression of oestrogen receptor (ER) and PR are under dual control of oestradiol and progesterone. The expression of endometrial sex steroid receptors [PR, ER and androgen receptor (AR)] varies both temporally and spatially across the menstrual cycle (Garcia et al. 1988; Lessey et al. 1988; Snijders et al.1992; Critchley et al. 2001b; Slayden et al. 2001).

Much insight has been gained about progesterone/progestogen action on endometrial function from the observations of pharmacological withdrawal of progesterone from the endometrium (Critchley et al. 2003). Hence, studies that address the actions of anti-progestins have informed understanding about the local mechanisms that are targeted in order to maximize the contragestive and abortifacient properties of these compounds. The anti-progestin, mifepristone (RU486), is known to exert its inhibitory effects by impairing the gene regulatory activity of the progesterone receptor (Baulieu 1989).

Utilization of a model of PR antagonism in vivo, that is, administration of the anti-progestin mifepristone, has made it possible to study local events in both non-pregnant endometrium and early pregnancy decidua. Antagonism of progesterone action at the level of its receptor results in an up-regulation of key local inflammatory media-

tors (chemokines and prostaglandins) and an influx of leukocytes (Cheng et al. 1993; Critchley et al. 1996).

In vivo studies where anti-progestins have been administered acutely in the luteal phase of the cycle or chronically at a low dose provide evidence for those functions in the non-pregnant endometrium regulated by progesterone. An increased expression of sex steroid receptors, ER, PR and AR in both the glandular and stromal compartments in mid-luteal phase endometrium after early luteal phase administration of anti-progestins has been described (Cameron et al. 1996; Berthois et al. 1991; Maentausta et al. 1993; Slayden et al. 2001). Administration of an anti-progestin in the early luteal phase will adversely affect local factors of potential importance to implantation, whereas administration in the mid-luteal phase will influence factors implicated in endometrial bleeding. The endometrial changes (including marked alterations in the endometrial vasculature; Johannisson et al. 1988) associated with withdrawal of progesterone and menstrual bleeding supports the involvement of vasoactive local mediators. Endometrial prostaglandin activity is modulated by progesterone and hence prostaglandins, with widely recognized vasoactive properties, are prime candidates for local modulation of progesterone action (Baird et al. 1996; Hapangama et al. 2002).

Chronic low dose oral administration of mifepristone has revealed the sensitivity of the endometrial morphology to anti-progestin exposure. Low dose daily administration of mifepristone inhibits ovulation and induces amenorrhoea or a marked reduction in endometrial bleeding (Brown et al. 2002). Chronic anti-progestin administration inhibits both endometrial secretion and proliferaton (endometrial anti-proliferative effects; Brenner et al. 2002 a).

4.4 Endometrial ERs

Steroid receptors are known to exist in more than a single isoform. Two structurally related subtypes of ER, known as alpha (ERα) and beta (ERβ) have been identified in the human (Green et al. 1986; Kuiper et al. 1996; Enmark et al. 1997). The function of ERβ in the uterus not yet been fully elucidated. Within the upper endometrial zones (functional layer) ERα expression increases in both glandular

and stromal cells in the proliferative phase and declines in the secretory phase due to suppression by progesterone. In the basal zone ERα is present in glandular and stromal cells across the cycle (Snijders et al. 1992; Garcia et al. 1988). In both the human and non-human 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 functional layer (Critchley et al. 2001 b). It is of particular interest that, unlike ERα, ERβ has been detected with both polyclonal and monoclonal anti-ERβ antibodies in the nuclei of the vascular endothelial cells. The presence of ERβ in endometrial endothelial cells suggests that oestrogen may act directly on endometrial blood vessels (Critchley et al. 2001 b; Leece et al. 2001; Critchley et al. 2002). There is thus the potential for direct effects of oestrogen in the process of endometrial angiogenesis and vascular permeability changes during the cycle. Albeit controversial, to date the PR has been described as absent from the vascular endothelium (Perrot-Applanat et al. 1994; Critchley et al. 2001 b) of the spiral arteries. Thus effects of progesterone or progestogens on endometrial vessels, which are likely to play a crucial role in endometrial bleeding, may be indirectly mediated by the PR-positive perivascular stromal cells.

4.5 Endometrial PRs

Two subtypes of the human PR have been described (Clarke and Sutherland 1990; Conneely and Lydon, 2000). PRA and PRB derive from a single gene and function as transcriptional regulators of progestin-responsive genes. PRA is the shorter subtype, missing 164 amino acids present at the N terminus of the B subtype. It is otherwise identical to the B subtype (Tung et al. 1993). A significant decline in PR expression in the glands of the functional layer of the endometrium, with the transition from the proliferative to the secretory phase of the cycle, has been reported. PR expression persists in the stroma in the upper functional zone, being particularly highly expressed in stromal cells in close proximity to the uterine vasculature. The basal zone is differentially regulated in that the glands and stroma of the deeper zones express PR throughout the cycle. Differences

between the superficial and basal zones of the endometrium are likely to be functionally important given that only the upper 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. For example, during the luteal phase the PRB subtype appears to decline in the stroma and PRA becomes the predominant form (Wang et al. 1998; Brosens et al. 1999).

4.6 Endometrial ARs

The endometrium is also a site for androgen action, either directly via the AR or indirectly via the ER after aromatization to oestrogen (Horie et al. 1992). Circulating concentrations of testosterone vary only minimally across the menstrual cycle. This is in contrast to the marked cyclical variations in oestradiol and progesterone concentrations. Testosterone concentrations are however approximately 10 times higher than those of oestradiol (Ribeiro et al. 1974: Goebelsman et al. 1974). AR has been spatially localized to human endometrial stroma with up-regulation in the oestrogen dominated proliferative phase followed by down-regulation in the late secretory phase. In the normal endometrium there is minimal expression in the glands (Mertens et al. 2001; Slayden et al. 2001; Burton et al. 2003). The physiological role of endometrial AR and the regulation of its expression is unknown. Androgens are, however, known to suppress oestrogen-dependent endometrial proliferation and Brenner and colleagues (2002 a; 2003) have hypothesized that the endometrial AR is involved with the anti-proliferative effects induced by anti-progestins. Indeed Brenner's group (Slayden and Brenner 2003) have demonstrated that in the rhesus macaque administration of an anti-androgen, flutamide, will counteract the suppressive effects produced by anti-progestins on endometrial thickness, stromal compaction and mitotic index. The endometrial AR may be a critical component of the mechanism by which anti-progestins induce endometrial proliferation in the presence of circulating oestrogens (Brenner et al. 2003).

4.7 Endometrial GRs

The role of glucocorticoids in endometrial physiology is not well understood. Bamberger and colleagues (2001) have described briefly the localization of the glucocorticoid receptor (GR) across the menstrual cycle. The GR was almost exclusively expressed in the stromal compartment and in endothelial and lymphoid cells. Recently Henderson et al. (2003) reported both at the mRNA and protein level that uterine NK cells express GR. The role of glucocorticoids in endometrial immune function has not yet been extensively studied.

4.8 Steroid Metabolism Within Endometrial Cells (Endometrial Intracrinology)

In reproductive tissues, the local actions of sex steroids, oestrogens, progestogens and androgens are modulated by hydroxysteroid dehydrogenase (HSD) enzymes. The various dehydrogenases are multigene families. By way of example, 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 (Peltoketo et al 1999; Labrie et al 2000). The type 2 enzyme (17β HSD-2) plays a major role in inactivation of oestradiol to oestrone (Mustonen et al. 1998). 17β HSD-2 is expressed in the endometrial glandular epithelium and is up-regulated by progesterone (Maentausta et al. 1993). Its activity decreases when progesterone concentrations decrease (as with luteal regression) or after anti-progestin administration (Mustonen et al. 1998; Maentausta et al. 1993).

4.9 Endometrial Leukocyte Populations

Leukocyte populations within the endometrial stroma vary across the menstrual cycle and throughout pregnancy. Endometrial leukocytes include T and B cells, mast cells, macrophages and neutrophils but it is the phenotypically unique uterine natural killer cells (uNK) that make up the majority of the leukocyte population in the late secre-

tory phase and early pregnancy (King 2000; Henderson et al. 2003). 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, oestrogen 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 present but their numbers increase from day luteinizing hormone surge (LH)+3 and markedly so in the mid-late secretory phase (day LH+11–13) where they are found in close contact with endometrial glands and spiral vessels (King et al. 1998; King 2000). It has not been 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 (Lanier et al. 1997). There is evidence for proliferation of uNK cells in the endometrium as the proliferation marker Ki67 has been co-localized within this cell type (King et al.1991; Kammerer et al. 1999). It has been proposed that oestrogen and progesterone may exert their effects on uNK cells indirectly via cytokines such as interleukin (IL)-15 and prolactin or other soluble factors (Verma et al. 2000; Dunn et al. 2002; Gubbay et al. 2002).

Recent quantitative real time RT–PCR studies have demonstrated an absence of ERα and PR mRNA in purified uNK cells (Henderson et al. 2003). In contrast mRNA for ERβ isoforms (ERβcx/β2, ERβ1) and GR have been demonstrated to be present in uNK cells (Henderson et al. 2003). Co-localization using specific monoclonal antibodies has confirmed that uNK cells are immunonegative for ERα and PR protein (King et al. 1996; Stewart et al. 1998; Henderson et al. 2003). These cells are also immunonegative for ERβcx/β2 but do express ERβ1 and GR proteins. These results have raised the possibility that oestro-

gens and glucocorticoids could be acting directly on uNK cells through ERβ and GR respectively, to influence gene transcription in the endometrium and decidua (Henderson et al. 2003). In this context it is interesting that uNK cells, which strongly express GR, have proposed roles in decidualization (King 2000) and have been shown to express the prolactin receptor (Gubbay et al. 2002).

The processes of endometrial differentiation, menstruation and placentation involve the remodelling of the endometrial vasculature. The angiogenic factor, vascular endothelial growth factor (VEGF)-A plays an important role in new blood vessel formation and induces endothelial cell proliferation, migration and differentiation in the endometrium. VEGF also influences vascular permeability. In isolated human endometrial cells in vitro oestradiol increases mRNA and protein levels of VEGF-A (Shifren et al. 1996). VEGF-A has also been localized to individual cells, presumably leukocytes, dispersed in the endometrial stroma. These cells have been identified as neutrophils through dual immunohistochemical staining by Mueller et al. (2000). VEGF-A expression has also been reported in uterine macrophages in the secretory phase of the cycle (Charnock-Jones et al. 2000). VEGF-C and other angiogenic factors, placenta growth factor (PlGF) and angiopoietin2 (Ang 2) mRNA are expressed in uNK cells (Li et al. 2001). VEGF-C was originally characterized as a growth factor for lymphatic vessels but it can also stimulate endothelial cell proliferation and migration (Olofsson et al. 1999). 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.

4.10 Endometrium and Effects of Progestogen Exposure

The endometrial morphology observed after exposure to exogenous progestogens will be the consequence of a perturbed local endocrine environment and reflect the route of administration, formulation and dose of steroid administered and the duration of therapy. The endometrium is exposed to the highest concentrations of progestogens if these compounds are delivered via an intrauterine route. The first hormone-releasing intrauterine system (IUS) developed was the Progestasert

(French and Guillebaud 2003). This device released 65 μg of progesterone daily to the uterine cavity. The life span of this IUS was 12 months. Levonorgestrel (LNG) is a potent progestagen, which also has a binding affinity for the androgen receptor (Kloosterboer et al. 1988), and is a common constituent of contraceptive preparations. The first progestogen-only implant marketed was Norplant (subdermal multi-system unit releasing LNG). The levonorgestrel releasing intrauterine system (LNG-IUS) comprises a T-shaped plastic frame, with a cylinder of the synthetic progestin LNG enclosed in a silastic sleeve that permits the slow and controlled release of 20 μg LNG over 24 h into the uterine cavity (Luukkainen 1990). The LNG-IUS provides effective contraception and reduces menstrual bleeding and dysmenorrhoea. It may also provide endometrial protection in combination with oestrogen as a mode of postmenopausal hormone replacement (Jensen 2002). Irregular or unscheduled breakthrough bleeding (BTB) is a frequent side effect of steroid contraception and is the most common reason for discontinuation of steroid contraceptive methods (Findlay 1996). BTB can occur in up to 53% of women after 3 months' use of the LNG-IUS (Irvine et al. 1998) and more than 50% of women using Norplant have been reported to request removal within the first year of use on account of menstrual disturbances (Peers et al. 1996). Hysteroscopic observations have suggested that the endometrial bleeding is focal in nature and is a consequence of exposure of the endometrium to both low and high dose progestogens (Hickey and d'Arcangues 2002). The pathogenesis of BTB remains inadequately explained despite extensive research over the last decade. The mechanisms implicated are likely to involve angiogenic factors, matrix metalloproteinase (MMP) activation, and altered haemostatic factors that result in increased endometrial vessel fragility. Such a pathogenesis may be directly related to high dose local LNG exposure. Alternatively, indirect actions of LNG may be responsible, such as the influence upon endometrial sex steroid receptor expression, or ligand availability in the endometrium.

It is essential to understand the effects of progestogen administration (and in the context here, particularly, LNG) on endometrial morphology and function (recently reviewed; Critchley 2003) if successful interventions are to be developed to overcome the problem of BTB.

4.11 Endometrial Morphology with Intrauterine Delivery of LNG

The intrauterine delivery of LNG induces a rapid and dramatic transformation of the endometrium, characterized by extensive decidualization (Nilssson et al. 1978; Silverberg et al. 1986; Critchley et al. 1998 a; Fig. 1). The observed morphological changes are consistent with progesterone-mediated differentiation as observed during the progesterone-dominated secretory phase and during pregnancy (King 2000; Buckley and Fox 1989). 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 a month of insertion of the LNG-IUS. Importantly, the morphology of the endometrium returns to normal within 1–3 months of removal and there is a complete return of previous fertility (Andersson et al. 1992). A summary of some of the documented endometrial responses to local LNG exposure is provided in Table 1 (modified from Critchley 2003).

Table 1. Morphological and functional features of the endometrium after exposure to intrauterine LNG

Extensive decidualization
Down-regulation of oestrogen receptor, progesterone receptor and androgen receptor
Expression of prolactin (stroma) and prolactin receptors (epithelium and isolated leukocytes in stroma), tissue factor (stroma) and IGFBP-1 (stroma)
Elevation of leukocyte infiltrate following insertion (uNK cells, macrophages)
Expression of local inflammatory mediators (cytokines and prostaglandins) enhanced after insertion
Altered endometrial and leukocyte MMP expression
Altered vessel fragility and perturbed angtogenesis
Continuous induction of PAI-1 may contribute to the therapeutic effect of LNG-IUS on reduction in menstrual blood loss
Thus, initially following LNG-IUS insertion sex steroid receptor content is decreased with consequent altered expression of local mediators which may play a role in aberrant bleeding episodes
No single factor yet identified to explain mechanism of breakthrough bleeding with progestin-only contraception

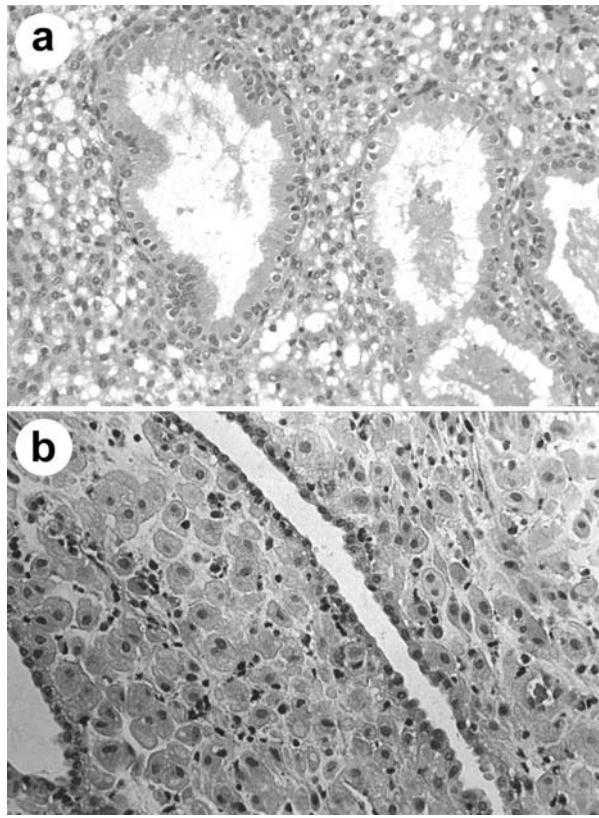


Fig. 1. Morphological appearances of (**a**) normal secretory phase endometrium and (**b**) the marked decidualization of the endometrium after exposure to intrauterine LNG. The glands are atrophic. (Reproduced with permission from Critchley 2003)

Coincident with the marked decidualization of the endometrium is the expression of local markers of decidualization including, prolactin, prolactin receptor (Jones et al. 1998; Critchley et al. 1998 a), insulin-like growth factor binding protein-1 (IGFBP-1; Pekonen et al. 1992; Rutanen et al. 1997) and tissue factor (Lockwood et al. 2004) in endometrium exposed to intrauterine LNG. Secretory activity of the epithelial glands is reduced. Evidence for the decrease in

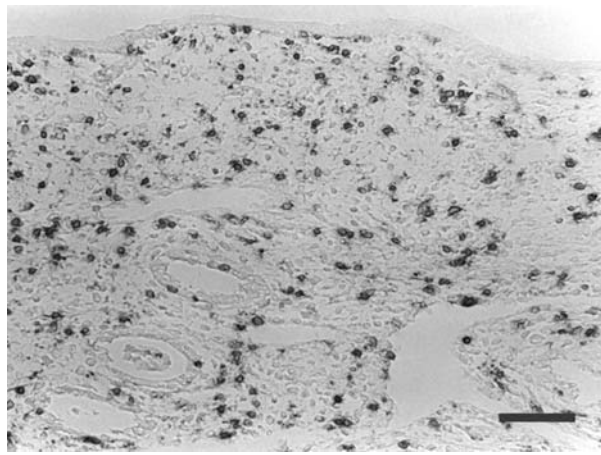


Fig. 2. Photomicrograph of endometrium exposed to intrauterine levonorgestrel. Note marked leukocyte infiltrate (leukocyte common antigen-LCA positive) Scale bar, 50 μm

cellular proliferation is supported by the report of a decrease in immunoreactivity of the cell proliferation marker, Ki67 (Hurskainen et al. 2000).

The characteristic morphological features observed include an infiltrate of white blood cells (leukocytes, mainly uterine NK cells and macrophages) into the decidualized stroma, atrophy of the glandular and surface epithelium and alterations in the vasculature (Fig. 2). There is no infiltrate of infection-associated plasma cells. An increase in number $CD68^+$ macrophages has been described in women using subdermal Norplant contraception who report abnormal bleeding patterns (Clark et al. 1996).

There appear to be abnormalities in the structure of endometrial microvessels in progestogen-only contraceptive users including intrauterine LNG. These structural changes appear to be associated with an increase in vessel fragility that may underlie the problem of BTB (Hickey and Fraser 2000). Since progesterone receptors are prominent in perivascular cells but absent in the endothelium (Koji et al. 1994; Critchley et al. 2001 b), LNG induced changes may be mediated through perivascular rather than endothelial cells (Roberts

et al. 1992). The subcutaneous delivery of LNG (Norplant) also has an impact on the vascular architecture as there is a reported decrease in expression of several components of the endothelial cell basement membrane (Hickey et al. 1999). The modulated extracellular matrix is likely to result in increased vascular fragility. Rutanen and colleagues (2000) demonstrated that tissue-plasminogen activator (t-PA) mRNA was constantly expressed in cycling endometrium during the proliferative and secretory phases and during menstrual bleeding. However the inhibitor of t-PA, that is, mRNA of plasminogen activator-inhibitor-1 (PAI-1) was expressed only in menstrual endometrium. Consequently endometrial haemostasis and remodelling appears to be important in the regulation of endometrial bleeding. However, no differences were observed between women with and without heavy menstrual blood loss. The authors noted that local intrauterine LNG administration maintained continuous decidualization and a similar balance in PAI-1/t-PA mRNA as detected during menstrual bleeding. It is plausible that the inhibition of fibrinolysis contributes to the therapeutic effect of the LNG-IUS for management of heavy menstrual blood loss (menorrhagia; Rutanen et al. 2000).

4.12 Down-Regulation of Endometrial Sex Steroid Receptors

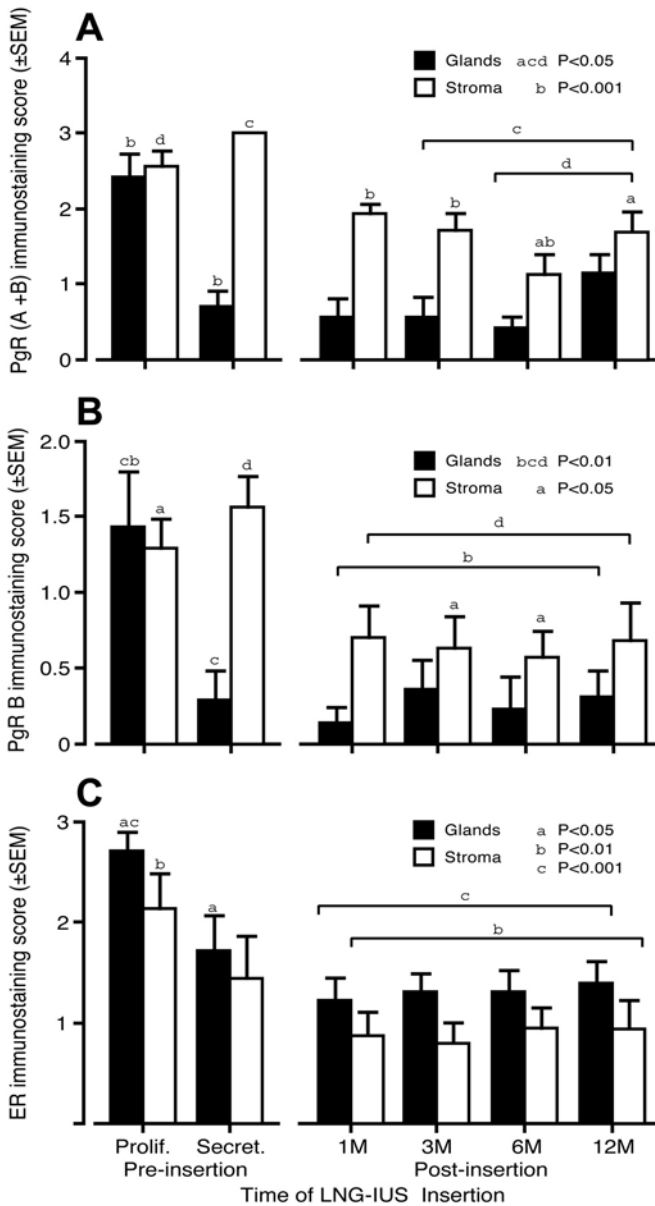
Short-term administration of synthetic progestogens decreases the progesterone receptor content of the endometrial stromal and epithelial cells in both pre-menopausal and post-menopausal women (Lane et al. 1988). The continuous delivery of LNG exerts an effect on endometrial PR expression that reflects the local endometrial concentrations of progestogen. In this context PR expression in endometrium exposed to intrauterine delivery of LNG differs from the expression of PR in endometrium of women using subdermal LNG (Norplant) for contraception. PR immunoreactivity has been reported to be persistently increased in Norplant exposed endometrium. The mechanism by which this observation may be explained and whether there is an increased number and concentration of functional PR is unknown (Critchley et al. 1993). There are consistently observed functional differences between local intrauterine and subdermal

LNG delivery (Pekonen et al. 1992). The differences in endometrial response are likely to reflect dose-dependency effects of LNG. The intrauterine delivery of LNG produces local endometrial LNG concentrations 1000 times greater than serum concentrations. The latter are of the same order of magnitude as those achieved with subdermal implants (Pekonen et al. 1992). It may be that Norplant users have relatively normal levels of oestradiol and low levels of progestogen.

Several research groups have described the down-regulation of PR and ER in LNG-IUS treated endometrium (Critchley et al. 1998 b; Hurskainen et al. 2000; Fig. 3).

In a longitudinal study that observed the effects of sex steroid receptor expression with intrauterine LNG delivery, endometrial biopsies were examined prior to insertion of a LNG-IUS and subsequently 1, 3, 6, and 12 months post IUS insertion. Prior to insertion of the LNG-IUS, ER and PR patterns of expression were as expected for either the proliferative or secretory phase of the cycle. Detailed study of ER, PR (utilizing an antibody that recognized both A and B forms of the PR) and PRB immunoreactivity demonstrated a down-regulation of both ER and PR in the glandular and stromal compartments of the endometrium (Critchley et al. 1998 b) compared to the proliferative phase of cycle, because PR and ER are

Fig. 3 A–C. Distribution of steroid receptors in endometrium following LNG-IUS insertion. Immunostaining scores (±SEM) in endometrium prior to and at 1, 3, 6 and 12 months after insertion of an LNG-IUS. **A** Progesterone receptor $(PR)_{A+B}$. Glandular and stromal PR immunostaining levels are significantly decreased following insertion of LNG-IUS. With continued exposure to progestin, PR increase in the glandular compartment (between 6 and 12 months post insertion). **B** PR_B. Lower levels of immunostaining for PR_B were detected in pre-insertion biopsies consistent with normal cycle data. A further down-regulation is observed after LNG-IUS insertion which was maintained throughout the course of the study. **C** ER. A significant down-regulation of ER is observed at all time points following LNG-IUS insertion in both glandular and stromal compartments. *Prolif.*, proliferative; *Secret.*, secretory; *M*, months. Significant differences between groups are denoted by *matching letters*. (Reproduced with permission from Critchley et al. 1998 a, © European Society of Human Reproduction and Embryology; Reproduced by permission of Oxford University Press/Human Reproduction)


A
PgR (A +B) immunostaining score (±SEM)
Glands acd P<0.05
Stroma b P<0.001
B
PgR B immunostaining score (±SEM)
Glands bcd P<0.01
Stroma a P<0.05
C
ER immunostaining score (±SEM)
Glands
Stroma
a P<0.05
b P<0.01
c P<0.001
Prolif.
Secret.
Pre-insertion
1M
3M
6M
12M
Post-insertion
Time of LNG-IUS Insertion

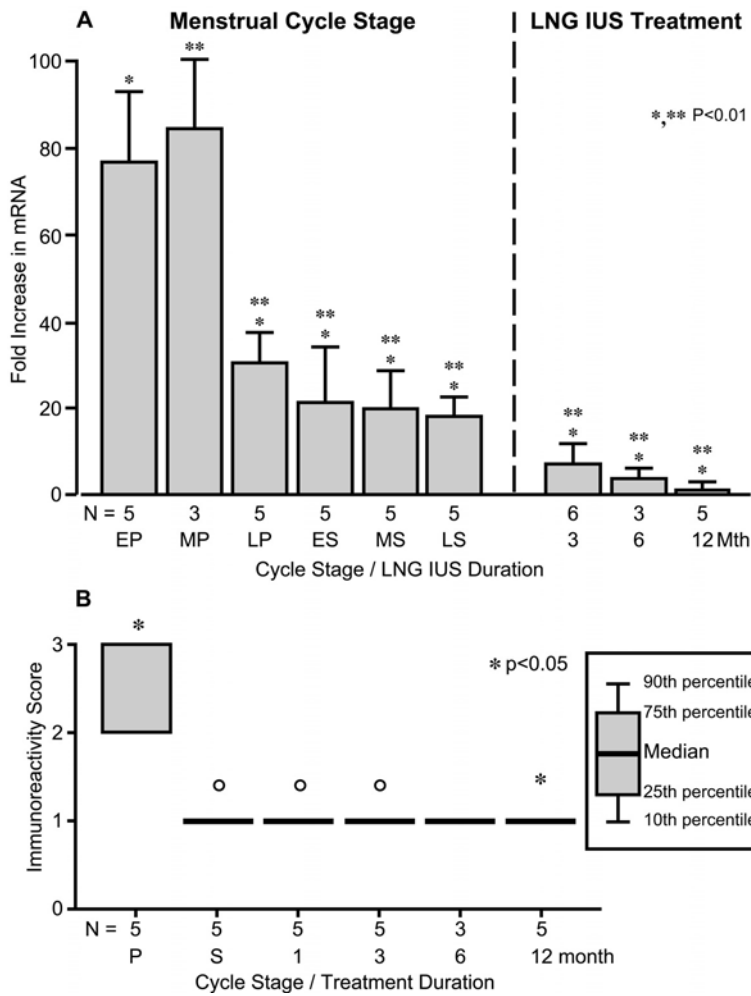


Fig. 4. A Quantitative evaluation of AR mRNA in endometrium across the stages of the menstrual cycle and following intrauterine delivery of levonorgestrel (LNG-IUS). All endometrial tissue samples were compared with an internal control (comparator) obtained during the early proliferative stage of the menstrual cycle. AR mRNA levels were high in the early and mid-proliferative samples but fell significantly in samples taken from the late proliferative and early-late secretory samples ($P<0.01$). Levels of AR mRNA

normally decreased in glands in the secretory phase. This pattern of sex steroid receptor expression persisted for at least 6 months. These immunohistochemical studies demonstrated a marked down-regulation of PRB isoform in both glandular and stromal cells. The data were interpreted that the few residual PR positive stromal cells remaining with local LNG exposure were likely to be PRA positive stromal cells. The glandular expression of PRB was minimal at all times after exposure of the endometrium to local LNG. The differential expression of PR subtypes is not understood. However, the predominance of the PRA subtype when the endometrium is decidualized either in the late secretory phase, in early pregnancy or with the decidualization caused by intrauterine LNG, suggests that the PRA subtype mediates decidual transformation (Critchley et al. 1998 b; Jones and Critchley 2000). Similar observations of decrease in ER and PR expression with local endometrial LNG exposure have been reported by other groups. Endometrial samples available for examination between 12 and 15 months post IUS insertion still exhibited a depressive action on both ER and PR (Zhu et al. 1999).

More recently down-regulation of the androgen receptor in LNG exposed endometrium has been reported (Burton et al. 2003). During the menstrual cycle AR mRNA was observed to be maximal in stroma during the proliferative phase and to decrease during the secretory phase. AR mRNA expression was lowest after intrauterine exposure to LNG. The AR protein was also demonstrated to be down-regulated by local intrauterine LNG administration (Fig. 4).

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were again low in the LNG-IUS-treated samples when compared with the (early and mid-) proliferative stages of the cycle ($P<0.01$). *EP*, early proliferative; *MP*, mid-proliferative; *LP*, late proliferative; *ES*, early secretory; *MS*, mid-secretory; *LS*, late secretory. **B** Androgen receptor (*AR*) immunoreactivity scores in endometrial stromal compartments throughout the menstrual cycle and following high dose intrauterine levonorgestrel exposure (1–12 months). Box-and-whisker plots: *box* represents the 25th and 75th percentiles and the *heavy bar* represents the median. (O) represents outliers; *P*, proliferative; *S*, secretory. (Reproduced with permission from Burton et al. 2003, © European Society of Human Reproduction and Embryology; Reproduced by permission of Oxford University Press/Human Reproduction)

4.13 Effects of Local LNG Delivery on Endometrial Intracrinology

Because 17β HSD-2 mRNA expression in human endometrium is high within the first months after LNG-IUS insertion and then declines to low/undetectable levels by 6 months of LNG-IUS exposure it is likely that the intracellular oestrogen concentrations are affected (Burton et al. 2003). Since the enzyme converts oestradiol to the weaker oestrogen, oestrone, endometrial glands would be exposed to high levels of weaker, oestrone and low levels of more potent, oestradiol during the initial 3 months of local LNG intrauterine exposure. This is the time period when women particularly complain of BTB episodes. As a consequence, any oestradiol-dependent products of the glands that have potential paracrine actions throughout the endometrium would be suppressed. Our hypothesis is that BTB is due in part, to an intracellular 'oestrogen deficiency' that either directly or indirectly results in blood vessel fragility (Burton et al. 2003). Since ERβ is continually expressed in endometrial vascular endothelium across the menstrual cycle (Critchley et al. 2001b) the effects of lowered endometrial oestradiol levels on vascular integrity may be mediated by ERβ.

The effect of anti-progestins on endometrium and suppression of endometrial bleeding have already been referred to above. The intermittent treatment of cynomolgus macaques with an anti-progestin at the time of low dose oestrogen plus progesterone therapy has been demonstrated to reduce BTB (Williams et al. 1997). Important in the present context is the knowledge that acute anti-progestin administration raises endometrial ER, PR (Slayden et al. 1993; Cameron et al. 1996; Maentausta et al. 1993) and AR (Slayden et al. 2001) and suppresses 17βHSD-2 expression (Mustonen et al. 1998; Maentausta et al. 1993). Thus among women using a LNG-IUS, short exposure to an anti-progestin would be expected to lower intracellular oestrone and elevate intracellular oestradiol concentrations and to elevate endometrial ER. Hence the endometrium then should be exposed to a more potent oestrogen in a local environment with steroid receptors, including ER elevated.

Importantly this hypothesis concerning morphological and functional changes within the endometrium is supported by data from

clinical studies. In a pilot study where a single dose of anti-progestin (mifepristone, RU486) was administered to women using Norplant (subdermal LNG) an up-regulation of endometrial ER was observed and a reduction in vaginal bleeding reported (Glasier et al. 2002). Moreover, a larger clinical study (double-blind, randomized, placebo-controlled trial among 100 women) undertaken in China (Cheng et al. 2000) demonstrated a decreased frequency in bleeding episodes. Women using mifepristone (50 mg taken once every 4 weeks) reported significantly shorter episodes of bleeding compared with women in the placebo group.

4.14 Effects of Local and Systemic Progestogens on Expression of Inflammatory Mediators (Cytokines, Prostaglandins) and Tissue Remodelling (MMPs)

The down-regulation of sex steroid receptor expression observed in response to local LNG administration is likely to influence endometrial cytokine release and may thus contribute to altered morphology and function and to the pathogenesis of BTB. In the early months after insertion of the LNG-IUS there is an elevated cytokine and prostaglandin production, and expression of MMPs (reviewed in Jones and Critchley 2000). For example, a high immunoexpression of the chemokine IL-8 has been reported 1 month following insertion of a LNG-IUS with a subsequent decrease in expression after 3 and 6 months of progestogen exposure. The relevance of such observations to leukocyte recruitment is not clear. It is well known that prostaglandins modulate vessel tone, inflammation and the immune system. The availability of prostaglandins (PGs) and PG metabolites is closely regulated by synthesizing and metabolizing enzymes. Local intrauterine delivery of LNG is associated with an initial reduction in glandular expression of the metabolizing enzyme, prostaglandin dehydrogenase, followed by a gradual return of activity after 6 months (Critchley et al. 1998 b). The initial elevation of these inflammatory mediators is coincident with the time when BTB complaints are most frequent. It is possible that prostaglandins and locally produced cytokines have important actions on the vasculature of LNG exposed endometrium. Moreover, the observed up-regula-

tion of inflammatory mediators lends support to a hypothesis that the reduction observed in PR expression post LNG-IUS insertion creates a local 'progestogen deprived' environment (Critchley et al. 1998 b, Jones and Critchley 2000). In accord with the reported improvement in bleeding patterns with continued use of the intrauterine system, a gradual reduction in all markers of inflammation is observed.

The MMPs are enzymes responsible for breakdown and remodelling of the extracellular matrix. The focal pattern of expression implicates local rather than hormonal regulation of the expression of MMPs. Leukocytes present in endometrium may be source of release of MMPs. There exist complex interactions between leukocytes, stromal and epithelial cells that induce and activate MMPs. MMPs display both temporal and spatial variation in expression across the menstrual cycle. There is an observed increase in expression upon progesterone withdrawal, premenstrually (Marbaix et al. 1995; Salamonsen and Woolley 1996; Brenner et al. 2002 b). Much attention has been paid to the role for MMP production in the aetiology of BTB with progestogen-only contraception (Skinner et al. 1999; Vincent et al. 1999; Hickey and d'Arcangues 2002). The balance between MMPs and their tissue inhibitors (TIMPs) determines the level of proteolytic action in the endometrium. By way of example MMP-9 (gelatinase B) is expressed by both glandular and stromal cells, and by a subpopulation of leukocytes (Skinner et al. 1999; Vincent et al. 1999). After insertion of an intrauterine LNG system MMP-9 is observed to be strongly expressed by the decidualized endometrial cells. Similarly, increased numbers of MMP-9 positive leukocytes have been demonstrated in the endometrium from women using subdermal Norplant (LNG) for contraception (Skinner et al. 1999; Vincent et al. 1999). MMP-9 expression is thus strongly expressed at time when significant tissue remodelling is likely (Jones and Critchley, 2000). In support of the role for MMPs in maintenance of endometrial morphology, users of depot medroxyprogesterone acetate (Depo-provera) have an observed influx of MMP-9 positive neutrophils and a decrease in immunoreactive endometrial TIMPs (Vincent et al. 2002). Observations such as these further support the accumulating evidence that MMP/TIMP balance is important for the maintenance/loss of endometrial integrity.

4.15 Effects of Progestogens on the Endometrial Vasculature

The disrupted bleeding patterns reported by women with the use of progestogen-only contraceptives are likely to indicate modifications in the endometrial vessels from which bleeding arises, including changes in vessel integrity and/or haemostasis. Moreover, it is probable that aberrant bleeding arises from a different vascular source than normal menstrual bleeding (Hickey and Fraser 2000). BTB arises mainly from capillaries and veins adjacent to the uterine lumen and is related to increased vessel fragility. During the hysteroscopic examination of women using subdermal LNG contraception (Norplant) it has become apparent that these superficial endometrial vessels are abnormally fragile (Hickey et al. 1998, 1999) and that the superficial vasculature is altered (Hickey and Fraser 2002).

The vascular changes in the endometrium that are observed with intrauterine LNG include thickening of uterine arteries, suppression of spiral artery formation and the presence of large distended vessels. The subcutaneous delivery of LNG (Norplant) also has a marked effect of the endometrial vasculature. A decreased expression of a number of components of the endothelial cell basement membrane is evident with Norplant administration (Hickey et al. 1999). 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 only part of the process that results in aberrant angiogenesis.

Local factors involved in the angiogenic process (new blood vessel formation) and the modulation of this crucial event by progestogens are being studied (Charnock-Jones et al. 2000; Krikun et al. 2002). In the normal menstrual cycle there is a positive correlation between the stromal expression of the angiogenic growth factor, VEGF and endothelial cell density. This correlation is less consistent in endometrium collected from women using Implanon (etonorgestrel) or a combined contraceptive preparation (ethinyl oestradiol plus desogestrel; Charnock-Jones et al. 2000). Observations such as these indicate that progestogen induced effects on the microvasculature of

the endometrium are dependant upon the route, dose and type of steroid delivery. Recently changes in the vascular morphology with LNG-IUS use have been described. Large thin-walled vessels have been observed in the decidualized superficial stroma of endometrium of short-term (between two and four menstrual cycles prior to hysterectomy) LNG-IUS users (McGavigan et al. 2003). The subjects in this study had received a LNG-IUS or constituted a control group prior to hysterectomy for menorrrhagia. It has been proposed that the vascular anomalies described above do not persist when the endometrium becomes atrophic and may thus be associated with improvement in BTB episodes. Indeed consideration has been given to pre-treatment regimens designed to ensure the endometrium is atrophic or to coincident administration of agents such as anti-progestogens or inhibitors of MMP activity (McGavigan et al. 2003). There are data (mentioned above) in this context to support an improvement in bleeding patterns for women using Norplant when a single dose of the anti-progestogen, mifepristone, was administered on a monthly basis (Cheng et al. 2000; Glasier et al. 2002).

Exogenous administration of sex steroids has been reported to influence uterine perfusion. Hickey and colleagues (2000) have demonstrated reduced endometrial perfusion in Norplant users in the early months of use.

Recent evidence has implicated a potential role for use of antioxidants (for example, vitamin E supplementation) as a strategy to counteract unscheduled bleeding with progestogen-only contraception. In this recent study progestogen-induced hypoxia/reperfusion and/or oxidative stress resulted in a dysregulation of the angiogenic factors, Ang-1 and Ang-2 gene activation with consequent activation of the signalling cascade in endometrial stromal and endothelial cells, the development of enlarged permeable blood vessels and aberrant bleeding (Krikun et al. 2002).

4.16 Conclusion

There is a large body of knowledge about the local morphological and functional effects on the endometrium with progestogen exposure. This review has particularly focused on the effects of intrauter-

ine LNG administration. It is disappointing that to date no single factor has emerged that directly correlates with the aberrant endometrial bleeding associated with any of the methods of progestogen-only contraception. It is therefore probable that several factors acting closely together will contribute to the pathogenesis of these abnormal bleeding patterns experienced by women. The eventual elucidation of such factors will facilitate the development of intervention strategies designed to overcome the BTB experienced with progestogen-only contraception. Thereafter progestogen-only contraceptive methods will gain increased user satisfaction and women may benefit from other health gains, especially if the progestogen is delivered locally to the uterine cavity.

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5 Role of Progesterone in the Structural and Biochemical Remodeling of the Primate Endometrium

O. D. Slayden, R. M. Brenner

5.1 Introduction

Progesterone (P) plays critical roles during female reproduction. It is involved in the control of ovulation (Hibbert et al. 1996), preparation of the uterus before and during embryo implantation (Ghosh and Sengupta 1989; Navot et al. 1986), and in the maintenance of pregnancy (Hodgen and Tullner 1975). In addition to its reproductive functions, P also acts on numerous other organ systems including the mammary gland, skeletal system, cardiovascular system, central nervous system (Graham and Clarke 1997), and urinogenital sys-

tem (Robinson et al. 1996). Like other steroid hormones, the genomic effects of P are mediated in target cells through interactions with specific intracellular P receptors (PR). Progesterone receptor modulators (PRMs) are compounds that bind PR and either mimic P action (progestins) or that block P action (antiprogestins; antagonistic PRMs). Currently, PRMs are used extensively for hormone-based therapies and contraception (Coelingh Bennink 2000; Giannoukos et al. 2001; Chwalisz et al. 2002). However, some PRMs have side effects due to lack of selectivity for PR and high cross reactivity for glucocorticoid receptor (GR) and androgen receptor (AR) (Giannoukos et al. 2001). Recently, new PRM compounds with improved PR selectivity (selective PRM; SPRM) have been produced (Giannoukos et al. 2001) that may provide therapy for reproductive tract diseases including endometriosis (Chwalisz et al. 2002) and breakthrough bleeding (Williams et al. 1997). However, it is very difficult to predict the in vivo effects of novel PRMs from their chemical structure (Giannoukos et al. 2001). In vivo studies of SPRM action require experimental models that accurately reflect hormone action in the human reproductive tract. For preclinical studies of endometrial effects, non-human primates are the animal models of choice (Cline et al. 2001). Ovariectomized macaques can be treated with implants of estradiol (E_2) and P to induce artificial cycles that mimic the natural cycle; this greatly facilitates the evaluation of new compounds as the hormonal environment is precisely controlled. In this manuscript we briefly review the effects of P on the endometrium in rhesus macaques as a model for preclinical studies of PRM action in women.

5.2 Natural and Artificial Cycles in Rhesus Macaques

Noyes and coworkers (Noyes et al. 1950) devised a scheme for morphologically dating the endometrium of women relative to an ideal 28-day cycle and although the precision of this method has been questioned (Murray et al. 2004), it has been used extensively in human clinical studies. In several reviews, we described analogous morphological changes within the macaque endometrium during the natural menstrual cycle (Brenner et al. 1990, 1991; Brenner and Slayden 1994a). In macaques, like women, E_2 secreted by the ovary

during the follicular phase stimulates cell proliferation in both the endometrial glands and stroma. Therefore, the follicular phase is also frequently referred to as the proliferative phase of the cycle. The length of the proliferative phase in the natural cycle is normally 10–14 days but can be highly variable (Gilardi et al. 1997). After ovulation, rising P levels induce secretory changes in the endometrium (the secretory phase) and prepare the uterus for embryo implantation. If fertilization and implantation do not occur, the luteal phase of a natural cycle usually lasts for 12–14 days. In macaques, like women, the decline of P at the end of the luteal phase induces menses and the upper third of the endometrium sloughs off as the next cycle begins (Corner 1963; Rogers 1999). We refer to the transition between cycles as the luteal follicular transition (LFT). Hodgen et al. (1983) reported that a secretory endometrium and pregnancy could be established in ovariectomized monkeys treated with silastic implants of E_2 and P in sequential fashion to recreate the pattern of serum E_2 and P that occurs during the fertile menstrual cycle. In such an artificial cycle, the withdrawal of P at the end of the cycle results in an LFT, including menses, that is essentially identical to that observed in the natural cycle. If P action is chronically blocked during these artificial cycles with an antagonistic PRM such as mifepristone (RU 486), secretory differentiation and subsequent menses are inhibited (Slayden et al. 2001 a). Therefore, this model provides a system for evaluating novel PRMs during specific hormonal states typical of the proliferative, secretory and LFT phases of the cycle.

5.3 General Methods

5.3.1 Animal Treatments

All the studies described in this review were approved by the Oregon National Primate Research Center (ONPRC) Animal Care and Use Committee, and supervised by the ONPRC veterinary staff. Sexually mature rhesus macaques (*Macaca mulatta*) were ovariectomized and artificial cycles (Rudolph-Owen et al. 1998; Slayden et al. 2001 a) were initiated in the animals by inserting a subcutaneous

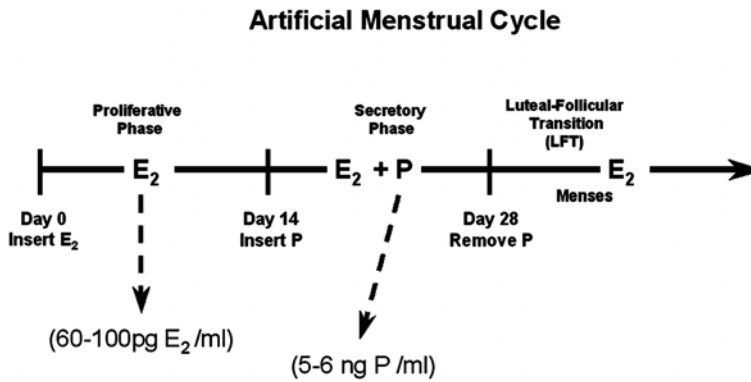


Fig. 1. A timeline depicting the induction of artificial menstrual cycles in ovariectomized rhesus macaques with silastic capsules that release E_2 and P

(s.c.) capsule that released E_2 to produce an artificial 14-day proliferative phase, followed by insertion of a P capsule s.c. to stimulate an artificial 14-day secretory phase. Removal of the P implant on day 28 completed the cycle. Serum samples were collected at intervals to confirm that the levels of E_2 and P produced were within the normal range for macaques during the natural cycle. Figure 1 depicts a timeline for these artificial menstrual cycles. We have analyzed the endometrium at the following artificial cycle phases: (1) the end of the proliferative phase; (2) the end of the secretory phase; and (3) during days 1–6 of the LFT after P withdrawal (Rudolph-Owen et al. 1998). We also evaluated the action of several antagonistic PRMs including mifepristone (Roussel UCLAF) ZK 137 316, and ZK 230 211 (Schering AG) administered chronically to artificially-cycled macaques (Hirst et al. 1992; Slayden et al. 1993; Slayden and Brenner 2003; Slayden et al. 2001 a).

5.3.2 Endometrial Dissection

The macaque uterus is anatomically similar to the human uterus, and consists of three parts, the fundus (dome-shaped top), the corpus (body) and the isthmus (neck), which leads into the cervix. We re-

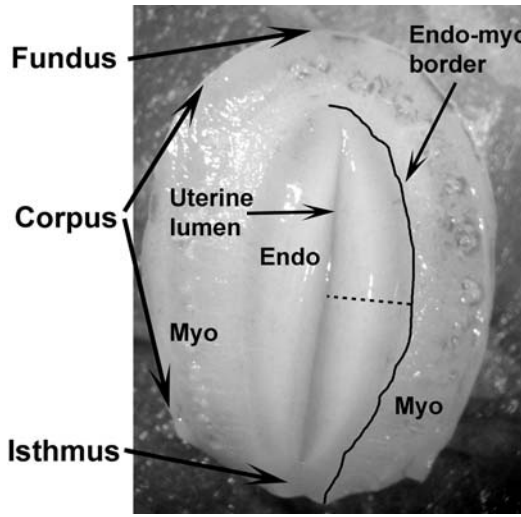


Fig. 2. Photograph showing half of a rhesus macaque uterus that has been cut along the longitudinal axis. A *solid black line* has been drawn to delineate the endometrial–myometrial border, and a *dashed line* has been drawn to show the plane for the histological specimens

moved the oviducts and separated the uterus from the cervix at the isthmus and cut it in half longitudinally through the corpus and fundus. The endometrium lines the uterine cavity and is surrounded by the muscular wall or myometrium. Figure 2 shows half of a representative rhesus macaque uterus that has been cut along the longitudinal axis. After the first longitudinal cut, the uterine halves were again cut longitudinally to create equal quarters. From two of these quarters, cross-sections (2-mm thick) that transect the full thickness of the endometrium, including all four zones, were then cut freehand with a razor blade and prepared for immunocytochemistry (ICC) and morphological study. In Fig. 2, a black line has been drawn to delineate the endometrial–myometrial border. A dashed line has been drawn to show the plane for the free-hand cut sections. All photomicrographs were prepared from full-thickness sections cut with this orientation and show all four zones of the endometrium (see below). The endometrium from the remaining two quarters of the uterus was

blunt dissected from the myometrium with iris scissors and prepared for RNA isolation or receptor binding studies. Separation of the uterus into quarters, as described, facilitates the precise dissection of endometrium from myometrium.

5.3.3 Histological and Immunocytochemical Methods

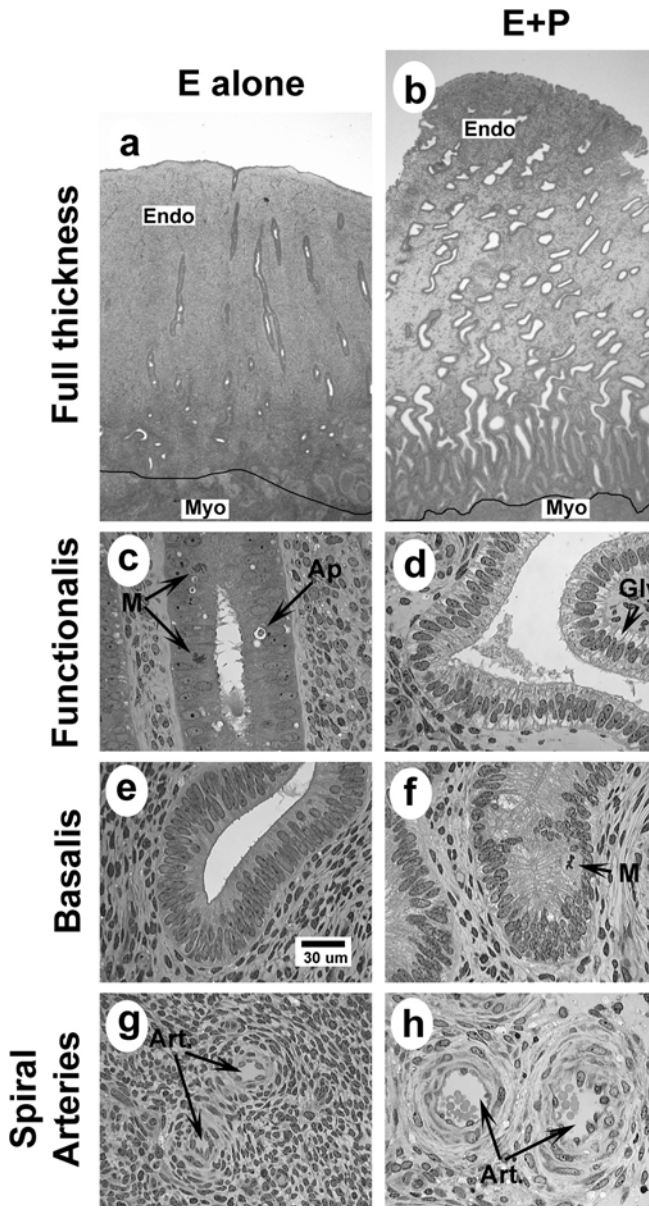
In our studies, tissue samples for morphological study were fixed in 2% glutaraldehyde and 3% paraformaldehyde, embedded in glycol methacrylate (GMA), sectioned (2 μm) and stained with Gill's hematoxylin. Samples of fresh tissue for ICC were microwave stabilized (Slayden et al. 1995) for 7 s in an Amana Radarrange Touchmatic microwave oven (Amana, Iowa), mounted in Tissue Tek II OCT (Miles Inc, Elkhart, IN), and frozen in liquid propane. The cryostat sections (5 μm) were thaw-mounted on Superfrost Plus (Fisher Scientific, Pittsburgh, PA) slides, placed on wet ice at 5 °C, and microwave irradiated again for 2 s. ICC for steroid receptors and markers of cell proliferation was carried out as recently described (Slayden et al. 1998, 2001 b). Briefly, microwave-treated sections were lightly fixed (0–2% picric acid, 2% paraformaldehyde in PBS) and the ICC was conducted with specific monoclonal or polyclonal antibodies. In each case primary antibody was reacted with biotinylated secondary antibody and detected with an avidin–biotin peroxidase kit (Vector Laboratories, Burlingame, CA). The monoclonal antibodies used in the studies presented in this review included: anti-ERα (1D-5; Biogenex, San Ramon, CA), anti-ERβ (ERM02; Novocastra, Norwell, MA), anti-PR (PR Ab-8; Neomarker Inc, Fremont, CA) anti-Ki-67 antigen (Dako Corp Carpinteria, CA); anti-bromodeoxyuridine [Br(d)U] (Cat# 69199, ICN Biomedicals Inc, Costa Mesa, CA), and anti-matrix metalloproteinase (MMP)-1 and MMP-3 (Oncogene Research Products, Cambridge, MA). A polyclonal antibody to phosphorylated histone H3 (phospho H3, a marker of mitotic cells) was purchased from Upstate Biotechnology, Waltham, MA (Cat 06-570) (Brenner et al. 2003).

Digital images were captured with an Optronics DEI-750 CCD camera through Zeiss planapochromatic lenses. In several studies we assessed the abundance of proliferating cells in the endometrium of

hormone-treated macaques by counting mitotic cells, Ki-67 positive and Br(d)U labeled cells (Slayden et al. 1998, 2003). These counts were determined by manual counting by a trained observer who used an ocular micrometer grid to define microscope fields, and more recently by computer assisted cell counting (Brenner et al. 2003). We further utilized computer image analysis with Image Proplus (Media Cybernetics Inc, USA) to quantify endometrial gland and arterial area (Slayden et al. 2000). In each case cell counts and morphometric values were analyzed by ANOVA followed by Fisher LSD (Petersen 1985).

5.4 Effects of Progesterone on Endometrial Morphology

In classical morphological studies, Bartelmez described four zones in the rhesus (Bartelmez 1951) and human (Bartelmez 1957) endometrium. Zone 1 was defined as inclusive of the surface epithelium and an underlying band of stromal cells. Slightly deeper, zone II contains glands that run perpendicular to the surface. Deeper still, zone III contains glands that are branched, and the deepest zone, zone IV, is the basal layer that is adjacent to the myometrium, where the glands terminate. Secretory differentiation and menses occur in zones I–III, and these zones combined are frequently termed the functionalis zone. In women, zone IV, the basalis zone, is relatively unresponsive to cyclic hormonal changes, but in the macaque, the basalis proliferates during the luteal phase under the influence of P (Padykula et al. 1989; Okulicz et al. 1993; Brenner et al. 2003). It has been proposed that the deepest endometrial zone consisting of endometrial basalis and the first few layers of myometrium should be considered a separate zone, the archimetra, because it is anatomically and biochemically different from other endometrial zones (Leyendecker et al. 1996, 1998, 2002). Figure 3 shows photomicrographs of hematoxylin and eosin-stained sections of rhesus macaque endometrium collected at the end of the proliferative phase (14 days of E_2 alone) and at the end of the secretory phases (14 days of E_2+P) of the artificial cycle. In both the artificial and natural cycle the proliferative phase begins when menstruation ends (see effects of P withdrawal below) and the glands and stroma begin a period of


E alone
E+P
Full thickness
Functionalis
Basalis
Spiral Arteries
a
b
c
d
e
f
g
h
Endo
Endo
Myo
Myo
M
Ap
Gly
M
30 um
Art.
Art.

E_2-dependent growth. After 14 days of E_2 priming the upper layers of endometrium display tubular glands with abundant mitotic cells. To detect and quantify endometrial cell proliferation we and others have used ICC for several proliferation-associated markers including: Ki-67 (Gerdes et al. 1984, 1991; Slayden et al. 1993; Okulicz et al. 1993), phospho H3 (Brenner et al. 2003) (a marker of mitotic cells) and in vivo labeling with Br(d)U (Apte 1990). Figure 4 shows representative sections stained with these markers. Counts of cells stained with these methods reveal that almost all of the epithelial cell proliferation during the late proliferative phase occurs in the mid- and upper functionalis zones; only minor proliferation occurs in the basalis (Brenner et al. 2003). However, the basalis zone displays an intense period of cell proliferation during the early luteal phase (Fig. 4d). Stromal cell proliferation occurs throughout the endometrium. After E_2 priming the upper zones also contain abundant apoptotic cells, suggesting that during the proliferative phase there is a balance of cell birth and cell death. New growth of small vessels in the upper functionalis zone appears to peak on day 8 of the proliferative phase (Nayak and Brenner 2002). In the proliferative phase, spiral arteries are found primarily in the deep zones.

In the artificial cycle, the effects of P on the epithelium become evident on day 3 of the artificial secretory phase. At this time, P gradually suppresses cell proliferation in the glandular epithelium of the functionalis and induces secretory changes including hypertrophy

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Fig. 3a–h. GMA-embedded, hematoxylin-stained sections showing the histological effects of E_2 and E_2 + P on the endometrium of artificially cycled monkeys. **a**, **b** Full thickness section of endometrium, a *dark line* has been drawn to show the myometrial border. Treatment with E_2+P induces expansion of the endometrial stroma and sacculation of the glands typical of the secretory phase. **c**, **d** Endometrial functionalis zone. Abundant mitotic figures (*arrow*) were observed in the glands during E_2 treatment (**c**; artificial proliferative phase) whereas treatment with P blocked mitosis and induced secretory differentiation (**d**). **e**, **f** Endometrial basalis zone. No mitotic cells were observed after 14 days of E_2 alone, whereas treatment with P stimulated mitotic activity in the basalis glands. **g**, **h** Spiral arteries. E_2+P treatment resulted in hypertrophy of the spiral arteries (**h**) compared to treatment with E_2 alone. The *bar* in (**e**) represents the magnification for **c**–**h**. *Endo*, endometrium; *Myo*, myometrium; *M*, mitosis; *Ap*, apoptosis; *Art*, spiral artery

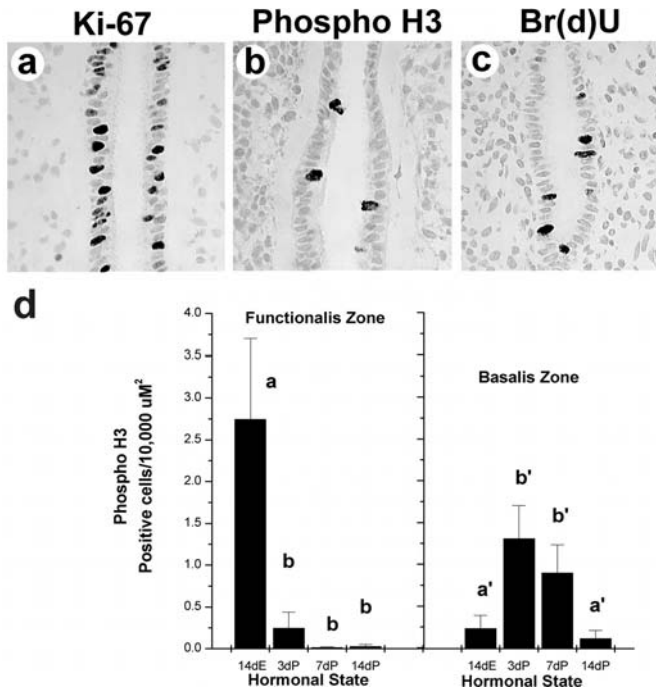


Fig. 4. Immunostaining for Ki-67 antigen (**a**), Phospho H3 (**b**), and Br(d)U (**c**) in macaque endometrium from an animal during the artificial proliferative phase. *Dark-stained* cells are positive. Counts of phospho H3 stained cells (**d**) revealed that cell proliferation in the functionalis zone occurs primarily during E_2 ($14dE_2 = E_2$ implant for 14 days) and decreases strikingly after P treatment (3dP, 7dP and 14dP = 3, 7, and 14 days of $E_2 + P$ treatment in the secretory phase). In contrast, mitotic activity in the basalis zone was suppressed by 14dE2 and increased during the early secretory phase (3dP and 7dP)

and accumulation of glycogen in the basal portions of the cells. As noted, the basalis zone behaves differently from the functionalis in macaques; P stimulates mitotic activity in this zone throughout the luteal phase. By day 14 of $E_2 + P$ treatment the functionalis glands are dilated and the epithelium has a saw-toothed, sacculated, appearance, with shrunken nuclei and jagged apical surfaces. While epithelial cell proliferation is suppressed by P treatment, P stimulates a

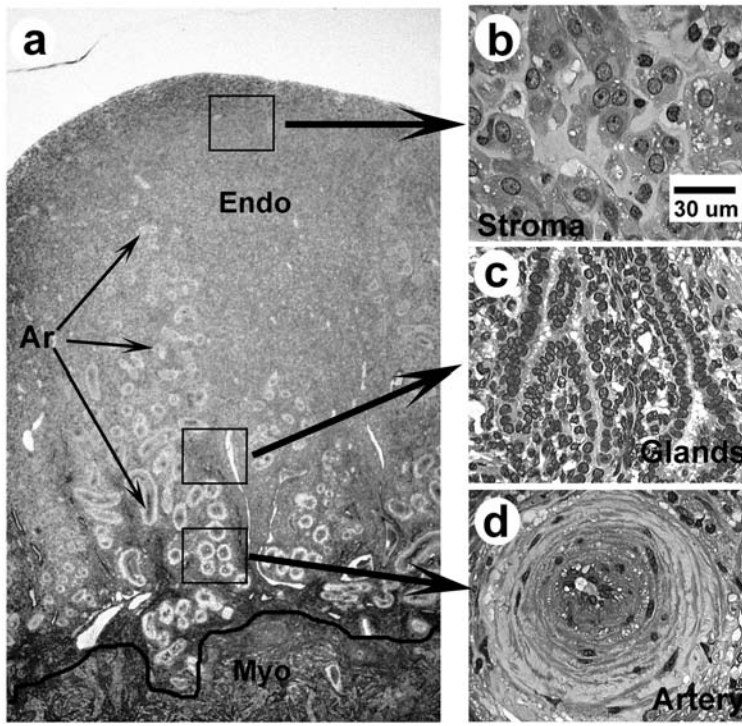


Fig. 5. GMA-embedded, hematoxylin-stained sections showing the histological effects of 5 months of E_2+P on the endometrium of rhesus macaques. Full thickness section of endometrium (**a**) a *dark line* has been drawn to show the myometrial border. The endometrium after extended E_2+P treatment shows a decidualized endometrial stroma (**b**) with atrophied glands (**c**) and greatly enlarged spiral arteries (**d**). *Endo*, endometrium; *Myo*, myometrium

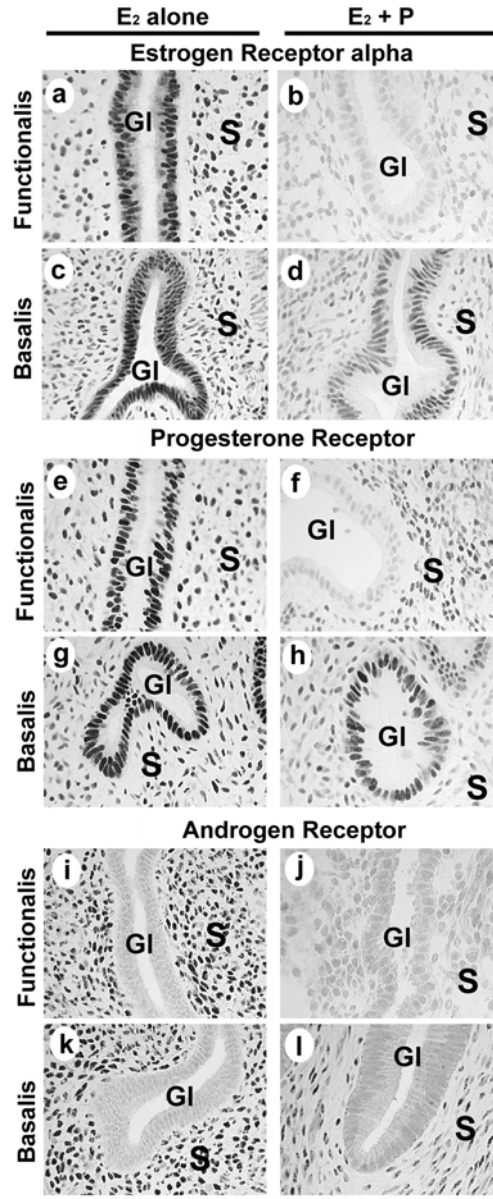
transient increase in stromal cell proliferation, which constitutes a striking predecidual event. By day 7 of E_2+P treatment the endometrial stroma becomes highly edematous and the spiral arterioles begin to enlarge (Fig. 3h). Spiral artery growth is associated with a striking P-dependent increase in cell proliferation in endothelial, smooth muscle and perivascular stromal cells. The macaque endometrium does not fully decidualize until either pregnancy occurs or

when P treatment is maintained for much longer than 14 days. Compared to animals treated with E_2+P for only 2 weeks in the artificial cycle (Fig. 3), treatment for 5 months with E_2+P induces a highly decidualized endometrium with enlarged stromal cells, excessive amounts of extracellular matrix in the functionalis, enlarged spiral arteries and a greatly reduced number of glands (Fig. 5). Decidualization of the endometrial stroma and glandular atrophy has also been reported in macaques treated for 14 weeks with intrauterine devices that released the synthetic progestin levonorgestrel (Wadsworth et al. 1979).

5.5 Effects of Progesterone on Steroid Receptors

Two functional estrogen receptors ERα and ERβ, transcribed from different genes, occur in the endometrium and other tissues. The two ERs are regulated differently and show cell type-specific expression in the endometrium. During the cycle, ERα mRNA and protein increase under E_2 influence and decrease under P influence in the endometrium of women and nonhuman primates (Brenner et al. 1990). Examples of ERα PR, and AR immunostaining are shown in Fig. 6. After E_2 treatment alone for 14 days, staining for ERα is strongly positive in the nucleus of glandular epithelium, and in the stroma in both the functionalis and basalis zones. P treatment during the artificial secretory phase (E_2+P) blocks estrogen upregulation of ERα, and ERα staining is minimal in the glands and stroma of the functio-

Fig. 6. Photomicrographs of endometrium stained for ERα PR and AR by ICC. **a–d** ERα. Strong staining was observed in the glands and stroma of the functionalis and basalis after E_2 treatment. After treatment with E_2+P, P blocked E_2 upregulation of ERα in the glands and stroma of functionalis zone, whereas some ERα staining was maintained in the basalis zone. **e–h** PR. After E_2 alone, strong PR staining was observed in the glands and stroma of both the functionalis and basalis zones. Cotreatment with E_2+P suppressed glandular PR in the functionalis zone only (compare **f** and **h**). Light PR staining was retained in the stroma after E_2+P treatment in both zones. **i–l** AR. Treatment with E_2 increased AR only in the stroma, co-treatment with E_2+P decreased stromal AR staining. *Gl*, glands; *S*, stroma


E2 alone
E2 + P
Estrogen Receptor alpha
Functionalis
Basalis
a
b
c
d
Gl
S
Progesterone Receptor
e
f
g
h
Androgen Receptor
i
j
k
l

nalis zone (compare Fig. 6 a–d), but does not inhibit ERα expression in the glands and stroma of the basalis zone. Compared to ERα, ERβ staining in the endometrial glands and stroma of the macaque (data not shown) change minimally during the cycle. However, it is reported that in women, ERβ in the endometrial glands, but not the endometrial stroma, is significantly decreased during the late luteal phase of the menstrual cycle (Critchley et al. 2000). While no ERα staining is detectable in the endothelium of endometrial vessels of the macaque (Fig. 7) or women (Critchley et al. 2001), there is strong staining for ERβ in the vascular endothelium throughout the follicular and luteal phases (Fig. 7). This means that E_2 could act on the vascular endothelium through endothelial ERβ during the whole cycle, uninfluenced by P. Two functional PRs (PR-A and PR-B) have also been identified. However, unlike the two ERs, both PR-A and PR-B are encoded by a single gene, and are differentially regulated by transcription of two distinct promoters and by translation at two distinct promoter sites. PR-B is a larger molecule than PR-A due to the presence of an additional 165 amino acids in the N-terminal domain, and these two isoforms appear to be differentially expressed in the human endometrium (Mangal et al. 1997). As shown by SDS–PAGE, between days 2 and 8 of the menstrual cycle PR-B is almost undetectable and the A : B ratio is $>10:1$. From day 9 to day 13, the ratio is about 5 : 1, and it is about 2 : 1 between days 14 and 16. Thereafter, PR-B dwindles rapidly and is virtually undetectable at the end of the cycle. Radioligand binding studies show that E_2 increases total PR binding in the macaque endometrium. ICC with antibodies that recognize both PR forms show that E_2 treatment increases PR nuclear staining in all cell types except vascular smooth muscle and endothelium. Figure 6 (e–h) shows staining for PR in the macaque endometrium. Strong staining is observed in both the glands and stroma during the proliferative phase. In the luteal phase or after cotreatment with P, glandular PR staining is strikingly decreased, but PR is retained in the functionalis stroma, basalis glands and basalis stroma. As noted, PR staining is strong in perivascular stromal cells, but there is no specific PR staining in either the endothelium or the smooth muscle cells of the endometrial vasculature (Fig. 7). Therefore, any effects of P on the vascular endothelium are likely to be indirect.

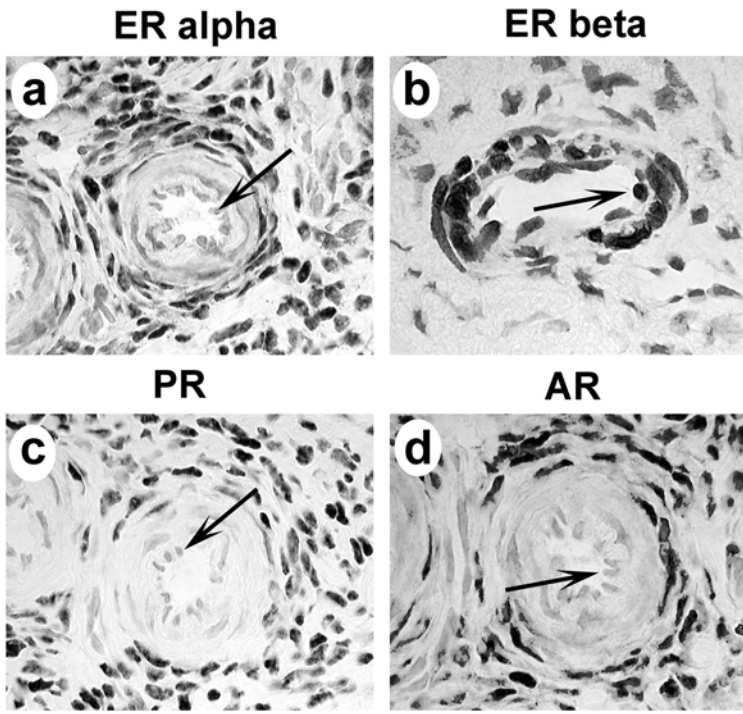


Fig. 7 a–d. Immunostaining of spiral arteries for ERα, ERβ, PR and AR. ERα, PR and AR staining was strong in perivascular stromal cells, but absent from endothelial cells. Strong ERβ staining was detected in both the endothelium smooth muscle and pericytes of the spiral arteries

Androgen receptor is also present in the macaque and human endometrium (West et al. 1990; Adesanya et al. 1999; Slayden et al. 2001 b). Like ERα and PR, E_2 increases and P decreases expression of endometrial AR (West et al. 1990) but normally, AR mRNA and protein are only expressed by endometrial stroma (Fig. 6). No AR is detectable in the endothelium or vascular smooth muscle of the spiral arteries (Fig. 7).

5.6 Effects of Progesterone Withdrawal on the Endometrium

In the endometrium, the most striking remodeling occurs when P levels fall at the end of the secretory phase. In artificial cycles, during the first 48 h after P implant removal, the endometrium shrinks, and by 72 h, the upper regions of the functionalis slough away as menstruation ensues. In the human and macaque endometrium several MMP enzymes that facilitate breakdown of the extracellular matrix increase dramatically in the functionalis zone before and during menses. Once the sloughing is complete, expression of these MMPs declines. The precise effects of the different MMPs on menstrual bleeding is not clearly established, but it is clear that many of them can break down collagen and other fibrillar components of the extracellular matrix (Marbaix et al. 1992, 1995) which undoubtedly contributes to the disruption of tissue integrity associated with sloughing (Galant et al. 2000). Figure 8 shows immunocytochemical localization of MMP-1 and MMP-3 in the macaque endometrium on day 3

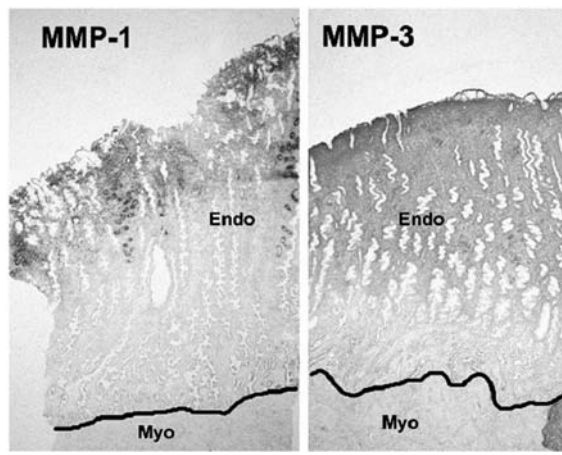


Fig. 8. Immunostaining for MMP-1 and MMP-3 in the endometrium on the day after P withdrawal during the luteal follicular transition. Note the strong gradient of expression for MMP-1 and -3 in the functionalis zone. No specific MMP staining was detected in the basalis zone

after P withdrawal. The gradient of MMP-1 and MMP-3 expression is strongest in the upper functionalis zones, the area of greatest fragmentation. Markee (1940) found that after P withdrawal, the spiral arteries that vascularize primarily the upper zones of endometrium undergo pulses of vasoconstriction that could lead to localized ischemic hypoxia in the superficial zones. Recently we reported that during the LFT in the macaque, hypoxia inducible factor 1 alpha (HIF-α) (Slayden et al. 2004) was elevated in the small vessels of the endometrial functionalis, supporting a role for hypoxia in the endometrium during this period.

Hypoxia is a strong regulator of vascular endothelial growth factor (VEGF) and our laboratory reported that both stromal and glandular VEGF were dramatically upregulated premenstrually in the macaque endometrium (Nayak and Brenner 2001). Similarly, there is marked upregulation of VEGF in human menstrual tissue (Sharkey et al. 2000). VEGF interacts with several receptors, including VEGFR-1 and VEGFR-2, located on the membranes of endothelial cells (Ferrara and Davis-Smyth 1997; Klagsbrun and D'Amore 1996). After P withdrawal VEGFR-2 is also strongly expressed in the stromal cells of the functionalis zone (Nayak et al. 2000). Because these stromal cells also express MMPs on P withdrawal, we postulated that VEGF, acting through VEGFR-2 could play a role in upregulating stromal MMPs (Nayak et al. 2000). This hypothesis remains to be physiologically tested.

There is growing evidence that the menstrual process occurs in two phases (Kelly et al. 2001). In the first phase, menses is reversible and can be blocked by replacing P, but in the second phase, P replacement cannot reverse the menstrual cascade (Slayden et al. 1999). One proposed mechanism for this event involves the Nf6B system. P normally suppresses the Nf6B system in PR-positive, endometrial stromal cells. When P levels fall at the end of the cycle Nf6B expression increases, inhibitory factors decrease, and NF6B activates transcription of various cytokine genes. These events occur first in PR-positive cells, and lead either directly or indirectly to an increase in cyclooxygenase-2 (COX-2), which results in increased synthesis of various prostaglandins that are potent vasoconstrictors. Also, with time, activated NF6B enhances the secretion of a variety of cytokines that affect vascular permeability, induce leukocyte inva-

sion, and further stimulate the secretion of prostaglandins and endothelins. Many leukocytic cell types secrete MMPs and other lytic enzymes, which would facilitate tissue breakdown and bleeding. Because endometrial leukocytes and macrophages appear to be PR negative, adding back P would not directly affect their synthetic activities. With time, endothelial cells, which are also PR negative, initiate synthesis of endothelins, and the invading leukocytes contribute their lytic effects to the menstrual cascade. Finally, prostaglandins affect the myometrium and induce contractions strong enough to expel the menstrual slough. Presumably, it is the upregulation and activation of Nf6B which leads to the second and irreversible phase of the menstrual process.

In our laboratory, we explored the time period associated with reversible and irreversible menstruation in macaques. At the end of an artificial cycle we withdrew P and then added it back at 12-h intervals. Adding back P at times up to 36 h of P withdrawal reliably blocked menstruation, but after 36 h, adding P back could not do so (Slayden et al. 1999). Adding back P before, but not after 36 h of P withdrawal, also blocked upregulation of VEGFR-2 and MMP-1 in the stromal cells of the upper functionalis (Nayak et al. 2000). These data support the hypothesis of a two-stage development of the menstrual cascade and indicate that, in macaques, the first phase of menstrual induction takes approximately 36 h.

The PR-positive perivascular cells that surround and support the spiral arterioles are major players in the menstrual cascade. Two cytokines that are generated specifically within the perivascular stroma are interleukin (IL)-8 and MCP-1. These are among the cytokines which attract multiple leukocytic cell types into the endometrium. By utilizing an experimental model of P withdrawal in women, Critchley et al. (1999) demonstrated that IL-8 and COX-2 mRNA and protein were substantially upregulated 48 h after P withdrawal. Furthermore, IL-8 mRNA expression, as demonstrated by in situ hybridization, was primarily expressed by the perivascular cells in the late secretory endometrium (Milne et al. 2001). This locus of IL-8 expression is likely to facilitate the attraction and inward migration of leukocytes into the endometrium through vascular pathways.

5.7 PR Blockade with Progesterone Antagonists

The effects of various regimens of mifepristone and some Schering antagonistic PRMs including ZK 137 316 and ZK 230 211 on the endometrium of rhesus macaques was recently reviewed (Brenner and Slayden 1994 b; Brenner et al. 2002). Administration of these compounds during the secretory phase blocks the action of P and induces menses similar to P withdrawal. Menstrual induction in E_2 + P primed macaques provides a sensitive bioassay for evaluating the effective doses of various antagonistic PRMs. For example, we have found that 2 days of frank menstrual bleeding could be induced by 0.15 mg ZK 137 316/kg (intramuscularly) whereas similar bleeding could be induced by 0.03 mg/kg of ZK 230 211. This suggests that ZK 230 211 is approximately fivefold more potent than ZK 137 316 (Slayden et al. 2001 a).

However, when menses-inducing doses of antagonistic PRMs are administered chronically throughout the entire menstrual cycle, beginning in the proliferative phase, the progestational effects of P are blocked, the endometrium remains in a suppressed state and there is no P-withdrawal bleeding when the P implant is removed at the end of an artificial cycle. Therefore, chronic administration of these compounds throughout the natural menstrual cycle provide women a means to avoid menstruation and may also serve as a treatment for some menstrual disorders, including dysfunctional uterine bleeding, menorrhagia, and irregular bleeding due to progestin-based contraceptives (Slayden et al. 2001 a).

The ability of antagonistic PRM to inhibit the natural menstrual cycle was first described in cynomologus monkeys. In that study, high doses of RU 486 (5 mg/day) administered on days 10–12 of the menstrual cycle, delayed the mid-cycle LH surge and lengthened the intermenstrual interval from the normal approximately 30 days, to 61 days, effectively blocking one menstrual cycle (Collins and Hodgen 1986). Similar results were reported for women treated with RU 486 during the follicular phase, where RU 486 disrupted follicle maturation and delayed progression of the menstrual cycle (Liu et al. 1987). A single high dose of RU 486 administered to women during the mid-luteal phase of the cycle also suppressed serum luteinizing

hormone (LH) pulse amplitude and frequency, was luteolytic and induced menstruation (Garzo et al. 1988).

We evaluated chronic administration of various Schering PRMs in naturally cycling rhesus macaques. Low doses (0.03 mg/kg) of ZK 137 316 inhibited endometrial development but still allowed menstrual and ovarian cyclicity in half of the animals treated, whereas higher doses inhibited menstrual cyclicity in all animals (Slayden et al. 1998; Zelinski-Wooten et al. 1998). Figure 9 shows the effect of two doses of ZK 230 211 on menstrual cyclicity serum steroid levels and menstruation in rhesus macaques (Slayden et al. 2001 a). When a subthreshold dose of ZK 230 211 (0.005 mg/kg) was administered daily there was no effect on either menstrual cyclicity or menstruation. However, a 10-fold increase (0.05 mg/kg) blocked ovulation, the development of a luteal phase, and menstruation, and serum E_2 concentrations remained at mid-follicular phase levels. This effect was fully reversible and menstrual cyclicity returned in the recovery period after treatment ended (Fig. 9). These studies indicate that antagonistic PRMs can act centrally to inhibit the menstrual cycle in intact macaques and women. However, we have also shown that chronic administration of these compounds effectively block menses in spayed-artificially cycled macaques as well, indicating that these chronic, low doses act directly on the endometrium to block the progestational effect of P. For example, Fig. 10 shows that treatment with the potent antagonistic PRM (ZK 230 211) inhibits progestational differentiation of the endometrium. Histologically the tissue takes on a generally proliferative appearance with tubular glands, and dense stroma. Antagonistic PRM treatment also results in striking increases in endometrial ER*a*, PR and AR. However, our laboratory and those of others have shown that, even though ER*a* is elevated, antagonistic PRMs block E_2-dependent endometrial proliferation, though the mechanism of this action has not been fully explained (Slayden et al. 1993, 1998, 2001 a; Slayden and Brenner 1994). Hodgen's laboratory was the first to report that high doses of mifepristone had an anti-endometrial effect in ovariectomized, E_2 treated macaques. Because mifepristone binds only weakly to ER (Hodgens' group called this action a 'noncompetitive antiestrogenic effect') (Wolf et al. 1989). Overall this results in a gross reduction of endometrial wet weight (Slayden et al.

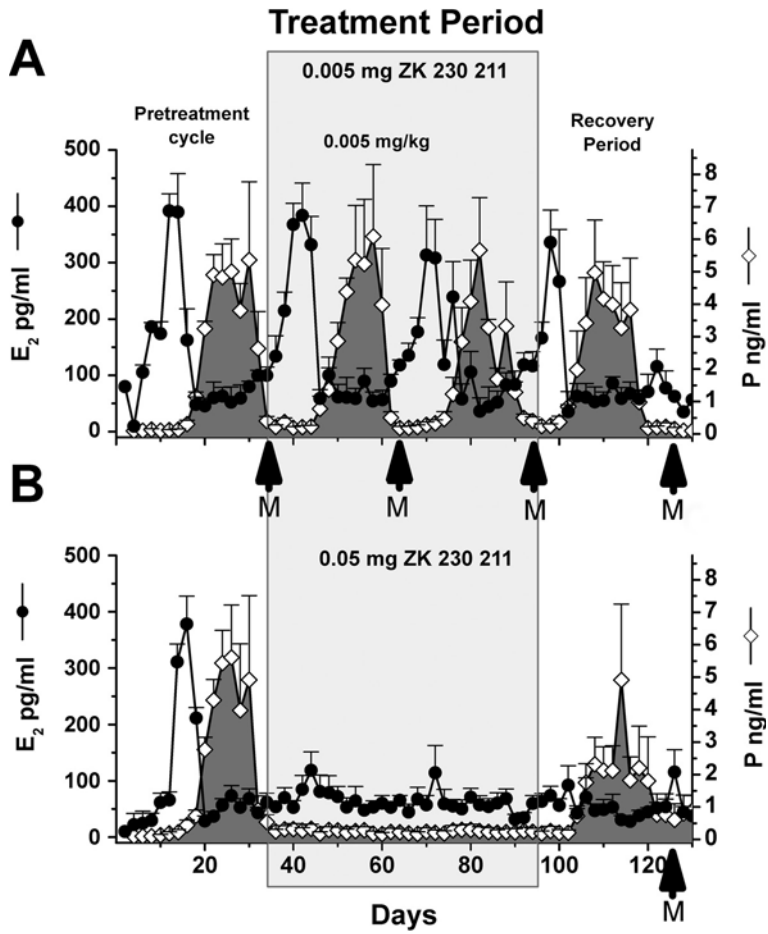


Fig. 9. Mean (*a*SE) serum E_2 and P levels in animals treated with 0.005 mg (**A**), and 0.05 mg/kg ZK 230 211 (**B**) for 60 days. *Vertical arrows* show times of menses (*M*). Treatment with 0.05 mg/kg ZK 230 211 reversibly suppressed ovulation (serum E_2 remained at mid follicular levels), the rise in luteal phase P, and menses

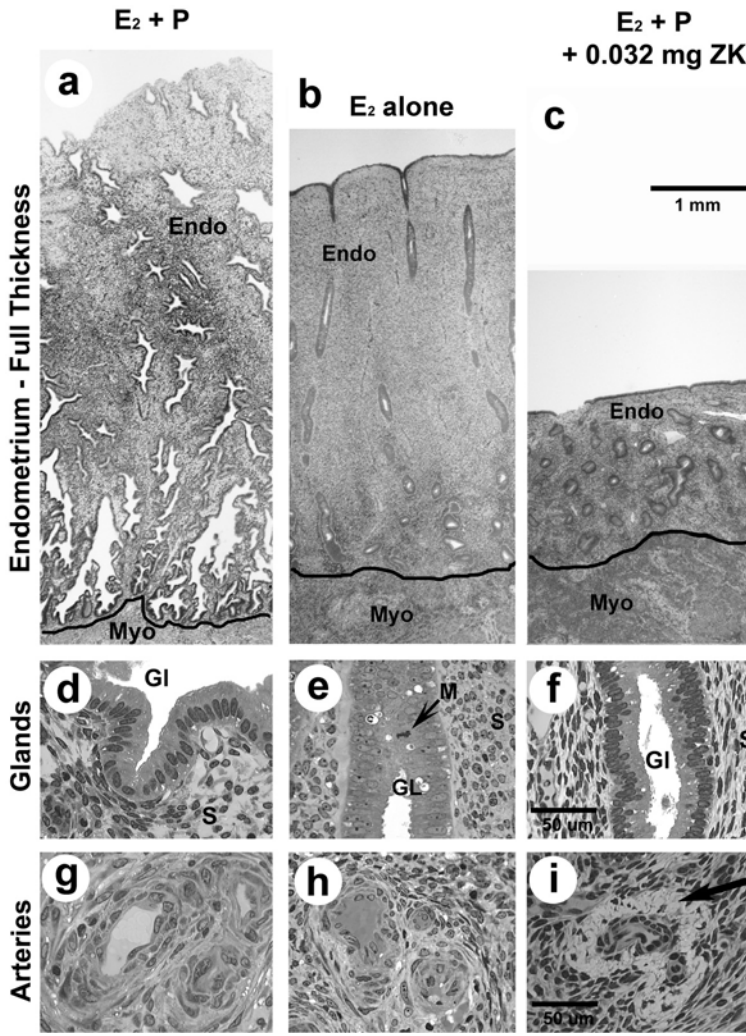


Fig. 10. Photographs of hematoxylin-stained sections of endometrium after treatment with E_2+P, E_2 alone or E_2+P+ the potent antagonistic PRM ZK 230 211. Full thickness macrophotos of endometrium (**a–c**) have a *black line* drawn to indicate the endometrial–myometrial border (*bar* shows scale). Treatment with ZK 230 211 blocked the effects of P and suppressed endometrial thickness to levels thinner than seen in animals treated with E_2

2001 a) and thickness (Fig. 10). In addition, antagonistic PRM treatment led to stromal cell atrophy, stromal compaction and hyalinizing degeneration of the spiral arteries, all in the presence of high serum E_2 levels and elevated ER. Similar effects were found after long-term treatment of intact cycling macaques with low doses of RU 486 for 8 years (Grow et al. 1996). Also, low doses of RU 486 administered chronically to women inhibited glandular mitosis and induced stromal compaction in the endometrium (Baird et al. 2003).

Several mechanisms have been proposed for the antiproliferative effects of antagonistic PRMS. For instance, Hodgen's laboratory reported that RU 486 treatment resulted in excessively elevated ER, and they suggested that elevated ER could produce a super-estrogenized state (Neulen et al. 1990, 1996). Since superphysiological doses of estrogen are reported to be inhibitory to endometrial growth (Neulen et al. 1987) then overexpression of ER could result in a similar antiproliferative action. However, as mentioned above, endometrial AR is also strikingly increased after treatment with antagonistic PRMs. Androgens are also reported to inhibit endometrial growth and elevated AR may play a role in the antiproliferative effect. We have extensively reviewed this possibility elsewhere (Brenner et al. 2002). It is well documented that antagonistic PRMs can induce degeneration of the spiral arteries. These vessels, which are unique to the primate endometrium, are primary targets for PRM action even though they lack ER*a*, PR and AR. Regardless of the primary mechanism, damage to the spiral arteries may ultimately lead to the major inhibitory actions of these compounds (Chwalisz et al. 2000).

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alone for 28 days. This reduction in endometrial thickness was associated with an increase in stromal compaction. Although the ZK 230 211 treated endometrium appeared to have a 'proliferative' appearance, there were no mitotic cells present in ZK 230 211 treated animals (compare **d–f**). Treatment with E_2+ZK 230 211 resulted in hyalinizing degeneration of the spiral arteries (compare **g–i,** see *arrow* in **i**). **k–m** ER*a* staining. *Ar*, arteries; *S*, stroma; *Endo*, endometrium; *Myo*, myometrium. High-power photos were made at ×250 original magnification (bar shows scale)

5.8 Conclusion/Future Directions

PRMs have great clinical potential to ameliorate various reproductive tract disorders, but novel compounds should be evaluated in nonhuman primates, as these species provide superior models of endometrial physiology. Such compounds could included both novel steroidal molecules like CDB-2914 (Passaro et al. 2003) and nonsteroidal compounds (Zhang et al. 2003). PRMs have long and short acting effects that differ in their end results: (1) if applied acutely during the luteal phase, after P has induced progestational changes in the endometrium, they block the ability of P to maintain the endometrium and bring on menstruation; (2) if applied chronically, and if ovulation occurs, they block the progestational effects of P so the endometrium never differentiates into a receptive state and never bleeds when luteolysis and P withdrawal occurs. The growth of the spiral arteries is also inhibited by chronic administration of antagonistic PRMs and this undoubtedly contributes to the reduction in endometrial bleeding. In addition, chronic administration of antagonistic PRM blocks the actions of unopposed estrogens on endometrial proliferation by a currently unexplained mechanism that may involve the AR. These effects, which are well documented in macaques and women, provide a sound basis for the development of new compounds of higher potency and selectivity that hopefully can greatly benefit women's health.

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6 Effects of Progestogens on Endometrial Maturation in the Implantation Phase

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6.1 Introduction

Progesterone is necessary for the establishment and maintenance of pregnancy in virtually all mammalian species. Ever since the biological role of progesterone was established, it has been recognized that the use of compounds, antiprogestins, which block or modulate progesterone action would mean a significant advance in human fertility

control. Progesterone receptor (PR) modulators also offer a way of studying progesterone-dependent mechanisms involved in the process of successful implantation, such as the cyclic development of uterine receptivity.

The endometrium is a complex tissue that contains a number of different cell types, e.g., epithelial, stromal and endothelial cells and cells of the immune system. During the luteal phase progesterone stimulates the development and the maturation of the endometrium. At the development of receptivity the endometrium undergoes specific changes and becomes able to respond to the signals from the blastocyst. The endometrium is only receptive for implantation during a very short time after which the endometrial environment becomes refractory to the blastocyst (Navot et al. 1991). The transient nature of the receptive stage has led to the concept of an 'implantation window'. It is likely that failure to develop a receptive endometrium contributes significantly to implantation failure in humans (reviewed in Sharkey and Smith 2003). A number of molecular and morphological markers, whose expression are cycle dependent and coincide with the implantation period, have been identified. Alterations in some of these factors have been found in the endometrium of women with unexplained infertility and endometriosis. In knockout experiments in mice, some of these factors have been shown to be essential for normal implantation to occur. However, there seems to be alternative pathways that compensate for the knockout of many cytokines, and a considerable heterogeneity in expression of local factors exists even in fertile women. So far no single marker of receptivity has been identified in humans. However, a common feature for all or most of the suggested markers of receptivity is that they are directly or indirectly regulated by progesterone. The action of progesterone in the endometrium is complex, involving direct, endocrine and indirect, paracrine, actions through induction and inhibition of gene expression.

Today there are several progestogens, selective progesterone receptor modulators or inhibitors available, but so far it is only mifepristone that has been studied in humans to any extent (Van Look and Bygdeman 1989). Mifepristone is a 19-nor steroid that binds with high affinity to the PR thus inhibiting the effect of endogenous progesterone. When treatment with mifepristone is given after ovula-

tion, or in regimens that do not inhibit ovulation, there seems to be a dose dependent effect on endometrial receptivity and implantation.

A better understanding of progesterone action and the nature of endometrial receptivity provide new means to diagnose and treat some forms of infertility and to develop methods to target the endometrium for contraceptive purposes.

6.2 The Role of Progesterone in Development of Receptivity

After ovulation, remarkable changes occur in the morphology of the endometrium, depending on the degree and exposure to progesterone. In the normal menstrual cycle, these changes occur with great regularity and precision, in particular during cycle days luteinizing hormone surge (LH) –3/–2 to LH +7/+8, i.e., between ovulation and implantation (Johannisson et al. 1987; Li et al. 1988; Dockery and Rogers 1989). The receptive phase in humans and primates has been shown to occur between cycle days LH +6 and +10 (Hertig and Rock 1956; Croxatto et al. 1978; Navot et al. 1991) coincident with the peak serum progesterone levels. The tissue progesterone concentration parallels the plasma concentration of progesterone (Runnebaum et al. 1978). Removal of the corpus luteum or blockage of progesterone synthesis results in subsequent decline in progesterone levels and pregnancy loss (Csapo and Pulkkinen 1978).

In humans postovulatory estradiol secretion by the corpus luteum does not seem to be necessary for the development of a normal secretory endometrium (Younis et al. 1994). However, estrogen produced by the endometrium or the embryo may have local effects (Edgar et al. 1993; Bulun et al. 2001). 17β-Hydroxysteroid dehydrogenase (17βHSD) is the major enzyme metabolizing estradiol in the endometrium (Liu and Tseng 1979). Endometrial estradiol is converted into biologically less active estrogen, estrone (Gurpide et al. 1977). 17βHSD is thought to be an important factor regulating exposure of endometrial cells to estrogens (Smith et al. 1975). The enzyme activity is induced by progesterone with peak concentrations in the mid-secretory endometrium (Tseng and Gurpide 1974). Thus progesterone can determine the influence of estrogen both by induction of 17βHSD and by reducing estrogen receptor (ER) in the endo-

metrium. Endometrial glandular epithelium exhibits the highest 17βHSD activity, although activity in stromal cells has also been observed (Holinka and Gurpide 1981).

The glycoprotein glycodelin (PP14) is progesterone induced major secretory product of the glandular epithelium. Glycodelin starts to appear in deep basal endometrial glands on day 5 after ovulation and is strongly expressed on day LH + 10. After ovarian hyperstimulation glycodelin appears earlier after ovulation and levels increase after administration of progesterone while no increase is found in anovulatory cycles and in infertile women (Seppälä et al. 1987, 1988; Julkunen et al. 1986; Tulppala et al. 1995). Glycodelin may also play a role in the immune response to pregnancy (Oheninger et al. 1995).

In the female reproductive tract progesterone has also been shown to play a synergistic role with nitric oxide (NO) (Chwalisz et al. 1999). This interaction is particularly important in reproductive processes such as implantation. Studies in mice have shown that both inducible NO synthase (NOS, iNOS) and endothelial NOS (eNOS) are upregulated in the implantation site (Gouge et al. 1988; Purcell et al. 1999). The administration of L-NAME, a NOS inhibitor during the preimplantation phase, has been shown to cause a reduction in the implantation rate in rats (Biswas et al. 1998; Novaro et al. 1997).

6.3 Progesterone Receptor Expression

ER and PR levels are high in the proliferative phase, reaching a maximum around the time of ovulation (Garcia et al. 1988; Lessey et al. 1988). Steroid receptor expression then decline in epithelial cells while the stromal cells maintain PR and retain progesterone responsiveness. Progesterone-induced paracrine factors produced in the stroma may act in a paracrine way on the expression of other factors. Under the influence of progesterone the stromal cells undergo decidualization. Downregulation of progesterone receptors in the epithelium has been shown to be highly associated with the development of endometrial receptivity (Gemzell-Danielsson et al. 1994).

The human PR is composed of two isoforms, PR-A (94 kDa) and PR-B (116 kDa) (Horwitz and Alexander 1983; Savouret et al.

1990). PR-A is a truncated form of PR-B lacking 164 amino acids from the N terminus. Both isoforms are products of a single gene and are translated from individual messenger ribonucleic acid species under the control of distinct promoters (Kastner et al. 1990). Differential expression of PR-A and PR-B has been shown in the human endometrium. Wang and colleagues demonstrated with immunhistochemistry that PR-B was present in glands and stroma in the proliferative phase, and was dramatically reduced in the glands during the secretory phase (Wang et al. 1998). Scores for PR-B immunostaining in stromal cells were slightly higher than that in glandular cells.

Pinopodes have been suggested to be an ultrastructural marker of receptivity but the function in humans remains largely unknown. Recently it was shown that downregulation of PR-B occurs at the onset of pinopode formation in the human endometrium (Stavreus-Evers et al. 2001).

6.4 Effects of Progestogens on Endometrial Development During the Implantation Phase

The importance of progesterone for endometrial development can be clearly demonstrated by the effect of antiprogesterones. Administration of mifepristone during the preovulatory phase of the menstrual cycle, after selection of the dominant follicle, either disrupts follicular development or inhibits ovulation (Croxatto et al. 1995). Alternatively the follicle may remain unruptured until the end of the cycle (Spitz et al. 1994). When the influence of mifepristone is over, ovulation resolves and the following luteal phase seems to be normal (Swahn et al. 1990; Marions et al. 2002). Normal endometrial function is also supported by observations in rhesus monkeys where delayed implantation may occur due to delay of ovulation following follicular phase administration of mifepristone (Nayak et al. 1997; Gosh et al. 1997).

Treatment with 200 mg of mifepristone immediately after ovulation on days LH +0 to +2 does not affect the normal rhythm of the menstrual cycle or the plasma levels of estrogen and progesterone but has profound effects on endometrial development with decreased

glandular diameter, increased glandular apoptosis, decreased number of vacuolated cells and increased number of stromal mitoses (Swahn et al. 1988; Critchley et al. 1999). Expression of 17βHSD in endometrial glandular and luminal epithelium will be prevented as will endometrial secretory activity measured by *Dolichos biflorus* agglutinin (DBA) lectin binding (Gemzell-Danielsson et al. 1994) and serum levels of glycodelin (Cameron et al. 1996). Treatment with mifepristone inhibited endometrial epithelial eNOS expression but did not affect the expression of endothelial eNOS.

Administration of mifepristone in the early luteal phase seems to induce a delay in glandular secretory differentiation in the human endometrium. However, in a recent study, administration of mifepristone on day LH +2 caused a significant increase in insulin-like growth factor binding protein-1 (IGFBP-1) protein and mRNA (Qui et al. 2002). A stimulating effect of an antiprogesterone is unexpected since accumulating evidence has demonstrated that progesterone is responsible for the production of this protein in the human endometrium (Qiu et al. 2002). Thus the administration of mifepristone in the early luteal phase does not simply retard endometrial development. Furthermore mifepristone seems to have diverse actions on glandular and stromal cells (Rutanen et al. 1986). These findings support an earlier observation that administration of mifepristone on day LH +2 to +6 had no demonstrable effect on the predecidual reaction (Li et al. 1988). It was observed that while glandular secretory activity appeared retarded, glandular mitotic count remained unchanged while still other parameters like stromal extravasation appeared advanced. In vitro, mifepristone causes a transient super induction of IGFBP-1 and prolactin secretion in human endometrial stromal cells when incubated with relaxin (Bell et al. 1991; Gao et al. 1999; Tseng et al. 1992). Thus there is the possibility of a switch from antagonistic property to agonist activity, depending on the intervention of other signaling pathways. It has also been shown that elevation of cAMP levels in a human breast cancer cell line leads to the functional reversal of progesterone antagonist action. cAMP amplifies the transcriptional signals of agonist-occupied steroid receptors. Additionally, in the case of progesterone antagonists at least, cAMP can switch the transcriptional phenotype to render them potent agonists (Brosens et al. 1999; Sartorius et al. 1993). A possible

explanation could be that treatment with mifepristone alters the signaling mechanisms that may affect cAMP activation in vivo leading to the unexpected observation of stimulation as opposed to suppression by the PR antagonist.

The endometrium seems to be more sensitive to antiprogesterone than the ovulatory process. Once weekly administration of 2.5 or 5 mg of mifepristone for 8 weeks or daily treatment with 0.5 mg for 12 weeks did not inhibit ovulation or significantly disturb the menstrual cycle compared to control cycles (Gemzell-Danielsson et al. 1996, 1997). The number of endometrial glands and glandular diameter decreased during treatment, but this decrease was not significant for the 2.5 mg group. Despite only minor effects on endometrial morphology, all regimens reduced secretory activity assessed by DBA lectin binding. Endometrial glandular expression of glycodelin was decreased during the third month of daily mifepristone treatment. There was no measurable effect on endometrial development following daily treatment with 0.1 mg of mifepristone.

Daily treatment with 2 mg of mifepristone for 30 days inhibited ovulation in four out of six subjects (Cameron et al. 1996). In the two women in whom ovulation did occur, secretory transformation was delayed. Despite anovulation and high estrogen levels there were no signs of hyperplasia.

When a single dose of 10 mg of mifepristone was administered on day LH +2, the observed effect on the endometrium was less pronounced than after treatment with 200 mg or repeated low doses (Marions et al. 2002). Following 10 mg the endometrium was slightly out of phase and four of six women had decreased DBA lectin binding.

6.5 Effect of Antiprogestins on Progesterone Receptor Expression

Interestingly, PR-A and PR-B proteins have been shown to respond differently to progestin antagonists (Giangrande and McDonnell 1999). In the endometrium, PR-B immunostaining and mRNA levels were increased after treatment with mifepristone. In contrast to the Fallopian tube, there was a predominant expression of the PR-B iso-

form in the glandular cells. In the Fallopian tube immunhistochemical staining localized the increased expression of PR-B to both epithelial and stromal cells, but it was more pronounced in the stromal cells (Sun et al. 2003).

Downregulation of PR is highly correlated to the development and onset of receptivity. Treatment with a single dose of 200 or 10 mg of mifepristone immediately postovulation inhibits the normal downregulation of PR (Marions et al. 2002; Gemzell-Danielsson et al. 1994). Treatment with low weekly doses (5 mg) of mifepristone for 2 months significantly increased PR during the second treatment month while low daily (0.5 mg) treatment for 3 months did not significantly affect PR expression (Gemzell-Danielsson et al. 1996, 1997). Still endometrial secretory function was decreased. Mifepristone also affects ER and androgen receptor expression (Critchley et al. 2002; Slayden et al. 2001).

6.6 Progesterone-Regulated Markers of Endometrial Receptivity

In addition to morphological markers several molecular markers of normal endometrial development have been described. It has become clear that normal morphological appearance does not necessarily reflect normal endometrial function or receptivity. The use of endometrial biomarkers has provided insight into the development of endometrial receptivity, suggesting that receptivity is initiated in the luminal epithelium followed by a shift to the stroma or decidua (Lessey 2003). However, to what extent changes in local markers reflect functional alterations and non-receptivity is still not clear (Sharkey and Smith 2003).

6.6.1 Pinopodes

Pinopodes are large cytoplasmatic protrusions from the epithelial surface, which develop under the influence of progesterone apparently absorbing luminal fluid, drawing the uterus closely around the embryo (Psychoyos and Martel 1985). Pinopodes are also developed

in human endometrium and have been reported to be present only during the receptive phase (Psychoyos and Martel 1985). The appearance of pinopodes in fertile women varies between days LH +5 and LH +8 but was shown to occur at the time of downregulation of PR-B (Stavreus-Evers et al. 2001). There are also conflicting data showing persistent pinopode expression throughout the luteal phase (Acosta et al. 2000).

The function of pinopodes in humans remains unknown. A decrease in the volume of uterine fluid has been observed also in humans at the time of endometrial receptivity (Gemzell-Danielsson and Hamberg 1994). In vitro blastocyst attachment to the luminal epithelium seems to occur to the pinopodes (Bentin-Ley et al. 1999). In mouse luminal epithelium heparin-binding epidermal growth factor-like growth factor (HB-EGF) was increased around the implantation site, which in turn promoted hatching of the blastocyst (Das et al. 1994). The expression of HB-EGF is elevated in the glandular and luminal epithelium during the secretory phase in the human endometrium (Leach et al. 2001), with the highest expression when pinopodes are present on the endometrial surface (Yoo et al. 1997). HB-EGF is present both inside the luminal epithelial cells and on the surface of pinopodes (Stavreus-Evers et al. 2002). It has also been found that HB-EGF is a potent inducer of avb3 integrin in the endometrial epithelium, a suggested biochemical marker of endometrium receptivity (Somkuti et al. 1997). Aberrant expression of pinopodes has been shown in the endometrium of women with unexplained infertility. Following treatment with 200 mg of mifepristone on LH +2, the number of pinopodes did not seem to be decreased (unpublished results) (Fig. 1). However, the amount of uterine fluid was significantly increased compared to controls, which could indicate an effect on pinopode function (Gemzell-Danielsson and Hamberg 1994). Expression of HB-EGF in luminal and glandular epithelial cells was decreased following treatment with mifepristone (unpublished observation).

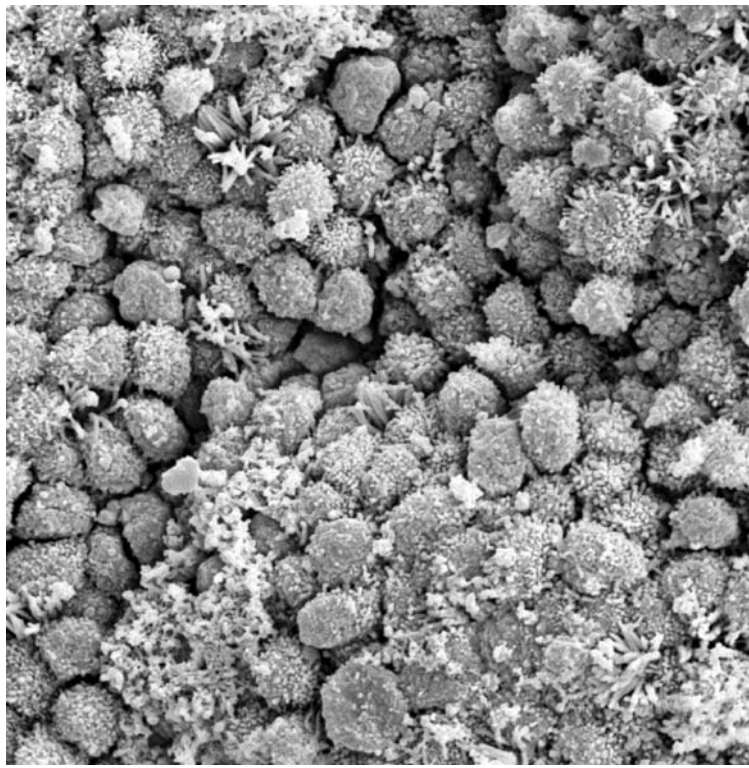

Fig. 1. Pinopode formation. Endometrial sample obtained on cycle day LH +7 (electron microscopy)

6.6.2 Integrins

Integrins are cell surface receptors for the extracellular matrix and connect extracellular cell adhesion proteins to cytoskeletal components. Certain integrin subunits appear to be regulated within the cyclic endometrium (Lessey et al. 1992), and specific alterations were shown in the preimplantation period suggesting a possible role for $\alpha 4$, $\beta 3$ and $\alpha v\beta 3$ subunits in the establishment of endometrial receptivity. The loss of $\alpha v\beta 3$ is highly associated with endometriosis, retarded endometrial development and infertility (Lessey et al. 1992,

1994). The expression of $\beta 3$ coincides with the presence of pinopodes (Stavreus-Evers et al. 2002) and is upregulated by interleukin (IL)-1α and IL-1β of embryonic origin (Simon et al. 1998), which appears to affect the ability of the blastocyst to adhere to the endometrium. Reduced immunostaining for $\alpha 4$ and $\beta 3$ subunit in glandular epithelium at the time of implantation was found after treatment with various doses of mifepristone (Marions et al. 1998 a, 2002). However, no effect of mifepristone could be demonstrated on $\alpha v\beta 3$. In contrast intermittent doses of onapristone, effective for contraception in bonnet monkeys, reduced expression of $\alpha v\beta 3$ even in low doses that did not affect PR expression (Puri et al. 2000).

6.6.3 Leukemia Inhibitory Factor

Endometrial expression of leukemia inhibitory factor (LIF) has been demonstrated to be necessary for successful implantation in mouse (Stewart et al. 1992). LIF is expressed in human epithelial cells during the implantation period. The expression was found to have decreased after treatment with 200 mg of mifepristone on LH +2 (Gemzell-Danielsson et al. 1997 a; Cameron et al. 1997). Reduced expression of LIF was also found following 5 mg mifepristone once weekly for 2 months or 0.5 mg daily for 3 months, while a single dose of 10 mg postovulatory had no effect on LIF expression (Gemzell-Danielsson et al. 1997 b; Marions et al. 2002).

Human preimplantation embryos have been shown to express both LIF and its receptor (Chen et al. 1999). Secretion of LIF by endometrial explants from women with infertility was found to be reduced compared to those of fertile women and LIF secretion increased by adding IL-1 (Delage et al. 1995).

6.6.4 Prostaglandins

Prostaglandins (PGs), either maternally or embryonally derived, have been thought to be involved in the initial phase of implantation. The principal role of PGs may be to create a mild inflammatory reaction in the endometrium. Cyclo-oxygenase is the rate limiting enzyme in

the synthesis of PGs and exits in two isoforms, COX-1 and COX-2. COX-1 deficient mice are fertile while mice lacking COX-2 are infertile due to both anovulation and impaired implantation. Immunostaining for COX-1 was found to be present mainly in the glandular and luminal epithelium while COX-2 was localized to the luminal epithelium and perivascular cells. Treatment with 200 mg mifepristone on day LH +2 significantly reduced the expression of COX-1 in glandular epithelium and COX-2 in luminal epithelium, and has been shown to reduce immunostaining for PG-dehydrogenase within endometrial glands (Marions et al. 1999; Cameron et al. 1997). Treatment with mifepristone was found to reduce uterine fluid PGF2*a* concentrations as effectively as the PG biosynthesis inhibitor naproxen in the midluteal phase (Gemzell-Danielsson and Hamberg 1994).

6.7 Inhibition of Endometrial Receptivity and Embryo Implantation

Preimplantation embryo growth on day 6 after ovulation was significantly affected in the morula–blastocyst transition stage in monkeys treated with mifepristone compared with that in control animals. Furthermore, treatment resulted in a 25% decrease in embryo recovery from the reproductive tract (Gosh et al. 1997). However, mifepristone had no direct effect on implantation ability of embryos of cynomolgus monkeys (Wolf et al. 1990). Corresponding data on embryo development are lacking from humans but it has been shown that mifepristone does not affect human fertilization rates in vitro (Messinis and Templeton 1988). Thus, although information on the situation in humans is scarce, available data indicate that mifepristone has no direct effect on embryo development and that the effect seen in vivo is likely to be secondary to changes in the tubal and/or uterine milieu.

The importance of progesterone for endometrial receptivity has been proven by the contraceptive effect of mifepristone. Twenty one women, all with proven fertility, were given a single dose of 200 mg of mifepristone on LH +2 for 1–12 months as their only contraceptive method (Gemzell-Danielsson et al. 1993). The women used a

rapid urinary test to detect the LH surge and to determine the day of treatment. A total of 161 cycles were studied. In 124 ovulatory cycles at least one act of intercourse occurred between 3 days before and 1 day after ovulation. One pregnancy occurred corresponding to a probability of pregnancy of 0.008. In a similar study 32 women used the contraceptive fertility monitor, Persona, to determine the day of treatment. Two-hundred milligrams of mifepristone were taken on day LH +0 to +3 (Hapangama et al. 2001). In a total of 178 cycles there were two pregnancies (probability of pregnancy, 0.01). The high efficacy of this regimen has also been confirmed in monkeys (Ghosh and Sengupta 1993).

The contraceptive efficacy of low daily or intermittent doses of mifepristone that did not inhibit ovulation but affected endometrial development or markers of endometrial receptivity was also investigated. When 5 mg was administered once weekly to 17 women during 60 cycles, three pregnancies occurred, corresponding to a probability of pregnancy of 0.067 (Marions et al. 1998b). Treatment with 0.5 mg mifepristone daily to 32 women during 141 cycles resulted in five pregnancies and a probability of pregnancy of 0.04 (Marions et al. 1999).

6.8 Conclusion

The development of uterine receptivity depends on the action of progesterone through the PR. Blocking or modulating the effect of progesterone through PR antagonists, modulators or agonists provide new insight into the role of progesterone as well as on the nature of endometrial receptivity and provides the ground for the development of new treatment options for infertility and contraception.

A number of biochemical or morphological markers, whose expression coincides with the downregulation of progesterone receptors and the development of receptivity, have been studied. The expression of some of these suggested that markers of receptivity are altered in women with unexplained infertility, delayed endometrial development or endometriosis. Some of these local factors have been shown to be necessary for implantation to occur in animal studies. PR modulators and PR blockers offer a tool to study the complex

nature of endometrial receptivity. Furthermore antiprogesterone like mifepristone has been shown to be highly effective to inhibit endometrial receptivity and implantation.

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7 Gene Expression During the Implantation Window: Microarray Analysis of Human Endometrial Samples

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7.1 Introduction

The endometrium is a complex tissue undergoing significant morphological and structural changes during the menstrual cycle (Brenner and Slayden 1994). These changes are regulated by the ovarian hormones estradiol and progesterone. The characteristic morphological features have been used for staging of endometrial biopsies according to Noyes et al. (1951). It is only during a short period of the

idealized 28-day cycle that the endometrium is receptive for an arriving blastocyst. This period is the so-called implantation window (Beier 1997). In humans, this receptive phase is limited to days 19–24 of a normal ovulatory cycle. Understanding the mechanisms underlying the development of a receptive phase endometrium has been the focus of gynecological research for a long time. In recent years a number of potential markers for endometrial receptivity has been studied by using a 'one-by-one' approach, thus allowing the identification of candidate proteins or protein families in animal models and human specimens (Stewart et al. 1992; Beier and Beier-Hellwig 1998; Giudice 1999; Cavagna and Mantese 2003). However, recent data show a high inter- and intra-individual variability of some of the proposed markers (Ordi et al. 2003) and the clinical relevance has generally not been shown.

With the recent progress made in the genomic area and the advent of large-scale analyses, powerful tools are now available to decipher the molecular mechanisms underlying physiological processes in different tissues and cell systems. Gene expression profiling by oligonucleotide or DNA array analysis has provided new insights into the complex cellular processes involved in growth and differentiation in various cell systems. It has also been applied to analyze endometrial cell differentiation (Popovici et al. 2000; Brar et al. 2001; Kao et al. 2002; Carson et al. 2002; Borthwick et al. 2003; Riesewijk et al. 2003; Tierney et al. 2003). We used oligonucleotide array expression analysis to study human samples of the early and mid-secretory endometrial phases in order to gain insight into the mechanisms involved in the receptivity of the endometrium. This should help in the identification of novel pathways and targets that may offer new approaches to female contraception. Additionally, endometrial specimens from endometriosis patients were analyzed in order to identify specifically regulated genes. This may pave the way for new treatment paradigms as well as for diagnostic approaches for endometriosis.

7.2 Analysis of Human Endometrial Samples: Data Collection

The endometrial samples utilized in this study were collected from three different groups of patients who visited assisted reproduction clinics. The samples came from healthy patients (with established male factor infertility) during: (1) the pre-receptive, early secretory phase [cycle day 16–18, luteinizing hormone surge (LH) +2 to 4]; (2) receptive, mid-secretory phase (cycle day 21–23, LH +7 to 9); and from (3) proven endometriosis patients during the receptive phase (cycle day 21–23, LH +7 to 9). Sample collection was performed based on the LH peak and initial analysis of staging was performed at the molecular level by PCR amplification of known receptivity genes such as leukemia inhibitory factor and calcitonin. Additionally, after array analysis, expression data were analyzed by principal component analysis (PCA), which allowed the identification of several outlier samples that did not align with other samples of the same group or of the same cycle phase. Analysis of the hormonal status of all specimens indicated that these outliers had low luteal phase progesterone levels. Consequently, only samples fitting all inclusion criteria, i.e., sampling according to the LH peak with a luteal phase progesterone concentration of ≥12 ng/ml were included in the data analysis for this study. Taken together, a total of 13 samples were analyzed, distributed into the following groups: (1) healthy pre-receptive ($n=4$); (2) healthy receptive ($n=4$); and (3) endometriosis, receptive ($n=5$).

Two different arrays have been used for the analysis of endometrial samples. One was the commercially available Affymetrix chip Hu95A which covers approximately 12,000 mRNAs. The second array had a proprietary design, as ordered by Schering AG. The probe sets present on this chip were selected based on in silico analysis of all human genomic data available, thereby providing a unique selection of potential target genes. This array focused on protein kinases, G protein-coupled receptors, proteases, protein phosphatases and nuclear receptors, which form potentially ‘drugable’ gene families. The GeneData Expressionist software as well as a proprietary software tool were used to analyze the expression data.

RNA from the endometrial biopsies of the present study as well as from a variety of other human organs was hybridized individually on both the Hu95A and the custom-designed chips. After normalization, data can be compared within the same tissue class (here the endometrial samples) but also with the different other tissues that were analyzed on the same chip. By this, the tissue expression level of each gene represented on the chip can be assessed. This database of tissue expression profiles thereby provides a very valuable tool to help in the evaluation of potential target genes of interest. A public database of molecular abundance profiles also compiled from hybridization array data generated during a variety of studies can be found at http://www.ncbi.nlm.nih.gov/projects/geo. Another public source is the expression atlas of the Genomics Institute of the Novartis Research Foundation, which provides data only for academic and not-for-profit use. It can be accessed at http://symatlas.gnf.org/SymAtlas/. These sources allow the evaluation of regulated genes not only in the context of the tissues analyzed but also with respect to their physiological distribution. However, one should bear in mind that data derived from expression analysis need to be confirmed by independent methods.

7.2.1 Data Analysis: Regulated Genes

The comparison of receptive and pre-receptive phase endometrium from healthy women resulted in the identification of 171 significantly up- and 220 significantly downregulated genes (*t*-test, $P \leq 0.01$) with more than twofold regulation when comparing the mean expression values of the pre-receptive and receptive phases.

Among the regulated genes, some had been described as markers for receptivity already, for instance osteopontin and glycodelin (Coutifaris et al. 1999; Mueller et al. 2000). The experimental confirmation by RT-PCR of the regulation of these two genes in individual endometrial samples is documented in Fig. 1 (top and middle panels). Glycodelin has already been identified as a product of the secretory phase endometrium by subtractive cDNA hybridization (Vaisse et al. 1990; for a review see Mueller et al. 2000). Indeed, almost all previous expression profiling studies conducted with endometrial samples in which either proliferative or early secretory phase

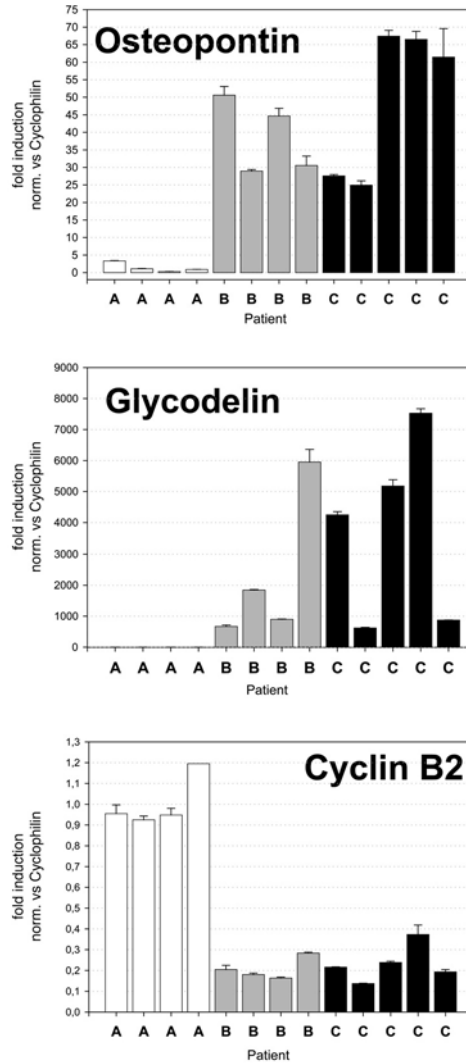

Fig. 1. Confirmation of the regulation of selected genes by quantitative PCR analysis of individual samples. The expression levels were standardized to those of cyclophilin A. **A** Healthy, pre-receptive, early secretory phase (cycle day 16–18). **B** Healthy, receptive, mid-secretory phase (cycle day 21–23). **C** Endometriosis patients, receptive phase (cycle day 21–23).

endometrium was compared to mid-secretory phase tissue reported that this gene was dramatically and consistently upregulated in the mid-luteal phase endometrium (Riesewijk et al. 2003; Kao et al. 2002). Glycodelin is believed to play a vital role in reproduction and endometrial receptivity (Mueller et al. 2000). It has been implicated in angiogenesis as well as in immune modulation. However, its specific mechanism of action is not yet understood.

The comparison of endometriosis versus healthy mid-secretory phase endometrium yielded only a small number of genes showing regulation. In addition, this regulation was usually very low (data not shown). This is in contrast to the results of Kao et al. (2003) who found several genes, including glycodelin, to be downregulated in the endometrium of endometriosis patients. As shown in Fig. 1 (middle panel), we found the upregulation of glycodelin in the mid-secretory phase, as compared to the early-secretory phase, to be similar in both healthy and endometriosis patients. We also noticed large variations between individuals. Indeed, marked differences in the expression levels of several proposed receptivity markers can be detected in a given individual, due to cycle-to-cycle variations (Ordi et al. 2003). This reflects the highly dynamic process underlying endometrial differentiation. The use of different staging methods might also account for the differences seen between studies. We characterized our tissue samples at the molecular level, via PCR and PCA of the array data. In addition we excluded samples with low luteal phase progesterone levels. On the other hand, our secretory phase biopsies yielded comparable gene expression patterns when compared to other studies, such as that of Riesewijk et al. (2003). This highlights the value and comparability of array studies in general, but at the same time shows that differences possibly due to technical variations as well as sample heterogeneity exist. Analysis of a much larger number of patient samples as well as individual gene analysis is now needed to extend and confirm these findings.

7.2.2 Data Analysis: Processes Potentially Involved in Endometrial Receptivity

The regulated genes found when comparing pre-receptive and receptive phase normal endometrium were classified into functional groups according to their annotation. The categories chosen were signaling, immunology, metabolism, growth and differentiation, gene expression, cell cycle, adhesion and cell interaction, angiogenesis, cell protection, cell structure as well as unknown. Genes found to be more than twofold regulated were grouped according to function and the results of this classification are shown in Fig. 2.

Interestingly, immunology-related genes were found to be mostly upregulated rather than downregulated in the implantation window. This highlights the importance of a specific immune modulatory environment in the receptive endometrium (Loke and King 1995; Pijnenborg 2002) and underscores the need for further investigation in this field. Furthermore, genes important for protection of the cell from oxidative stress, e.g., glutathione peroxidase-3 or superoxide dismutase-2, were upregulated in the receptive phase endometrium. On the other hand, a large number of cell cycle control genes were downregulated, consistent with the decreased proliferation seen in the secretory phase endometrium. One example is cyclin B2. Figure 1 (bottom panel) shows the confirmation of the downregulation of cyclin B2 in the implantation window by quantitative PCR analysis of endometrial samples. Concerning angiogenesis, we found activators to be upregulated in the implantation window, whereas inhibitors were downregulated, consistent with increased blood vessel formation during the secretory phase. An interpretation of the gene regulation patterns observed in gene families may give important hints about pathways and processes involved. However, individual analysis of genes and of their expression in the cellular context is additionally needed for valid interpretation. This is especially true for a complex tissue such as the endometrium, as biopsies are a mixture of different cell types with varying distribution during the menstrual cycle.

In order to provide deeper insight into the complex processes involved in the transition to a receptive phase endometrium, three examples of regulated pathways will be discussed below in more de-

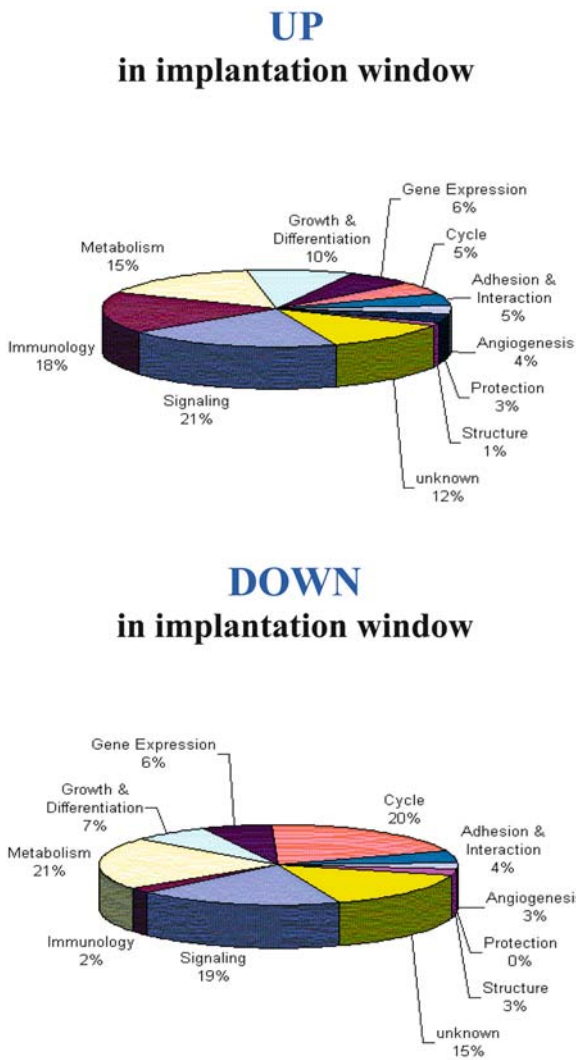

Fig. 2. Pie chart classification of genes regulated in the endometrium during the implantation window

tail. Table 1 lists all the genes discussed herein with their regulation factor between the pre-receptive and receptive phase endometrium. As an example for the changes taking place in immunology-related genes, the complement pathway will be discussed in detail. Another pathway that shows a clear regulation in the secretory phase endometrium is the coagulation cascade. Finally, changes in the Wnt pathway and of the expression levels of some of its members will be

Table 1. List of selected genes found to be regulated in the secretory phase endometrium. The regulation factor is the fold induction detected in the receptive phase endometrium compared to the pre-receptive endometrium (*t*-test, $P \leq 0.01$)

Gene description	Regulation factor	Category
Defensin, beta 1 (DEFB1)	49.5	Immunology
Glutathione peroxidase 3	48.7	Cell protection
Complement C4 binding protein	48.0	Immunology
Glycodelin (placental protein 14)	22.8	Immunology/ angiogenesis
Clusterin (complement lysis inhibitor, apolipoprotein J)	22.3	Immunology
Decay accelerating factor (DAF)	18.9	Immunology
Osteopontin (secreted phosphoprotein 1)	8.9	Immunology
Mn^{+} superoxide dismutase 2 (SOD2)	8.5	Cell protection
Plasma protease (C1) inhibitor	7.3	Immunology
Complement component 3 (C3)	6.4	Immunology
Thrombomodulin	5.7	Signaling (coagulation)
Dickkopf (*Xenopus laevis*) homolog 1 (DKK1)	5.0	Signaling (wnt pathway)
Protein S	3.5	Signaling (coagulation)
Complement C1r-like protease precursor	2.8	Immunology
Interleukin-15	2.7	Immunology
Complement component C1r	2.2	Immunology
Complement component C1s	2.2	Immunology
Secreted frizzled-related protein 4 (SFRP4)	–4.3	Signaling (wnt pathway)
Cyclin B2	–8.6	Cell cycle

discussed, as this pathway has recently been described to be implicated in endometrial differentiation.

7.3 Detailed Analysis of Selected Pathways

7.3.1 The Complement Pathway

The complement system is part of the innate immune system and represents an efficient effector system. The complement uses a cascade of serum proteases to finally assemble a membrane attack complex that introduces pores into the membrane of pathogens such as bacteria, thus building the first-line defense system. This efficient system cannot only lyse pathogens, but can also attack the host cells. It therefore has to be tightly regulated and controlled. In our study, both positive and negative modulators of this cascade were regulated. Both C1r and C1s as part of the initiation step of the classical pathway were found to be slightly upregulated in the receptive phase endometrium. However, their main inhibitor, C1 inhibitor, was also strongly upregulated, indicating a strict control of this activation arm of the complement pathway. Another player in the complement system, C3, has already been shown to be expressed in the glandular epithelium of the endometrium and its expression is increased in the luteal phase of the endometrium (Hasty et al. 1994). Our study confirmed the upregulation of this activator of the complement. However, several inhibitors of the cascade were strongly upregulated. Decay-accelerating factor (DAF) was one of the most strongly upregulated genes in our study. Together with C4 binding protein (C4BP), which was also upregulated in the mid-secretory phase endometrium, it prevents the formation of the active C3 convertase both in the classical as well as in the alternative pathway, thereby effectively inhibiting the cascade. DAF expression has previously been localized to the luteal glandular epithelial cells of the human endometrium (Hasty et al. 1994). Additionally, clusterin (apolipoprotein J) was found to be upregulated in the receptive phase endometrium by us as well as by others (Riesewijk et al. 2003; Kao et al. 2002). Clusterin is a ubiquitous, multifunctional glycoprotein, which has also been implicated in complement regulation by blocking the

membrane attack complex (Rosenberg and Silkensen 1995; Calero et al. 2000). Altogether, the data show that several components of the complement system are expressed in the endometrium and are specifically upregulated in the implantation window. This tight control of effectors of the complement system suggests that beside its role in protection from pathogens, this system is also implicated in reproduction. The hypothesis that strict regulation of the complement system is essential for normal implantation and pregnancy is further supported by the recent finding that mice lacking Crry, a cell-surface protein which protects tissues against misdirected complement activation, lose their fetuses (Xu et al. 2000; Molina 2002). Furthermore, Pennesi et al. (1998) could show a statistically significant increase of a C4B variant allele in women with recurrent spontaneous abortions, indicating that an imbalance of complement factors and their activity might indeed have detrimental effects on implantation and pregnancy in humans. The finding that these factors are markedly regulated, together with the upregulation of other immune modulators such as interleukin-15 or β-defensin, another player in the innate immune response, are in line with this. More studies aimed at investigating the involvement of the immune system in receptivity and implantation are likely to yield interesting new data soon.

7.3.2 The Coagulation Cascade

The process considered to be the major control mechanism of coagulation is the protein C anticoagulant pathway. It involves thrombomodulin, a receptor localized at the surface of endothelial cells which binds to thrombin and thereby activates protein C. Activated protein C (APC), together with its cofactor protein S, degrades factors Va and VIIIa, thereby inhibiting the cascade (for a review see Norris 2003). Interestingly, thrombomodulin was found to be a strongly upregulated gene in the receptive phase endometrium not only in our, but also in other studies (Borthwick et al. 2003; Carson et al. 2002), indicating a strong inhibitory regulation of the coagulation cascade in the endometrium, especially during the receptive phase. This is further supported by the upregulation of protein S, a cofactor of APC. However, the upregulation of thrombomodulin

might also be indicative of the increased endothelial cell content of the mid-secretory as compared to the early secretory phase endometrium. Coagulation control in the endometrium is apparently not only important to avoid tissue ischemia but it might also play a role in early pregnancy and potentially in implantation. It has already been shown by Isermann et al. (2003) that the thrombomodulin-protein C system is essential for the maintenance of pregnancy, as thrombomodulin-deficient mouse embryos do not survive beyond 8.5 days postcoitum. The same group showed that one underlying mechanism is the induction of giant trophoblast cell death by fibrin and fibrin degradation products, consistent with the reported association between fibrin deposits and trophoblast apoptosis in pathological human pregnancies (Toki et al. 1999; Ratts et al. 2000).

Additionally, besides its anti-coagulatory effects, the thrombomodulin-protein C-protein S system has direct anti-apoptotic and anti-inflammatory effects. The molecular mechanisms are not yet completely understood and there seems to be a link between the coagulation cascade and the complement system via the interaction of C4BP and protein S (Rezende et al. 2004). Concerning the receptive endometrium, upregulation of thrombomodulin, together with protein S, might not only prevent coagulation, but may also produce an anti-inflammatory environment with a reduced fibrin concentration beneficial for embryo implantation. The regulation of the immune-modulatory environment in the receptive phase endometrium has been described above.

7.3.3 The Wnt Signaling Pathway

The Wnt genes encode a large family of highly conserved, secreted proteins that plays a key role in cell proliferation, epithelial–mesenchymal communication and embryogenesis (reviewed in Nusse and Varmus 1992; Wodarz and Nusse 1998; Cadigan 2002). Multiple pathways have been identified for Wnt protein signaling. The expression of several members, activators as well as inhibitors of this pathway, was regulated during the transition from pre-receptive to receptive phase endometrium. Our study revealed a significant upregulation of the pathway inhibitor dickkopf-1 (Dkk-1) which acts

by interacting with the low-density lipoprotein receptor-related protein Lrp, a cofactor of Frizzled. Interestingly, another inhibitor of the pathway, secreted frizzled-related protein 4 (SFRP4) was significantly downregulated in the receptive phase endometrium. SFRP4 blocks the pathway by directly interacting with Wnt, thereby antagonizing its action. Endometrial regulation of several members of the Wnt pathway has already been reported by other groups (Kao et al. 2002; Carson et al. 2002; Borthwick et al. 2003), based on expression profiling experiments. Tulac et al. (2003) experimentally confirmed the expression and regulation of Dkk-1 and SFRP4 in the human endometrium using PCR and in situ hybridization, and expression of these proteins has been localized to the stromal cells. We were furthermore able to detect Wnt7a, Wnt5a, Wnt4 and Wnt2 expression in human endometrium, although no significant regulation was found between the early and mid-secretory phases, in line with published literature (Tulac et al. 2003; Bui et al. 1997). Wnt7a is important for female reproduction, as documented by the fact that Wnt7a knockout mice display disturbed postnatal uterine development (Parr and McMahon 1998; Miller and Sassoon 1998). Wnt5a has recently been implicated in epithelial–mesenchymal interactions in the mouse uterus (Mericskay et al. 2004). There is also increasing evidence that the blastocyst expresses a specific set of Wnt genes (Mohamed et al. 2004), which further underscores the importance of this pathway in reproduction. Taken together, these data support the hypothesis that the Wnt pathway is an important regulator not only of development but also of embryo–maternal interactions and of implantation.

7.4 Conclusions

The recent progress made in array expression profiling and their bioinformatic analysis generated a wealth of data on gene expression patterns of numerous tissues and cells. Using this approach, several data sets comparing different human endometrial biopsies originating from healthy or diseased patients (Kao et al. 2002; Carson et al. 2002; Borthwick et al. 2003; Riesewijk et al. 2003; Dominguez et al. 2003; Absenger et al. 2004), as well as from uterine/endometrial

tissues from model organisms such as rodents, sheep or primates (Andrade et al. 2002; Kim et al. 2003; Tan et al. 2003; Qin et al. 2003; Allan et al. 2003) have been collected. These data all document a very complex network of signaling pathways and cascades that converge to build a unique endometrial environment for blastocyst arrival and implantation. The genes identified belong to various classes including immune modulation, adhesion molecules, cytoskeletal proteins, genes involved in oxidative stress, angiogenesis and coagulation, and many more. When analyzing and interpreting expression profiling data, one has to remember that such data provide only a static snapshot of the dynamic processes taking place in the endometrium. Also, since the information coming from an interacting embryo is lacking, no direct insight into the embryo–maternal communication can be gained. For model species such as the mouse, analysis of implantation and inter-implantation sites has already revealed the huge impact of blastocyst signaling on the endometrium (Reese et al. 2001). Using in vitro approaches, the dynamics of gene expression after in vitro decidualization of stromal cells was investigated, revealing a complex temporal regulation of genes during the process of decidualization (Popovici et al. 2000; Brar et al. 2001; Tierney et al. 2003). A confirmation of these gene expression data in endometrial biopsies is now needed for better interpretation.

The steroid hormones estrogen and progesterone are the key regulators of the changes in the endometrium. However, only a few of the genes we identified as upregulated during the implantation are known to be directly controlled by these hormones. Using in silico promoter analysis, Borthwick et al. (2003) searched for estrogen-response elements (ERE) and progesterone-response elements (PRE) present in regulatory regions of genes expressed in the endometrium in order to obtain hints on a potentially direct steroid regulation during the menstrual cycle. Many of the genes that showed increased expression in the mid-secretory phase also possess a number of potential response elements. For instance thrombomodulin has four candidate ERE and seven PRE, and is strongly induced in the mid-secretory, progesterone-dominated phase. The functionality of these steroid response elements has however not been proven yet. For instance, Young et al. (2002) recently published data indicating an indirect regulation of endometrial DAF expression by heparin-binding

epidermal growth factor-like growth factor, rather than directly by steroids, despite the fact that Borthwick et al. (2003) identified three candidate ERE and 16 PRE in the promoter region of this gene. Adding to the complexity are studies showing that progesterone-dependent regulation of target genes in the primate endometrium exhibits a temporal pattern (Okulicz and Ace 2003). A classical 'one-by-one' experimental approach is therefore needed to find out which of the regulated genes is controlled by female sexual hormones, directly or indirectly.

Gene expression profiling of human endometrial samples can give insight into the complex network of pathways, signals and processes preparing the endometrium for blastocyst implantation. Our study revealed a striking regulation of immunology-related genes, highlighting the unique environment in the receptive endometrium. Comparison of receptive phase endometrium in dependence of the secretory phase progesterone level also shows that a sufficient progesterone concentration is needed to get a unique pattern of gene expression in the endometrium. Understanding the molecular basis of a receptive endometrium and the underlying regulating mechanisms might also open the way for new contraceptive approaches as well as for the treatment of infertility.

Expression profiling data, together with studies of in vitro cultures of endometrial cells and trophoblast-endometrial co-cultures, as well as animal studies and transgenic mice, provide a wealth of data on endometrial physiology and pathophysiology. They represent the start point of many further studies aiming at evaluating the functional relevance of individual genes in this complex network.

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8 Role of EBAF/Lefty in Implantation and Uterine Bleeding

S. Tabibzadeh

8.1 Introduction

Human endometrium undergoes sequential morphological, biochemical and molecular changes in each menstrual cycle in preparation for implantation. This includes a period of proliferation (proliferative phase) followed by changes that collectively characterize a progesterone (P) dominant secretory phase. These changes are initiated immediately after ovulation and prepare the endometrium for implantation several days later.

In humans, the ovum is fertilized in outer third of the fallopian tube. Shortly after fertilization, the fertilized ovum starts to divide, migrates through the fallopian tube and enters the uterine cavity around day 3–4 after ovulation. The blastocyst remains free floating within the endometrial cavity for a day but it starts to implant on day 5–10 after ovulation (Hertig et al. 1956; Formigli et al. 1987; Rogers and Murphy 1989; Navot et al. 1989, 1991).

If implantation does not take place, the fall in the serum level of P in the late secretory phase leads to menstrual bleeding. Under these conditions, shedding of the endometrium and bleeding is a normal physiologic response. In some conditions, however, bleeding from endometrium is abnormal. In these situations, menstrual bleeding may increase in amount (menorrhagia), duration, or frequency. In other instances, the patient may experience intermenstrual spotting or breakthrough bleeding. The endometrial bleeding in these conditions is also associated with dissolution of endometrial extracellular matrix (ECM). In order to establish sound therapeutic approaches for treatment of abnormal uterine bleeding, infertility or abortion, a full insight of the molecular repertoire that participates in endometrial tissue remodeling is required.

The available data demonstrate that the robust and generalized tissue breakdown of endometrium during menstruation results from secretion and activation of a class of proteinases which digest the ECM constituents. It is becoming increasingly clear, however, that the same enzymes contribute to a discrete digestion of endometrial tissue which is essential to the blastocyst implantation. The activation of these enzymes appears to be driven by Lefty during implantation and tissue shedding.

8.2 Role of P and P Withdrawal in Implantation and Endometrial Bleeding

The molecular events that keep the secretion and activation of matrix metalloproteases (MMPs) in check and prevent endometrial tissue shedding during the 'implantation window' remained obscure until recently. The current operative hypothesis is that P greatly suppresses the expression of MMPs during the implantation window and that upon P withdrawal, these enzymes are secreted by endometrial cells and lead to tissue dissolution (Schatz et al. 1994; Irwin et al. 1996; Osteen et al. 1994; Bruner et al. 1995). Using endometrial explants, Marbaix et al. showed that breakdown of endometrial matrix upon sex steroid hormone withdrawal was completely and reversibly inhibited at all stages of the menstrual cycle by specific inhibitors of MMPs, but not by inhibitors of cysteine and serine proteinases (Marbaix et al. 1996). Others have provided more direct evidence that P inhibits MMP expression and that P withdrawal leads to secretion of MMPs. P inhibited activation of latent MMP-2 by membrane-type 1 MMP (Zhang et al. 1996) and P withdrawal increased MMP-2 in endometrium and led to the dissolution of ECM and dissociation of stromal cells (Irwin et al. 1996). Using endometrial explants, Osteen et al. showed that the expression of MMP-7 is under the control of P (Osteen et al. 1994). Consistent with these *in vitro* findings, the expression of endometrial MMP-7 was suppressed when the level of P was experimentally elevated in ovariectomized rhesus monkeys (Rudolph-Owen et al. 1998). It was subsequently reported that administration of anti-progestins to reproductively intact baboons increased endometrial MMP-7 during the mid-secretory phase (Cox et al. 2000). Once released and activated, MMPs degrade the ECM, which is comprised of collagens, fibronectin, gelatins, proteoglycans, elastin and basement membrane components. Collectively, these findings show that MMPs are crucial to endometrial tissue shedding and bleeding (Kokorine et al. 1996; Liotta 1996; Salamonsen and Woolley 1999; Marbaix et al. 2000).

The initial clue to what accounts for endometrial tissue shedding and bleeding was gleaned over half a century ago. By transplanting endometrial tissues into the anterior chamber of the eyes of rhesus monkeys, Markee showed that menstrual tissue shedding and bleed-

ing is initiated by steroid hormone withdrawal (Markee 1940). It was later realized that abnormal uterine bleeding also results from endogenous estrogenic activities unopposed by progesterone (P), progestogenic activities, and by exogenous administration of steroid hormones such as that occurring during the use of contraceptives made of female steroid hormones (Bayer and DeCherney 1993; Fraser 1990; Biswas et al. 1996; Said et al. 1996; Coll Capdevila 1997; Affandi 1998; Benagiano et al. 2000). Markee hypothesized that the endometrial tissue shedding and bleeding induced by steroid hormones is controlled by local factors. This idea has received significant support from various basic research and clinical studies. The identity of some of the local factors that participate in these processes has been unraveled during the past several years and appears to include a molecular repertoire comprised of cytokines, namely, transforming growth factor beta (TGF-β), EBAF/Lefty, as well as MMPs and their inhibitors, TIMPs.

8.3 Role of MMPs in Implantation and Endometrial Bleeding

8.3.1 Role of MMPs in Implantation

In mammals, blastocysts invade the endometrial tissue in a manner similar to invasion of tissues by tumors. It is becoming increasingly clear that MMPs are primary contributors of the tissue invasion both by blastocysts and tumors. These enzymes, by virtue of inducing a localized dissolution of endometrial tissue, facilitate the implantation of blastocyst. The ECM in endometrium is comprised of an argyrophilic network of so-called reticular fibers and collagen (Woessner 1991; Stenback 1989; Iwahashi et al. 1996). This network undergoes remodeling in response to decidualization and implantation. The process of decidualization is associated with a vanishing collagen type I, III, V, and VI content in the pregnant rat uteri (Mullholland et al. 1992; Hurst et al. 1994). Although, the dissolution of ECM in decidualized stroma is independent of an embryo (Mullholland et al. 1992), the collagen concentration is reduced more around the implanting blastocyst as compared to the nonimplanting sites (Hurst et

al. 1994). Collagen type I is virtually absent around the rat embryos on day 5 of pregnancy but re-appears on day 8 around the developing placenta (Clark et al. 1996). These modifications of tissue occur in a background of significant alterations in protein synthesis and secretion which are associated with a positive protamine blue reaction at the site of embryo implantation (Weitlauf and Suda-Hartman 1988). Thus, the implantation requires both modification of synthesis as well as degradation of proteins. The expression of MMPs also shows distinct changes throughout pregnancy in ovine, mouse, rat and human endometrium (Salamonsen et al. 1995; Alexander et al. 1999; Hurst and Palmay 1999; Polette et al. 1994). MMP-1 immunoreactivity can be detected locally in close proximity of implanting embryo (Hurst and Palmay 1999). The mRNA encoding MMP-9 is detected in uteri which are undergoing oil-induced decidualization and immunoreactivity for MMP-9 and MMP-2 is detectable during early pregnancy and in oil induced decidualized endometrium (Bany et al. 2000). Injection of peptide hydroxamate, which is MMP inhibitor, retards the decidual development. Similarly, the development of decidua is inhibited in transgenic mice that overexpress TIMP-1 (Alexander et al. 1996). Besides the secretion of MMPs by endometrium, the invasion of blastocyst is further enhanced by the release of the same enzymes by trophoblasts which is stringently controlled in terms of time and space by TIMPs (Bischof et al. 2000). When placed in culture, mouse blastocysts adhere to ECM, and trophoblast giant cells invade and degrade ECM (Behrendtsen et al. 1992). Collectively, these findings show that the dual release of MMPs by the endometrium and the embryo jointly drives the implantation process.

8.3.2 Role of MMPs in Endometrial Bleeding

Although a localized expression of MMPs is essential to implantation, it appears that aberrant, dysregulated or superfluous expression of MMPs around the time of implantation by endometrium or blastocyst would be an undesirable event. A generalized activation of these enzymes in human endometrium can lead to menstruation or abortion causing significant tissue shedding, so that by the end of the process, most of functionalis is lost. These are predictable out-

comes if implantation fails to proceed normally or does not occur at all. For these reasons, to insure that the endometrial tissue breakdown does not occur unnecessarily or excessively, the function of MMPs such as MMP-3, MMP-7 and MMP-9 is controlled at two levels. First, their expression and secretion is controlled by local factors such as Lefty as detailed below. Additionally, once secreted, the action of MMPs is controlled by a group of inhibitors, the so-called TIMPs. Transcripts of these TIMPs (TIMP-1, TIMP-2, TIMP-3) can be detected throughout the pre- and peri-implantation development in the mouse uteri (Harvey et al. 1995). Careful analysis has shown that these inhibitors have a distinct temporal pattern of expression. For example, inhibitory activity is highest on day 1 of pregnancy in mouse endometrium and this activity progressively decreases so that minimal levels are detectable on day 12 (Nothnick et al. 1995). Most of such alterations are attributable to the changes in the expression of TIMP-1 while TIMP-2 expression levels do not change but TIMP-3 expression progressively increases during the same time period (Nothnick et al. 1995).

8.4 Endometrial Bleeding Is Associated with Dissolution of ECM by MMPs

If implantation does not take place or if it proceeds abnormally, endometrium is shed. This tissue shedding and associated bleeding occurs in the context of normal menstruation or proceeds during abortive processes. Abnormal uterine bleeding is also observed during the reproductive years as well as around the time of menarche or menopause (Bayer and DeCherney 1993; Biswas et al. 1996; Affandi 1998; Benagiano et al. 2000; Wathen et al. 1995; Hillard et al. 1992; Archer et al. 1994; Ober 1977). Menstrual breakdown of endometrium has been traditionally viewed to result from vasospasm of endometrial spiral arteries followed by tissue ischemia and lysis (Fraser 1990). However, the molecular events that underlie this tissue breakdown remained obscure until recently. Human endometrium contains an argyrophilic network of so-called reticular fibers which contain both type I and type III collagen (Woessner 1991; Stenback 1989; Iwahashi et al. 1996). When the serum concentration of progesterone falls at the end of the men-

strual cycle, this interstitial fibrillar meshwork undergoes focal breakdown and is extensively lysed at menstruation. Basement membranes around glands and blood vessels also undergo fragmentation and disruption. Breakdown and collapse of this framework leads to shrinkage of the endometrium, piecemeal loss of the functionalis layer, and subsequent bleeding (Woessner 1991). Regression of endometrium also occurs in mammals that do not menstruate. For example, in cycling rats, the wet weight and the collagen content of endometrium decreases during metestrus to 20% of their proestrus values (Yochim and Blahna 1976). These findings indicate that proteolysis is an essential feature of the endometrial cycle (Eeckhout 1990). The involvement of lysosomal enzymes in proteolytic events at menstruation was first proposed in late 1960s and early 1970s (Wood et al. 1969; Henzl et al. 1972). However, more recent studies have failed to provide conclusive evidence in support of this hypothesis (Cornelli 1990). A significant body of work shows that MMPs that degrade collagen, fibronectin, gelatin, elastin as well as the constituents of basement membrane including laminin, participate in digestion and breakdown of the endometrial matrix during menses and abnormal uterine bleeding (Salamonsen 1994; Tabibzadeh and Babaknia 1995). Various lines of evidence form the basis of this belief. These include: (1) spatio-temporal expression of MMPs during normal menstrual and abnormal uterine bleeding; (2) their secretion from endometrial cells in culture; and (3) the evidence that MMPs degrade endometrial extracellular matrix.

The first key evidence for the role of MMPs in endometrial tissue shedding is their spatio-temporal expression during menstruation. Virtually all known MMPs are expressed in human endometrium during menstrual bleeding (Salamonsen 1994; Tabibzadeh and Babaknia 1995), and expression of some endometrial MMPs such as MMP-1, MMP-2, MMP-3, MMP-7, MMP-9 and MMP-10 is enhanced around the time of menstrual bleeding (Rodgers 1994; Irwin et al. 1996; Kokorine et al. 1996). While the expression of some potent stromelysins such as MMP-7 is maintained at a low level in human endometrium during early and mid-secretory phases, its expression is significantly increased during late secretory and menstrual phases (Rodgers et al. 1993). In a related species with menstrual cycle such as baboons on artificial cycles, MMP-3 and MMP-7 is also expressed during menses (Cox et al. 2000). These studies show that

the expression of MMPs in human endometrium is exquisitely controlled during the menstrual cycle and that the expression of key MMPs is increased during menses. Moreover, MMPs are also highly expressed in the endometria of patients who experience steroid hormone-induced bleeding (Vincent and Salamonsen 2000; Vincent et al. 2000; Skinner et al. 1999). In women using progestin-only contraceptives, endometrial MMP-1 and MMP-3 were found to be highly expressed to the extent present in the endometria of menstrual phase controls (Vincent et al. 2000). In patients using subdermally implanted slow-release levonorgestrel (Norplant), positive MMP-9 immunostaining was observed extracellularly in areas of tissue lysis and in the surrounding stromal and intravascular leukocytes (Vincent et al. 2000). The number of MMP-9 positive cells was significantly increased in the endometrial biopsies of normal control subjects at menstruation and endometria of Norplant users which displayed a shedding morphology (Vincent and Salamonsen 2000). The close temporal and spatial correlation between the expression of MMPs and endometrial tissue shedding provides supportive evidence for the role of MMPs in tissue breakdown at menstruation. Once released and activated following P withdrawal, MMPs degrade the extracellular matrix and for this reason, it is believed that MMPs are crucial to endometrial tissue shedding and bleeding (Marbaix et al. 1996, 2000; Kokorine et al. 1996; Liotta 1996; Salamonsen and Woolley 1999).

Besides the endometrial extracellular matrix, a second target which participates in endometrial bleeding is the superficial endometrial arteries. Ultrastructural analysis have shown that the endometrial vessels undergo significant changes during the menstrual cycle (Roberts et al. 1992). In the early proliferative phase, the basal lamina of endometrial capillaries is loosely formed and discontinuous. In the late proliferative phase, the basal lamina becomes more distinct and in the early to mid-secretory phase and whorled extensions that include pericytes develop. In the late secretory phase, the endometrial stroma and the basal lamina undergo widespread degeneration, and the cell-to-cell contacts sharply decrease. Collectively, these events make vessels fragile and susceptible to bleeding. Essentially, similar changes occur in the endometria of patients who use progesterone-containing contraceptives such as Norplant. The vessels in

these patients appear to be extremely fragile and bleed easily during hysteroscopic examination (Hickey et al. 1999, 2000). Petechiae and ecchymoses are frequently observed in the endometria of these patients (Hickey et al. 2000). In the initial months of exposure to Norplant, when bleeding problems are most common (Hickey et al. 1999), the amount of basement membrane components such as laminin, collagen type IV, and heparan sulfate proteoglycan is reduced in the endometrial vessels (Hickey et al. 1999; Palmer et al. 1996). The degradation of the extracellular matrix components in these patients appears to be due to higher MMP activity (Vincent et al. 2000). Collagenase-1 mRNA, focal stromal breakdown, and lysis of collagen fibers are evidenced in most bleeding endometria, but never in the nonbleeding ones. In the areas showing breakdown, immunolabeling for gelatinase A is strongly increased (Galant et al. 2000). The number of perivascular mural cells which contain smooth muscle actin also decreases in patients using progestin-only contraceptives further leading to the vessel fragility (Rogers et al. 2000). The underlying basis of such changes appears to be a state of P withdrawal not necessarily due to reduced progesterone but presumably due to reduction of both progesterone receptor A and progesterone receptor B isoforms (Rogers et al. 2000; Lockwood et al. 2000).

8.5 Role of TGF-β in Implantation and Endometrial Bleeding

The current operative hypothesis is that P controls the expression of MMPs through local endometrial cytokines (Osteen et al. 1994, 1999; Bruner et al. 1995; Keller et al. 2000). Keller et al. recently showed that progesterone inhibits the interleukin-1 mediated stimulation of MMP-3 (Keller et al. 2000) and Bruner et al. identified TGF-β as the principal mediator of MMP-7 (matrilysin) suppression by progesterone (Bruner et al. 1995). During the secretory phase, endometrial stromal cells were found to be the source of this TGF-β (Bruner et al. 1995). On days 1–4 of pregnancy, a major portion of this TGF-β resides in the epithelium but the decidual cells and ECM also remain a substantial source of TGF-β in the mouse and rat endometria (Tamada et al. 1990; Das et al. 1992; Chen et al. 1993).

Likewise, in pregnant mares, TGF-β mRNA is found in the epithelial cells (Lennard et al. 1995) and in humans, TGF-β is found in the stromal and decidual cells and within ECM (Bruner et al. 1995; Lysiak et al. 1995; Ando et al. 1998). Taken together, these findings show that TGF-β supports implantation by regulating the expression of MMPs. Since TGF-β knockout was embryolethal, Das et al. studied the effect of the TGF-β by downregulating its receptors in the uteri of TGF-α transgenic mice (Das et al. 1997). This downregulation delayed the blastocyst attachment reaction and delayed parturition. These findings support the view that TGF-β plays important roles in homeostasis of ECM of endometrium and in implantation.

TGF-β is one of the leading cytokines that regulates the homeostasis of ECM. TGF-β controls the tissue integrity by two complementary mechanisms, one involving upregulation of collagen mRNA, and the other by inhibition of activity of MMPs (Massague 1990; Slack et al. 1993). TGF-β increases the ECM accumulation by signaling along the Smad pathway and by inducing connective tissue growth factor (CTGF). CTGF, a heparin binding 38-kDa cysteine-rich peptide, is considered to be an immediate early growth responsive gene and a downstream mediator of TGF-β actions in fibroblasts (Grotendorst 1997). CTGF induces collagen synthesis, in a number of *in vitro* and *in vivo* models (Paradis et al. 1999; Duncan et al. 1999; Moussad and Brigstock 2000). CTGF is present in epithelial cells of human endometrium throughout the entire menstrual cycle but its expression in the stroma coincides with the duration of the implantation window (Uzumcu 2000). Thus, expression of CTGF in human endometrium appears to be part of the molecular pathways involved in TGF-β actions and required for the implantation (Querfeld et al. 2000; Wenger et al. 1999; Kubo et al. 1998; Frazier and Grotendorst 1997). These findings have provided clues as how the fate of ECM in endometrium during the critical period of implantation needs to be controlled both by regulation of expression of collagen and the key MMPs. The last but critical final event in the intricate circuit that regulates the homeostasis of tissue matrix might rely on control of TGF-β expression and/or function. We have identified EBAF/Lefty, a novel member of the TGF-β superfamily, that suppresses TGF-β activity by inhibiting the phosphorylation of Smad2 (Kothapalli et al. 1997; Tabibzadeh and Satyaswaroop 1998;

Tabibzadeh et al. 2000; Ulloa et al. 2001; Ulloa and Tabibzadeh 2001). Based on this important evidence coupled with other data reviewed below which show that Lefty inhibits both CTGF and collagen mRNA synthesis and enhances secretion of collagenase, we have formulated the following hypothesis: Lefty is the key endometrial factor that regulates the breakdown of ECM both by inhibition of collagen synthesis and enhancement of its degradation by MMPs. These actions of Lefty are mediated by inhibition of TGF-β through the Smad signaling pathway.

8.6 Lefty Is an Endometrial Bleeding Associated Factor

Abnormal uterine bleeding is one of the most common disorders that occurs in women throughout the span of their reproductive years. During this time period, nearly 20% of women experience menorrhagia (Bayer and DeCherney 1993; Jacobs and Butler 1965; Hallberg et al. 1966) and virtually every woman, at some point in her lifetime, experiences episodes of abnormal uterine bleeding (Jacobs and Butler 1965). Extensive bleeding from endometrium accounts for 70% of the hysterectomies performed annually (Stirrat 1999). One of the more effective contraceptives is made of progestins. A high incidence of irregular uterine bleeding is the primary patient complaint that has limited the long-term use of progestin-only contraceptive agents (Biswas 1996; Affandi 1998; Benagiano et al. 2000; Alvarez-Sanchez et al. 1996). This is the major reason for discontinuation of use and acceptability of these common contraceptives (Rosenberg et al. 1996; Rees and Turnball 1989; Barnhart et al. 1997). Abnormal uterine bleeding is a major medical problem not only for women but also for their families and health care services (Coll Capdevila 1997). In spite of the widely appreciated magnitude of the problem, and the understanding that endometrial bleeding is directly related to the effect of steroid hormones on endometrium, it is not clear that when progesterone level falls what local mediator(s) triggers endometrial tissue shedding and bleeding.

We argued that factors that participate in endometrial bleeding should be maximally expressed in human endometrium around the time of menstruation. Moreover, we reasoned that the endometrial

factor(s) that is responsible for conferring a state of nonresponsiveness to endometrium and induces MMPs should be detectable at a high level in late secretory and menstrual phase endometrial tissues. To identify such an endometrial bleeding associated factor, we carried out differential display on RNA of endometrial tissues from all phases of the menstrual cycle of normal healthy menstruating women. The amplified products of differential display were resolved in sequencing gels. Amongst hundreds of amplified bands examined, the intensity of one band representing a novel human gene was found to be most intense during late secretory and menstrual phases. For this reason, we named this factor EBAF (endometrial bleeding associated factor) (Kothapalli et al. 1997). The band was cloned and sequenced. A GenBank search identified a mouse gene that showed a significant degree of homology to the human gene. In view of its asymmetric expression in the left side of the mouse embryo, the mouse gene was designated Lefty (Lefty-1) (Meno et al. 1996). EBAF is also named Lefty-A by Kosaki et al. who showed mutation in the gene in individuals with L–R axis malformation (Kosaki et al. 1999). For simplicity, we will refer to EBAF as Lefty. We argued that if Lefty is involved in endometrial tissue breakdown, it should be maximally expressed in human endometrium around the time of menstruation. Northern blot analysis of endometrial tissues of various phases of menstrual cycle gave credence to this line of reasoning (Kothapalli et al. 1997). In situ hybridization was used to identify the cells that express Lefty in human endometrium. Lefty was expressed both in the endometrial stromal fibroblasts and epithelial cells (Kothapalli et al. 1997; Tabibzadeh and Satyaswaroop 1988; Tabibzadeh et al. 1997). Careful examination of expression of Lefty in endometria of women with abnormal uterine bleeding showed that Lefty was also highly expressed in these endometria (Kothapalli et al. 1997). Taken together, these data provide support for the hypothesis that Lefty is an endometrial bleeding associated factor.

8.7 Lefty Expression Is Negatively Controlled by Steroid Hormones

We reasoned that demonstration of negative regulation of Lefty expression by steroid hormones would be supporting evidence of Lefty being the cause of endometrial bleeding. In other words, steroid hormones are negative regulators of Lefty and when the negative regulation is removed, Lefty induces endometrial bleeding. We showed that the expression of Lefty mRNA is negatively regulated by steroid hormones including P alone (Cornet et al. 2002). We quantified the concentration of Lefty mRNA during menstrual cycle *in vivo* and in organ cultures of endometrium. In noncultured endometria, Lefty mRNA concentration was dramatically (100-fold) increased in tissues which exhibited signs of matrix breakdown. A similar increase was seen in proliferative endometria, cultured for 24 h in the absence of ovarian steroids. This increase was inhibited by the addition of progesterone, alone or in combination with estradiol. In this model system, increase in Lefty mRNA preceded the release of MMPs. These findings show that Lefty expression is negatively regulated by P.

8.8 Lefty Is a Cytokine

We identified Lefty protein in the endometrial tissue, endometrial fluid, serum and urine of women around the time of menstrual bleeding. Three protein bands were immunoreactive with an affinity purified rabbit polyclonal antibodies generated against a peptide at the C terminus of Lefty protein (Tabibzadeh et al. 2000; Ulloa et al. 2001). To gain additional insight into the identity of these bands, we characterized the mode of processing and post-translational modification of Lefty proteins. Presence of a signal peptide suggested that Lefty might be a secreted protein (Kothapalli et al. 1997). Two potential cleavage sites exist within the Lefty precursor. Transfection of 293, BALB/3T3 and other mammalian cells with Lefty showed synthesis and release of proteins bands of 42 kDa, 34 kDa and 28 kDa into the culture media of transfected cells (Ulloa et al. 2001). Transduction of GP+E86 fibroblasts with retroviral vectors

transducing Lefty also showed presence of the same bands in the culture medium (Mason et al. 2002). Thus, it seems that the processing of Lefty does not depend on the cell type that expresses the protein. Using transfection and a number of mutations introduced into Lefty, we showed that Lefty is synthesized as a 42-kDa precursor and is cleaved at Lys^{77} and Arg^{135} to produce 34 kDa and 28 kDa mature secreted proteins (Ulloa et al. 2001).

The deduced amino acid sequence of Lefty showed a great amount of identity and similarity with the known members of the TGF-β superfamily. A motif search revealed that the predicted Lefty protein contains most of the conserved cysteine residues of the TGF-β related proteins (Kothapalli et al. 1997) which are necessary for the formation of the cysteine knot structure (Kingsley 1994; Daopin et al. 1992). The Lefty sequence contains an additional cysteine residue, 12 amino acids upstream from the first conserved cysteine residue. The only other family members, known to contain an additional cysteine residue, are TGF-β, inhibins and GDF-3 (McDonald and Hendrickson 1993). Lefty, similar to GDF-3/Vgr2 and GDF-9, lacks the cysteine residue necessary for the formation of an intermolecular disulfide bond (McDonald and Hendrickson 1993; McPherron and Lee 1993). Whereas the C-terminus of the TGF-β family is usually CX1CX1, Lefty has a longer C-terminal sequence, CX1CX19 (Kothapalli et al. 1997). Therefore, Lefty appears to be an additional member of the TGF-β superfamily with an unpaired cysteine residue which may not exist as a dimer.

8.9 Lefty Is an Inhibitor of TGF-β Signaling

Lefty and its related proteins are poised to act as inhibitors of TGF-β family members (Meno et al. 1996, 1997, 1998; Thisse and Thisse 1999; Schier and Shen 2000). Thus, we considered that Lefty might cause extracellular matrix remodeling by inhibiting TGF-β actions. A number of carefully executed studies showed the validity of this hypothesis (Ulloa and Tabibzadeh 2001). Lefty perturbs the TGF-β signaling by inhibiting the phosphorylation of Smad2 following activation of the TGF-β receptor (Ulloa and Tabibzadeh 2001). Moreover, Lefty inhibits the events which lie downstream from R-Smad

phosphorylation including heterodimerization of R-Smads with Smad4 and nuclear translocation of R-Smad–Smad4 complex. Lefty opposes the effect of TGF-β on the expression of reporter genes for major cell cycle factors p21 and Cdc25. Smad3 and Smad4, both have domains that bind the 5′-TCTGAGAC-3′ termed Smad binding element (SBE). Lefty inhibits the TGF-β induced promoter activity driven by SBE or promoter activity of a constitutively active TGF-β RI (Ulloa and Tabibzadeh 2001). Moreover, it has been recently shown that the expression of CTGF that induces proliferation of fibroblasts and collagen synthesis is mediated by Smad3 and Smad4 (Holmes 2001). Lefty is also capable of inhibiting the promoter activity of CTGF mediated by TGF-β (Ulloa and Tabibzadeh 2001). Thus, Lefty provides a repressed state of TGF-β responsive genes and participates in negative modulation of TGF-β signaling by inhibition of phosphorylation of R-Smads (Ulloa and Tabibzadeh 2001).

8.10 Lefty Inhibits Deposition of Collagen *In Vivo*

TGF-β is a profibrogenic cytokine that induces collagen synthesis and suppresses MMPs. Since Lefty inhibits TGF-β signaling, we entertained the possibility that Lefty might oppose the TGF-β actions *in vivo* and lead to the loss of ECM. Using *in situ* hybridization, we showed that Lefty is markedly expressed in endometrial stromal fibroblasts around the time of menstrual bleeding (Kothapalli et al. 1997). To simulate this *in vivo* expression for characterizing the actions of Lefty, we constructed two retroviral vectors, a control vector (LG) enabling cells to express green fluorescent protein (GFP) and the vector, LEIG, that transduces the expression of both GFP and Lefty-A (Mason et al. 2001). The success of these transduction experiments was assessed by analysis of GFP fluorescence, immunostaining and demonstrating that Lefty is secreted by the LEIG transduced and not LG transduced cells (Mason et al. 2001). These cells were then subcutaneously introduced into athymic mice and the amount of collagen deposited in the stroma of the tumors developed from the fibroblasts was quantified. As expected, the LG tumors contained abundant collagen fibers. In contrast, in the LEIG tumors there was little intervening stroma containing collagen. To further

validate that the extracellular matrix observed in these tumors was collagen, sections were stained with trichrome, which in view of its affinity casts a blue color onto collagen fibers. While collagen fibers were detected in large amounts both at the center and at the periphery of the LG tumors, the LEIG tumors exhibited a paucity of these fibers. These findings show that Lefty suppresses collagen synthesis and prevents collagen deposition.

8.11 Lefty Inhibits Collagen and CTGF mRNA Expression *In Vivo*

The RNA from LG and LEIG transduced cultured cells and tumors derived from them were subjected to reverse transcription (RT) followed by polymerase chain reaction (PCR) for mouse collagen type I (Mason et al. 2002). Although the collagen type I mRNA was detected in both LG and LEIG transduced cells *in vitro*, the LG tumors had more collagen mRNA that found in the LEIG tumors (Mason et al. 2001). We attribute lack of effect of Lefty *in vitro* to the absence of any stimulating signal such as TGF-β that Lefty is able to inhibit. The inhibitory actions of Lefty can be observed in the presence of a stimulating signal such as TGF-β which is ubiquitously expressed *in vivo*. Persuaded by these observations, we then carried out real-time quantitative RT-PCR to determine the amount of CTGF and collagen type I mRNA in the same tissues. These studies confirmed the results of RT-PCR and showed a 4.7-fold reduction in the expression of collagen type I mRNA in the tumors derived from Lefty$^+$ cells. Moreover, there was 2.8-fold reduction of CTGF mRNA expression in the same tumors as compared with the control tumors. These results are consistent with the histologic data on reduced deposition of collagen in tissue sections of Lefty$^+$ tumors and show that such reduction is the result of reduced CTGF and collagen mRNA transcription. Collectively, the available data support the hypothesis that Lefty inhibits collagen deposition *in vivo*. The findings show that Lefty, by acting on the CTGF promoter, inhibits expression of CTGF and consequently reduces the deposition of collagen by a mechanism which includes transcriptional control of collagen type I mRNA expression.

8.12 Lefty Induces Collagenolytic and Elastolytic Activities *In Vivo*

A critical step in building the hypothesis that Lefty is involved in tissue dissolution would be induction of collagenolytic activity by Lefty. Proteolytic activity was not significantly different in the tissue culture media of LG and LEIG transduced cells. However, *in vivo*, there was a significant increase in the proteolytic activities in the LEIG transduced tumors. This difference was noted using collagen, gelatin or elastin as substrates (Mason et al. 2001). At least five different species of enzyme, induced by Lefty causing collagenolysis and caseinolysis, were seen by zymography. These findings show that Lefty actively participates in the dissolution of ECM by inducing collagenolysis and elastolysis.

8.13 Induction of MMPs by Lefty Is Suppressible by Steroid Hormones

The above findings provide the evidence that Lefty causes the breakdown of the ECM components by induction of collagenolytic, gelatinolytic and elastolytic activities. Since such activities are inducible by MMP-3 and MMP-7, two major enzymes that degrade a wide variety of ECM components such as laminin, fibronectin, gelatin, proteoglycan, procollagenase and collagen type IV, V, IX, and X (reviewed in Tabibzadeh and Babaknia 1995), we measured the ability of Lefty to induce these enzymes in endometrial explants. Lefty induced significant levels of both MMP-3 and MMP-7 in the explants (Cornet et al. 2002). These findings suggest that Lefty causes degradation of ECM by induction of MMPs. The extent that steroid hormones counteract the MMP inducing effect of Lefty was also studied. Endometrial explant cultures were treated with Lefty in the absence and presence of steroid hormones and the amount of MMP-3 and MMP-7 in the culture media was analyzed by Western blotting. The experiments showed that steroid hormones significantly downregulate the amount of MMPs induced by Lefty (Cornet et al. 2002).

8.14 Lefty Is Overexpressed in Endometria of Patients with Abnormal Uterine Bleeding and Some Forms of Infertility

Using Northern blot analysis, we have demonstrated that during normal menstrual cycles Lefty is highly expressed in human endometrium around the time of menstruation and during menstrual bleeding (Kothapalli et al. 1997; Tabibzadeh and Satyaswaroop 1988). Moreover, we showed that Lefty is also highly expressed in the endometria of women with abnormal uterine bleeding. These data suggest that Lefty is involved in tissue shedding and bleeding (Kothapalli et al. 1997; Tabibzadeh and Satyaswaroop 1988). Additional evidence suggest that Lefty might also be implicated in infertility (Tabibzadeh et al. 1997),

Infertility is a common clinical problem. As shown in the classic Guttmacher's table, about 7% of couples can be considered infertile after they have tried for 2 years to attain pregnancy (Guttmacher 1956). In the US, in 1982, nearly one in five married women of reproductive age reported that during their lifetime they had sought professional help for infertility (Mosher and Pratt 1991) and in 1988, 8.4% (a total of 4.9 million) of women, ages 15–44, had impaired fertility (Mosher and Pratt 1993). After all the standard clinical investigations are done and known causes of infertility attributable to other identifiable pathologies are ruled out, a substantial number (10%) of infertility cases remain of unknown etiology ('unexplained infertility') (Gurgan 1995).

In normal endometrium a state of nonresponsiveness is attained after day 10 postovulation shortly before menstruation begins. Nearly 50% of women who become pregnant after day 10 postovulation lose their embryos (Baird et al. 1991). The possibility exists that in some infertile women, the endometrium attains this state of nonresponsiveness earlier as compared with normal fertile women, causing a state of infertility. A second possible scenario is that infertile women might become pregnant but might lose the blastocyst by abortion. This is also a likely possibility since about 30% of normal women experience a subclinical pregnancy which is ended with abortion (Wilcox et al. 1985; Chard 1991). An accentuation of this event could potentially be the cause of infertility in some patients

(Bulletti et al. 1996). Thus, it appears that there is a close relationship between the attainment of a nonreceptive state in endometrium in the secretory phase and a subsequent menstruation or loss of embryo by abortion (Barbieri 1996; Olive and Haney 1986; Arumugam and Lim 1997; Vercellini 1997).

There are conditions which are associated with lesions in the molecular repertoire that drives the secretory endometrium. For example, during an anovulatory cycle, the production of the systemic steroid hormones is aberrant. In luteal phase defects, this aberrancy is subtle and leads to a lag in the maturation of endometrium. The means by which the disease processes such as endometriosis or pathologies within the fallopian tube lead to infertility are not well understood. However, in women with infertility, the treatment of these processes increases the chance of conception, suggesting the endometrium as the target organ for the effects of these diseases. In some instances, the underlying basis for the infertility remains unclear (unexplained infertility). From the members of the molecular repertoire of the 'endometrial receptivity' period, with the exception of $\alpha_v\beta_3$ integrin (Lessey et al. 1992), no other gene has been described thus far whose aberrant expression is associated with or results in infertility. It has been suggested that the expression of the immunoreactivity for $\alpha_v\beta_1$ and $\alpha_v\beta_3$ integrins coincides with the implantation window (Lessey et al. 1994). Immunostaining for αv increased throughout the menstrual cycle, while the β subunit appeared abruptly on cycle day 20 on luminal and glandular epithelial cells (Lessey et al. 1992). Discordant luteal phase biopsies (β_3 days out of phase) from infertile patients exhibited delayed epithelial beta$_3$ integrin immunostaining (Biswas et al. 1996). Later, the abnormal beta$_3$ immunostaining was also found in infertility associated with tubal factor (Dahlen et al. 1998) and unexplained infertility (Ye et al. 1989).

We reasoned that because Lefty was highly expressed at the time that endometrium is nonreceptive, Lefty overexpression might be a molecular lesion in the endometria of patients with infertility. In normal fertile subjects, Lefty proteins were present at a low level in endometrium during the implantation window, and high levels could be found only around the time of menstruation (Kothapalli et al. 1997; Tabibzadeh and Satyaswaroop 1988; Tabibzadeh et al. 2000; Cornet et al. 2002). Consistent with this hypothesis, Northern blot analysis

showed that the expression of the Lefty mRNA was upregulated in the endometria of infertile patients during the implantation window. In more than 50% (14/26) of endometria from infertile patients, Lefty mRNA was upregulated during the 'endometrial receptivity period'. The infertility in these women was associated with endometriosis, polycystic ovary, bilateral tube occlusion, anovulatory cycle, luteal phase defect, premature ovarian failure and habitual abortion. In some women, the underlying basis of infertility remained unknown (unexplained infertility). Therefore, the dysregulated expression of the Lefty mRNA in endometrium seems to be a common event in diverse types of infertility. An additional, smaller Lefty mRNA was also detectable in endometria of some of infertile women. Consistent with these findings, the production/secretion of the endometrial Lefty was found to be perturbed in the endometria of infertile women. As compared to the amounts present in the endometria of normal fertile women, there was relatively more Lefty protein in the endometria of infertile women during the implantation window. Such dysregulated expression of Lefty by the endometrium could be reversed by an appropriate treatment strategy. When such expression was optimally reversed four out of four patients became pregnant. However, both women in whom the treatment failed to adequately suppress the dysregulated expression of Lefty by the endometrium, failed to become pregnant. Therefore, dysregulated expression of Lefty mRNA and protein in infertility patients during the endometrial receptivity period may result in premature closure of the implantation window and may account for their inability to allow the embryo to implant.

8.15 Lefty Overexpression Leads to Implantation Failure

The production and/or release of Lefty protein in human endometrium and serum was dependent on the phase of the menstrual cycle. The amount of the protein was lowest during the implantation window. Based on these findings, we speculated that successful implantation may occur in presence of a low level of Lefty protein in human endometrium and that implantation cannot take place shortly before menstrual bleeding when Lefty expression is high. Because Lefty causes collagenolysis, we thought that Lefty overexpression might be incon-

sistent with embryo implantation. For this reason, we injected the LG and LEIG retroviral particles into the endometrial cavity of mice on day 1 of pregnancy. Nine days later, there was no viable embryo in the uterine cavities of animals injected with LEIG retroviral particles whereas the uterine cavities of control mice and mice injected with LG retroviral particles showed the expected number of viable embryos (unpublished results). *In vivo* transfection of uterine horns of pregnant mice with pcDNA 3.1 Lefty expression vector also led to a reduced number of embryos (unpublished results). These findings show that Lefty overexpression is inconsistent with implantation and that Lefty overexpression can lead to infertility.

8.16 Conclusions

The body of the work reviewed here shows that many different conditions including implantation, abortion, menstruation and abnormal uterine bleeding, are all associated with remodeling of endometrial

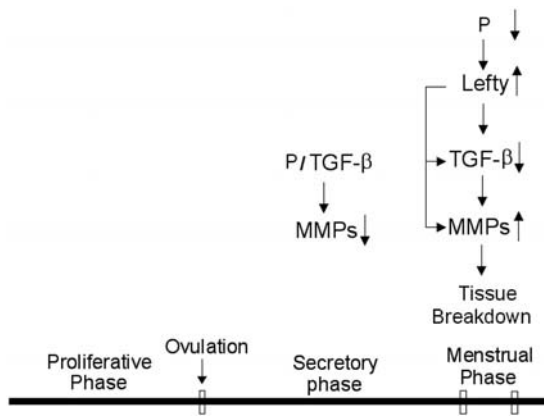

Fig. 1. A model for regulation of MMPs and tissue shedding and bleeding in endometrium. During the secretory phase, progesterone (*P*) and TGF-β suppress expression of MMPs. Controlled expression of MMPs drive localized tissue remodeling during implantation. P withdrawal during the late secretory phase or in bleeding conditions leads to upregulated expression of endometrial Lefty which leads to menstrual shedding and bleeding. (Adapted from Tabibzadeh 2002, with permission)

ECM. This remodeling, by and large, is due to either discrete or disseminated secretion and activation of MMPs in human endometrium. This activation, which might be due to P withdrawal or steroid hormone treatment, targets the ECM by virtue of participation of a major signaling molecule, EBAF/Lefty. The actions of this protein appears to be partly mediated by controlling the expression and activation of MMPs which are involved in endometrial tissue integrity. Excessive Lefty and subsequent release and secretion of MMPs leads to ECM remodeling, a process indispensable to implantation, menstruation as well as abortion and abnormal uterine bleeding (Fig. 1).

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9 Mechanisms Underlying Menstrual Bleeding Disturbances with Progestogens

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9.1 Introduction

Modern progestogenic contraceptives are able to provide women with safe, long-acting fertility control which is rapidly reversible. Long-term progestogen only contraceptives (LTPOC) release progestogens over 1–5 years and act at a number of different levels to effectively prevent conception. Levonorgestrel contraceptive implants act mainly by changing the quality of cervical mucus and inhibiting normal sperm penetration (Croxatto et al. 1987). Etonogestrel implants act primarily by inhibition of ovulation. Abnormal endometrial development will prevent implantation, should fertilization occur. Ovulation and luteal function may also be affected (Meckstroth and Darney 2000) though these effects may vary between women and in the same user over time. Intrauterine progestogens act by inducing profound endometrial suppression and thickening cervical mucus without usually inhibiting ovulation.

9.2 Contraceptive Implants

The first progestogen-only contraceptive implant placed on the market was Norplant, a multiunit system releasing levonorgestrel. Innovations have only recently led to a wider choice with new implants that offer easier insertion and removal and other advantages depending on the type of progestogen. Jadelle is similar to Norplant but consists of only two, rather than six, silastic rods to simplify insertion and removal; nevertheless, levonorgestrel serum levels are identical, and performance is the same for the two systems. The currently available single implant systems are: Implanon which releases etonogestrel over three years; Nestorone implants for breast feeding and nonbreast-feeding women lasting up to 2 years; and Uniplant, which is effective for 1 year and releases nomegestrel acetate (Croxatto 2000).

9.3 Intrauterine Progestogens

The levonorgestrel intrauterine system (LNG IUS) releases 20 μg/24 h of levonorgestrel from a polymer cylinder mounted on a T-shaped

frame and covered with a release rate-controlling membrane. It is approved for 5-year use. The most outstanding features of LNG IUS are its high contraceptive efficacy and reduction of menstrual blood flow. No single mode of action can account for its contraceptive efficacy. The endometrium becomes thin and inactive, and the cervical mucus turns scanty and viscous. Although ovulation may be disturbed to some degree, estradiol production continues normally. The Pearl index for LNG IUS from large clinical trials is 0.1. Extrauterine pregnancies occur in 1 in 5,000 users per year. There are also additional health benefits secondary to the inactivation of the endometrium: increased hemoglobin, decreased dysmenorrhea, a possible decrease in pelvic inflammatory disease. LNG IUS may also decrease the growth of fibroids. LNG IUS is well accepted by users, with typical annual continuation rates above 80% in clinical studies (Lahteenmaki et al. 2000).

9.4 Vaginal Bleeding Patterns

Disturbances of vaginal bleeding patterns are almost inevitable in users of LTPOC, and there are no devices which can guarantee regular bleeding or even amenorrhea. These bleeding disturbances are not known to threaten the health of implant users, though they may lead to further investigations to rule out cervical or even endometrial pathology. Their major significance is the degree to which bleeding disturbances are disliked by women, leading to rejection or discontinuation of these methods

Recent advances in understanding the mechanisms of this irregular bleeding will be discussed. These are mostly drawn from studies of levonorgestrel-releasing systems. In addition, the efficacy of current therapies and potential for future therapies based on direct interruption of these mechanisms will be mentioned, as well as future directions for research.

In the great majority of Norplant users vaginal bleeding pattern disturbances are most common in the initial months of use, and tend to diminish over time (Sivin 1994). This progressive improvement reflects the decrease in release rate of levonorgestrel which allows the return of ovulatory-like cycles. During the first year, most women experience a pattern characterized by prolonged bleeding occur-

ring at irregular intervals, while a quarter of users have regular bleeding and about 10% experience at least 3 months of amenorrhea (Frazer et al. 1987). In subsequent years, the number of bleeding episodes and of bleeding days and the length of bleeding episodes decrease (Sivan 1984). By the fifth year, two-thirds of Norplant users experience regular vaginal bleeding patterns, one-third an irregular pattern and amenorrhea is very uncommon. In comparative clinical trials, no significant differences were observed between the vaginal bleeding patterns induced by Norplant and those induced by Jadelle (Indian Council of Medical Research 1986).

Like Norplant, Implanon induces significant vaginal bleeding irregularities (Edwards and Moore 1999). The incidence of amenorrhea increases to 30–40% during the first 3 months and remains at that level thereafter. About 50% of the users experience infrequent bleeding during the first 3 months but this percentage decreases to 30% by 6 months. The incidence of prolonged bleeding, 30% during the first 3 months, declines to 10–20% subsequently. In trials comparing Implanon to Norplant (Affandi 1998; Zheng et al. 1990), Implanon was consistently shown to induce more amenorrhea and infrequent bleeding but less frequent bleeding than Norplant. The incidence of prolonged bleeding is similar with both devices.

Vaginal bleeding disturbances induced by Uniplant tend to improve over the 1-year lifespan of the device. An analysis of the diaries of 1803 Uniplant users (Coutinho et al. 1996) showed that, during the 3 months following insertion, 42% of women experienced a normal regular bleeding pattern, 30% experienced irregular bleeding, 13% infrequent bleeding, 13% prolonged bleeding, 7% frequent bleeding and 3% amenorrhea. By the end of the year, the percentage of women experiencing a normal regular pattern had increased to 67%, while the incidence of all other patterns had decreased. Amenorrhea and frequent or prolonged bleeding remained uncommon patterns during use. Clinical trials comparing Uniplant with other implants have not been conducted, thus these data cannot be directly compared with those described above as the method of diary analysis may have differed.

Changes in menstrual bleeding patterns are experienced by 98% of Mirena users (Baldaszti et al. 2003). With intrauterine levonorgestrel both the volume of menstrual blood loss and the number of

bleeding days are reduced. During the first year of use, up to 40% of women become amenorrheic and this is maintained at 3 years. Spotting affects up to 25% at 6 months and tends to decrease over time, although persistent spotting may occur (Hidalgo et al. 2002; Baldaszti et al. 2003). The variation between individuals is wide and unpredictable.

9.5 Vaginal Bleeding Patterns and Ovulatory Function

A number of studies have been carried out in progestogen users to investigate the relationship between vaginal bleeding patterns, ovarian function, endometrial thickness and levonorgestrel serum levels. Longitudinal studies have suggested wide variations in bleeding patterns in similar hormonal environments. For example low estradiol levels and absence of luteal activity could be associated with amenorrhea, frequent bleeding or prolonged bleeding patterns (Faundes et al. 1991, 1998; Kaewrudee et al. 1999). Conversely, women who had regular vaginal bleeding patterns may have ultrasound findings and estradiol and progesterone levels compatible with normal ovulation, luteinized unruptured follicles, persistent enlarged follicles without ovulation, or no follicular development (Olsson et al. 1990; Shoupe et al. 1991).

However, in two studies, one in Norplant users (Faundes et al. 1987), the other in Nestorone users (Faundes et al. 1991), Faundes and co-workers observed that fluctuations in estradiol levels did influence vaginal bleeding patterns. Bleeding occurred frequently after a rise and fall in serum estradiol or both estradiol and progesterone. Periods of prolonged bleeding often started concomitantly with a fall in estradiol levels and ended with a rise in estradiol. Among Nestorone users (Faundes et al. 1991), they found that in anovulatory cycles the duration of bleeding was directly and significantly associated with the highest concentration of estradiol in the previous 15 days. They concluded that despite the downregulation of steroid receptors by exogenous progestogen (Gurpide et al. 1985) endogenous hormone production influences endometrial bleeding patterns.

In most studies, Norplant users are found to have a thin endometrium, regardless of bleeding patterns or estradiol/progesterone levels

(Exacoustos et al. 1999; Coutinho et al. 1996) and hysteroscopic appearances are compatible with endometrial atrophy (Hickey et al. 1996). However, some authors report a positive association between endometrial thickness, peripheral estradiol levels and number of bleeding days in the 90 days preceding the endometrial biopsy (Hadisputra et al. 1996), and in a study of 18 5-year users of Norplant implants, Darney and co-workers (Darney et al. 1996) found that proliferative endometrium was associated with abnormal bleeding. Although higher doses of progestogens tend to be associated with an increased incidence of ovarian suppression and amenorrhea, there is no clear relationship between the levels of levonorgestrel provided by Norplant implants and vaginal bleeding patterns (d'Arcangues et al. 1992).

9.6 Vaginal Blood Loss

There are limited data available on the amount of vaginal blood loss experienced during progestogen implant use. In one study (Nilsson and Holma 1981) of 13 Norplant users, blood loss was measured during a control cycle, and during the whole first, sixth and twelfth months of implant use. Although the mean number of bleeding days during the treatment cycles was significantly greater than in the control cycle, the mean blood loss was similar during the four periods of observation.

In a study conducted in China, vaginal blood loss was measured before and during progestogen implant use in 89 women randomly allocated to three levonorgestrel-releasing implants: Norplant, Domestic implant I (similar to Norplant) and Domestic implant II (similar to Jadelle) (Han et al. 1999). In all three groups, women experienced similar bleeding patterns and had significantly less blood loss during implant use than during the control cycle. Some women continued to have heavy bleeding during progestogen use, but at a reduced level compared to their baseline.

In a recent study (Fraser et al. 2000), three groups of women were compared: 29 Norplant users, 18 users of an implant containing 120 mg of nestorone and 30 users of an implant containing 80 mg of nestorone. Vaginal blood loss was measured during two

control cycles and during weeks 1–8, 23–30 and 45–52 of implant use. With time, all users experienced less prolonged and irregular bleeding, and more oligomenorrhea or regular patterns. However, the incidence of amenorrhea decreased with time among Norplant users, while it increased in nestorone implant users. In all three groups, total vaginal blood loss measured during each of the three collection periods was decreased to less than half of that measured during control cycles. The highest blood loss measured on a single day during treatment was significantly reduced in all three groups, compared to control cycles. Considerable variation in the percentage of blood loss reduction was observed between women, but the few women who experienced actual increases in blood loss during implant use had light bleeding during their control cycles.

In the same study, in all three groups, hemoglobin levels measured at 1 year of implant use were unchanged compared to those measured during control cycles. Previous longitudinal studies of Norplant users reported similar findings (Fakeye and Balogh 1989; Shaaban et al. 1983; Olsson et al. 1988; Lubis et al. 1983), or reported small increases in hemoglobin over time in the majority of users (Han et al. 1999; Sivin et al. 1983; Gu et al. 1988).

In cross-sectional studies, Norplant users were found to have higher hemoglobin levels (Task force for Epidemiological Research in Reproductive Health 1998), and similar or higher ferritin levels (Task force for Epidemiological Research in Reproductive Health 1998; Faundes et al. 1987) than noncontraceptors. However, in one of these studies (Faundes et al. 1987), a subgroup of users who had increased bleeding and requested removal of the implants was found to have higher mean hematocrit but lower mean ferritin levels than controls, indicating that some Norplant users can have decreased iron stores.

Several studies have addressed vaginal blood loss in Mirena users. In women using the device for the treatment of menorrhagia, menstrual blood loss is reduced by up to 86% at 3 months and up to 97% at 1 year (Andersson and Rybo 1990; Crosignani et al. 1997).

In those using Mirena for contraception, menstrual blood loss has not been objectively measured, but appears to fall dramatically.

9.7 Implications of Vaginal Bleeding Patterns

Regular patterns of vaginal bleeding are central to beliefs concerning fertility, absence of pregnancy and reproductive health for women from many cultures. In addition, for some women the presence of irregular or unpredictable bleeding is a barrier to social, sexual and cultural activities and hence represents a major disruption to their life. Since irregular bleeding may also be a feature of infection of the genital tract, and (rarely) of malignancy, this symptom may also prompt additional investigations such as high vaginal and endocervical bacteriology, cervical cytology, colposcopy or even hyesteroscopy.

There are further potentially serious implications of prolonged and irregular bleeding in terms of sexually transmitted infection (STI). It appears that the transmission of STI, including HIV, is increased in men who are exposed to menstrual blood (Malamba et al. 1994) and in women who are currently menstruating (Tanfer and Aral 1996). Over 60% of HIV-positive women in one recent study complained of irregular bleeding whilst using Norplant implants (Taneepanichskul et al. 1997). It is not known whether this breakthrough bleeding (BTB) might affect HIV transmission, but as long-acting progestogen contraceptives are widely used and promoted in countries where these infections are common and increasing, it is vital that this issue be resolved.

Many women are keenly aware of the pattern of bleeding, but also the duration and amount of blood loss, as well as subtle factors such as the appearance and smell of fluid passed. A certain amount of variation in this is expected, particularly when exogenous hormones are used, but major changes in vaginal bleeding are a source of concern for many women. Amenorrhea may be a convenience for some, but for others deprives them of the regular reassurance that they are not pregnant and may have other negative cultural connotations that are currently poorly understood.

The need to wear a pad or tampon at all times is uncomfortable and becomes expensive when bleeding is prolonged. Hence it is not surprising that disturbances of menstrual bleeding are consistently the most common stated reason for patients to discontinue implantable contraceptives. Whilst irregular bleeding is tolerated by many

women, some are prepared to accept amenorrhea in the context of adequate support and explanation. Approximately 20 million women worldwide use progestogen-only contraceptives and an increasing number of these long-acting systems (d'Arcangues 2000). It is thought that the expectation of disturbed bleeding patterns dissuades many women from using these devices, and a thorough explanation of the likely patterns of bleeding should be intrinsic to any pre-insertion counseling program. The maximum disruption of bleeding occurs in the early months of use, with between 40% and 70% of discontinuations in clinical trials due to these disturbances. The extent to which bleeding disturbances lead to discontinuation of progestogen contraceptives in normal clinical practice is less clear, but it is likely that the extent of dissatisfaction with bleeding patterns in users exceeds that of actual removals, since many women will tolerate irregular bleeding because of the other advantages of these methods (Sangi-Haghpeykar et al. 2000).

9.8 Mechanisms Underlying Menstrual Bleeding Disturbances

Since endometrial vascular and epithelial breakdown must occur before vaginal bleeding is seen, the focus of recent investigations into progestogen-related irregular bleeding has focused on endometrial blood vessels and the local control of their growth, breakdown and repair.

Hysteroscopic observations have suggested that endometrial bleeding is focal following exposure to both low- and high-dose progestogens (Hickey et al. 1996; Lockwood et al. 2000). There is insufficient information about the pattern of vessel breakdown in normal menstrual cycles to know whether focal bleeding usually occurs or is peculiar to BTB. Focal bleeding suggests that the agents precipitating endometrial breakdown may be unevenly activated in the tissue, or that endometrial vessels respond differently to these agents. Lockwood et al. (2000) were able to sample endometrium from bleeding sites in women using Depo-provera and found that expression of tissue factor, the primary initiator of hemostasis was reduced at these sites, and that progesterone receptors A and B were

downregulated in these regions. Studies sampling endometrium from the same site during bleeding and nonbleeding episodes may throw further light on this issue. Hysteroscopic appearances in Mirena users have not yet been reported.

Current understanding is that BTB arises from abnormal endometrial vessels situated in an abnormal endometrial environment. The nature of these endometrial changes and the reasons that they predispose towards irregular vaginal bleeding will be discussed below.

9.9 Abnormal Endometrial Vessels

9.9.1 Reduced Vascular Structural Integrity

Based largely on primate endometrial explant studies, normal menstrual bleeding is thought to arise primarily from the spiral arterioles and to be controlled initially by vasoconstriction (Markee 1940). In contrast, hysteroscopic and immunohistochemical studies have suggested that BTB arises from small capillaries and veins on the endometrial surface (Markee 1940; Johannisson et al. 1991; Hickey et al. 2000). These small vessels are composed only of endothelial cells, their surrounding basement membrane and pericytes, and there is evidence that their integrity is compromised by progestogens. During the first months of exposure to Norplant, the number of endometrial vessels surrounded by the basement membrane components laminin, collagen IV and heparan sulfate proteoglycan are reduced (Hickey et al. 1999). This is likely to make these small vessels more fragile and the initial months of progestogen use is the time when BTB is most common. Vascular support from pericytes is also compromised by a reduction in vascular smooth muscle α-actin expression in progestogen-exposed endometrium (Rogers et al. 2000).

The hypothesis of vascular fragility is supported by the profuse subepithelial hemorrhages seen in progestogen users (Hickey et al. 1996, 1998). These are often seen at hysteroscopy when overt bleeding had not been noted by the progestogen user, suggesting that integrity of the epithelium is also vital in determining whether BTB is seen. Norplant is known to interfere with cytokeratin deposition in the endometrial epithelium (Wonodiresko et al. 1996) and hence

may increase the tendency of fragile vessels to leak into the endometrial cavity and lead to vaginal bleeding. Loss of epithelial integrity may also compromise vessel repair and hemostasis.

Intrauterine LNG induces a dramatic transformation of the endometrium, characterized by extensive decidualization. This is associated with strong expression of local factors associated with decidualization, including prolactin receptor and insulin-like growth factor binding protein-1. Estrogen and progesterone receptors are downregulated after insertion of the LNG-IUS, with a gradual return between 6 and 12 months postinsertion. An elevated leukocyte infiltrate is observed 1 month after insertion of the device, comprising large granular lymphocytes and macrophages. There is also evidence of abnormal chemokine expression following initial insertion with increased interleukin-8 and cyclooxygenase-2 and a reduction in PG dehydrogenase suggesting higher local concentrations of prostaglandins are present in the initial period of local LNG exposure (Jones and Critchley 2000).

9.9.2 Altered Endometrial Angiogenesis

Angiogenesis is the development of new microvessels from existing vessels, a process that involves microvascular endothelial cells. Physiological angiogenesis rarely occurs in adults except in the ovary and endometrium.

Endometrial microvascular appearance is altered by implant exposure. Superficial vascular dilatation (Hickey et al. 1996, 1998; Runic et al. 1997; Manconi et al. 2001) and neovascular formations (Hickey et al. 1996) suggest that progestogen contraceptives use interferes with endometrial angiogenesis. Dilated vessels are also seen on the endometrial surface following intrauterine progestogen exposure (McGavigan et al. 2003).

It is likely that the systemic dose and also the local endometrial concentration of progestogen will be of importance. In addition, low-dose progestogens are associated with a relative increase in endometrial vascular density (Rogers et al. 1993; Hickey et al. 1999). Increased vessel density with reduced stromal support may contribute to vascular fragility.

Vascular endothelial growth factor (VEGF) contributes to vascular permeability, provokes dilatation and promotes angiogenesis. Increased VEGF activity has been observed in Norplant users (Lau et al. 1999). Irregular bleeding with progestogens is also characterized by focal reductions in the expression of angiopoietin-1 (Ang-1), a vessel stabilization and maturation agent, and excess production of the potent angiogenic agents, VEGF and angiopoietin-2 (Ang-2). This seems to occur as a result of hypoxia/reperfusion-induced free radicals that directly damage vessels (Krikun et al. 2002). These findings suggest a link between the dysregulation of endometrial perfusion and aberrant angiogenesis.

Abnormal expression of angiogenic growth factors has also been observed in the endometrium of Mirena users (Roopa et al. 2003; McGavigan et al. 2003).

The regulation of other angiogenic factors such as basic fibroblast growth factor α and β, VEGF, transforming growth factor α, angiogenin and cytokines, may also be disturbed and contribute to abnormal endometrial vascular morphology.

9.10 Abnormal Endometrial Environment

9.10.1 Changes in Endometrial Hemostasis

Constriction of the endometrial spiral arterioles is thought to be an essential early mechanism of menstrual hemostasis (Markee 1940). Spiral arteriole development is suppressed by progestogens (Johannisson et al. 1991), and the bleeding arising from other vessels may result in compromised hemostasis and hence prolonged bleeding. In addition, the powerful vasoconstrictor molecules endothelins (ET) are reduced in progestogen users (Marsh et al. 1995), and the ET metabolizing enzyme neutral endopeptidase is increased (Salamonsen et al. 1999). Reduced ET activity may compromise vascular repair as well as hemostasis. Tissue factor (TF) is a primary initiator of endometrial hemostasis. TF mRNA is reduced in progestogen users compared to normal controls in the secretory phase of the cycle (Runic et al. 1997). TF is also a potent mediator of angiogenesis (Zhang et al. 1994).

Nitric oxide (NO) is a major paracrine mediator of various vascular functions and of inflammation. In primates, NO may be involved in the initiation and maintenance of menstrual bleeding by inducing tissue breakdown and vascular relaxation as well as by inhibiting platelet aggregation (Chwalisz and Garfield 2000). Although no systematic studies have investigated nitric oxide synthetase (NOS) activity in the human endometrium in women experiencing abnormal bleeding, it is possible that NO may play a central role in the initiation and maintenance of BTB. NO is a powerful vasodilator and progestogen exposed endometrium commonly demonstrates dilated vessels on the surface (Hickey et al. 1996, 1998). The inhibition of platelet aggregation may undermine efficient hemostasis in progestogen-related BTB. In addition, inducible NOS-derived NO can promote tissue destruction by activating matrix metalloproteinases (MMP) and inducing apoptosis.

9.10.2 Increased Capacity for Breakdown of Endometrial Vessels and the Extracellular Matrix

Normal menstruation is preceded by an increased statement and activation of proteolytic substances able to rapidly break down the extracellular matrix, and contribute to endometrial vascular repair via the activation of pro-angiogenic factors. Leukocytes are an integral component of the endometrium and display variation in type, number and site across the menstrual cycle (Salamonsen and Lathbury 2000). Menstruation has been likened to an inflammatory process because of the central role of leukocytes in the breakdown and remodeling of the endometrium. Salamonsen et al. (2000) propose that the leukocytes release MMP at this time and also that interactions between leukocytes and the stromal and epithelial cells of the endometrium induce and activate MMP.

Endometrial leukocytes produce a range of regulatory molecules including cytokines and proteases and are likely to respond to chemokines elaborated by endometrial epithelial, endothelial and stromal cells (Salamonsen and Lathbury 2000). Alterations in the number and proportions of endometrial leukocytes have been observed in women taking progestogens and complaining of BTB (Finn and

Pope 1984; Salamonsen et al. 2000; Song et al. 1996). How leukocytes enter the endometrium is not understood but a role for chemokines has been proposed. Little is known of how the leukocytes become activated (Salamonsen et al. 2000). Hampton et al. (2001) have recently investigated the role of monocyte chemotactic proteins (MCP) which act to recruit and activate monocytes into sites of inflammation in the endometrium. They failed to demonstrate a role for these proteins in the migration of macrophages into the endometrium, and their function thus remains unclear.

MMPs are present in the endometrium and display temporal and spatial variation throughout the menstrual cycle with an increase observed premenstrually, located particularly around endometrial blood vessels (Korkorine et al. 1996). MMPs are postulated to be responsible for the endometrial breakdown observed at menstruation (Marbaix et al. 1995; Salamonsen and Woolley 1996, 1999). The proteolytic action of MMP depends on the tissue balance between MMP and their tissue inhibitors (TIMPs). Exposure to low-dose progestogens is associated with a relative increase in expression of MMP-9 (Skinnet et al. 1999; Vincent et al. 1999). This phenomenon has recently been seen in postmenopausal hormone replacement therapy users who also commonly suffer from BTB (Hickey et al. 2001). MMP-9 shows substrate specificity for vascular basement membrane, known to be deficient in the endometrium of Norplant users with BTB (Hickey et al. 1999). In women with an LNG-IUS, MMP-9 is highly expressed in both endometrial glandular and stromal cells, and in the vasculature (in endothelial and perivascular cells) suggesting that MMP may contribute to irregular bleeding and endometrial remodeling in these women. A significant increase in mast cell numbers is seen in Mirena users with BTB compared to those in women with no reported bleeding. Mast cells contain MMP-1 and hence may contribute to endometrial breakdown and bleeding (Milne et al. 2001).

Lysosomes are intracellular membrane-bound vacuoles containing destructive hydrolytic enzymes. They are vital to tissue remodeling and regeneration and are present in all animal tissues (De Duve and Waittaux 1966). The progressive accumulation and release of these enzymes in secretory endometrium may be a principal mechanism in cell separation, endometrial bleeding, remodeling and subsequent re-

generation (Christiaens et al. 1982; Henzl et al. 1972). Instability of lysosomal enzymes with the release of various hydrolases may also contribute to endometrial vessel breakdown and BTB.

Specific MMP inhibitors are currently in clinical trials for use in periodontal disease and cancers.

9.10.3 Changes in Endometrial Perfusion and Oxygenation

Changes in exogenous estrogens and progestogens are known to affect uterine perfusion, as assessed from uterine artery Doppler ultrasound (Exacoustos et al. 1999). Altered endometrial perfusion accompanies spontaneous dysfunctional uterine bleeding (Fraser et al. 1998). Vasomotion describes the spontaneous and rhythmic dilation and constriction of microvessels. Pilot studies using laser-Doppler in early Norplant users show reduced endometrial perfusion in early users and profound alteration of normal vasomotion patterns with an almost total loss of short-term vasomotion (Hickey et al. 2000).

It is possible that reduced endometrial perfusion leads to relative endometrial hypoxia. Hypoxia is a potent vascular destabilizer compromising endothelial integrity (Ali et al. 1998), inducing vascular breakdown and stimulating angiogenesis via VEGF release (Smith 1997). Endometrial oxygenation is difficult to assess, but recent studies of the transcription factor hypoxia-inducible factor in the endometrium of normal cycling women has shown inconsistent detection, and cell culture studies have shown that hypoxia decreased (rather than increased) MMPs, hence making it unlikely that hypoxia is a direct stimulant of endometrial vascular breakdown via this mechanism (Zhang and Salamonsen 2002).

9.10.4 Changes in Endometrial Responsiveness to Sex Steroids

Conflicting results have been obtained regarding endometrial stromal sex steroid expression in Norplant users (Critchley et al. 1993; Lau et al. 1996). In women using the 20-μg levonorgestrel-releasing intrauterine system (Mirena), progesterone receptor expression is reduced, rendering the endometrium functionally unresponsive to en-

dogenous and exogenous progestogens (Critchley et al. 1998). Withdrawal of progesterone is known to initiate events leading to endometrial breakdown, and this receptor downregulation may produce a similar effect, perhaps via leukocyte or cytokine activation.

A likely cause of this differential receptor expression may be the variations in endometrial concentration of LNG seen in the contraceptive systems studied. Intrauterine LNG produces endometrial LNG levels 1000 times greater than serum concentrations (Pekonen et al. 1992).

It had previously been held that the endometrial microvessels themselves do not display receptors for estrogen and progesterone, and hence that the effects of these sex steroids must be indirect (Perrot-Applanat et al. 1988). Recent observations in Norplant users suggest that progesterone receptors are expressed in this tissue and that long-term progesterone exposure leads to suppression of endothelial cell proliferation, inhibition of migration and increased expression of MMP-9 (Rodriguez-Manzaneque et al. 2000).

The functional role of these microvessel receptors requires further clarification, but the overall effect of these vascular changes may be to increase endometrial vascular fragility. If the endometrium contains comparatively more microvessels with comparatively less structural support, and increased expression and activation of proteolytic molecules able to break down vessels, in conjunction with compromised haemostatic mechanisms, this may induce and perpetuate BTB. This process of vascular breakdown may also act as a stimulus to vascular repair and angiogenesis.

9.10.5 Sequence of Events Leading to BTB

BTB is likely to arise as the final point in a complex and multi-factorial process that may be activated by exposure to exogenous sex steroids, particularly progestogens. This represents possible schema for the onset of BTB in low-dose progestogen users. However, there are still relatively little good data to support these mechanisms and much of this is speculative. Also, clinical trials have been based on users of particular progestogens, particularly levonorgestrel in the Norplant system, and it is not known whether observations in Nor-

plant users can be applied to users of other progestogens. There is marked variation between individuals in bleeding patterns in response to the same progestogen exposure and within the same user over time. These can only partly be explained by variations in ovarian activity, and it is not known whether this variation is due to endometrial, ovarian, hematological or genetic factors.

9.11 The Management of BTB

To date, efforts to prevent or limit BTB in women using sex steroids have largely been unsuccessful. However, these interventions have mostly been empirical, and the improved understanding of the underlying mechanisms of BTB opens the possibility of directed therapies to reduce vascular fragility. Supplemental estrogens have been given to women using progestogen-only contraceptives to try and improve bleeding patterns. However, there is no evidence that estrogens improve bleeding patterns beyond the duration of their use (d'Arcangues 2000). The natural history of BTB is to gradually improve over time, and improvements in bleeding pattern observed following alterations in the type and dose of progestogen are likely to reflect this rather than a consistent therapeutic effect. There is an urgent need to identify and target specific points in the cascade of vascular breakdown that can be selectively inhibited, resulting in vaginal bleeding not occurring or being contained beneath the epithelium such that it is not seen by the contraceptive user.

9.12 Potentially Effective Treatment Approaches

9.12.1 Increasing Vascular Stability

In other organ systems, such as the human retina, loss of vascular stability is associated with oxidative stress and the release of free radicals (Kowluru et al. 1999). Increased free radical expression has also been seen in association with BTB (Krikun et al. 2002). At a molecular level, this vascular fragility is associated with reduced integrity of endothelial cell tight junctions and vascular basement

membrane competence and clinically leads to vascular breakdown and retinal bleeding (Martin et al. 1988). Flavonoids, part of the vitamin B complex, have been shown in controlled trials to increase peripheral capillary resistance and to improve the systemic symptoms of capillary fragility such as epistaxes, petechiae and conjunctival hemorrhages (Galley and Thiollet 1993). Recent pilot data (Subakir et al. 2000) have suggested that oral vitamin E (an antioxidant) given during bleeding episodes may reduce bleeding in users of low-dose progestogens. However these results were not confirmed in a multicenter study involving 500 users of Norplant (d'Arcangues et al. in press).

The prostaglandin synthetase inhibitor mefenamic acid has been used to control irregular bleeding secondary to Norplant use. In a double-blind placebo controlled study, 34 women who took mefenamic acid were significantly more likely to stop bleeding and had longer bleed-free intervals than the placebo group (Kaewrudee et al. 1999).

9.12.2 Reduce Vessel Destabilizers

The introduction of agents specifically targeted to block molecules stimulating breakdown of endometrial vessels and extracellular matrix may help to reduce BTB. MMP activity could be antagonized by selective use of TIMPs.

9.12.3 Improve Epithelial Integrity

Hysteroscopic studies in Norplant users strongly suggest that subepithelial bleeds (seen as petechiae and ecchymoses) are common in these women when vaginal bleeding has not been observed by the patient (Hickey et al. 2000). Norplant use appears to reduce epithelial integrity by interfering with cytokeratin deposition (Wonodiresko et al. 1996). Since endometrial bleeding is not problematic unless it manifests as vaginal bleeding, agents which maintain epithelial integrity may also act to contain bleeding. Estrogens induce endometrial epithelial proliferation and may thus effectively terminate pro-

longed bleeding episodes in progestogen users. Estrogens may also act to maintain endothelial cell junctional integrity, but there is currently little known about the regulation of these tight junctions in the endometrium. Since the addition of estrogens to progestogen-only contraception essentially undermines many of the advantages of these preparations, non-estrogenic agents to maintain epithelial integrity are needed. Current selective estrogen receptor modifying preparations (SERMs) aim to avoid endometrial receptor targets. There may be a role for SERMs acting to selectively stimulate the endometrium but not other tissues (Grow and Reece 2000).

Monthly administration of 50 mg of mifepristone has recently been shown to significantly improve bleeding patterns in Norplant users (Cheng et al. 2000), with no evidence that contraceptive efficacy is compromised. It is currently unclear how mifepristone works in these circumstances, but this therapy warrants further investigation, if only to improve bleeding patterns during the early months of progestogen use when bleeding patterns are most troublesome. A recent study demonstrated that a 50-mg dose of mifepristone taken every 2 weeks decreased the incidence of BTB in new starters of DMPA without compromising contraceptive efficacy (Jain et al. 2003).

9.13 Conclusion

Progestogen contraceptives are almost inevitably associated with disruptions in menstrual bleeding patterns. In many cases this amounts to bleeding that is frequent and/or prolonged. Evidence from studies of all available preparations suggests that menstrual disturbance is one of the most common reasons for discontinuation of these methods. This is particularly unfortunate since many progestogen users are women who have been unable to find other suitable methods and may now have very limited choices of safe contraception available to them.

Currently, there is no effective long-term management for bleeding disturbances and effective and acceptable treatments are unlikely to be developed without a fuller understanding of the factors under-

lying this bleeding. Recent information has greatly advanced understanding of the vascular and endometrial changes associated with progestogen use, but a number of areas require further study before the mechanisms of BTB can be defined. In addition, further information is required from women using these preparations in developing and developed countries regarding the perception of bleeding disturbances and the relative tolerability of varying bleeding patterns and of amenorrhea.

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10 Human Endocervical Mucins

I. K. Gipson

10.1 Introduction

As with all wet-surfaced epithelia of the body, mucus plays an important role in the defense of the female reproductive tract, but it plays a unique role as well-facilitation of reproductive function (Hafez 1980). The major structural components of mucus that give it its rheological properties, are mucins, the heavily glycosylated glycoproteins secreted by professional secretory cells of epithelia. Also within mucus are shed extracellular domains of the membrane-associated mucins (for review see below) that are present along apical

membranes of all the wet-surfaced epithelia of the body. Reviews of mucins (Gendler and Spicer 1995; Moniaux et al. 1999) and the expression of specific mucins by reproductive tract epithelia are available (Carson et al. 1998; Gipson 2001; Idris and Carraway 1999; Lagow et al. 1999). This review will draw from our previous reviews (Gipson and Argueso 2004; Gipson 2001) and will update expression patterns of newly discovered mucins in the reproductive tract. The main focus will be on the mucins of the endocervix as the cervical epithelium is the primary source of secreted mucus in the female reproductive tract.

10.2 Structural Properties of Mucins

Mucins are high molecular weight glycoproteins, the major mass of which is carbohydrate. Sequencing of mucin genes has extended the definition of mucins, and they are now defined (in addition to having a major mass of carbohydrate) as having the common feature of the presence of tandem repeats of amino acids rich in serine, threonine and proline in the central domain of the mucin core peptide (Gendler and Spicer 1995; Moniaux et al. 2001). Serine and threonine provide O-linked glycosylation sites in great number along the tandem repeat domain of each mucin. Each mucin gene has a unique number of amino acids per tandem repeat, and the number of tandem repeats per allele varies (Table 1). Alleles of different sizes are co-dominantly expressed, thus, mucin genes and resultant protein are polymorphic (Fowler et al. 2001; Swallow et al. 1987; Vinall et al. 1998). In addition, several mucins have been reported to exhibit splice variants (Moniaux et al. 2000). These variations as well as genetic and tissue variation in post-translational processing of mucins (e.g., glycosylation) yield mucin species of different size and character within and between individuals.

Based on sequence data, two categories of mucins have been identified – secreted mucins and membrane-associated mucins (Gendler and Spicer 1995; Moniaux et al. 2001). Of the mucins identified, six are considered secreted mucins, eight are membrane associated, and several remain uncharacterized, as there is insufficient sequence data to categorize them. Table 1 summarizes the mu-

Table 1. Characteristics of human epithelial mucins and their distribution/ presence in female reproductive tract epithelia

Name	Category	Chromo-some mapping	Amino acids in tandem repeat	Presence in female reproduc-tive tract[a]	References
MUC1	Membrane associated	1q21q24	20	ft, ut, en, ec, v	Gendler et al. 1987; Gipson et al. 1997; Lan et al. 1990
MUC2	Gel forming	11p15.5	23	2 of 15 en	Gipson et al. 1997; Gum et al. 1989
MUC3A/ 3B	Membrane associated	7q22	17	–	Gipson et al. 1997; Gum et al. 1990; Pratt et al. 2000
MUC4	Membrane associated	3q29	16	en, ec, v	Gipson et al. 1997; Nollet et al. 1998; Porchet et al. 1991
MUC5AC	Gel forming	11p15.5	8	en	Gipson et al. 1997; Guyonnet Duperat et al. 1995; Meer-zaman et al. 1994
MUC5B	Gel forming	11p15.5	29	en	Dufosse et al. 1993; Gipson et al. 1997; Keates et al. 1997
MUC6	Gel forming	11p15.5	169	en	Gipson et al. 1997; Toribara et al. 1993
MUC7	Soluble	4q13-q21	23	–	Bobek et al. 1993; Gipson et al. 1997
MUC8	ND	12q24.3	13	ut, en	D'Cruz et al. 1996; Shankar et al. 1994, 1997
MUC9	Soluble	1p13115	15	ft	Lapensee et al. 1997
MUC11	ND	7122	28	ut	Williams et al. 1999
MUC12	Membrane associated	7122	28	ut (weakly)	Williams et al. 1999

Table 1 (continued)

Name	Category	Chromosome mapping	Amino acids in tandem repeat	Presence in female reproductive tract[a]	References
MUC13	Membrane associated	3q13.3	15	ND	Williams et al. 2001
MUC15	Membrane associated	11p14.3	None	ov	Pallesen et al. 2002
MUC16	Membrane associated	19p13.2	156	ft	Yin and Lloyd 2001
MUC17	Membrane associated	7q22	59	ft, ut, en, ec, v	Gum et al. 2002
MUC19	Gel forming	12q12	ND	ND	Chen et al. 2003
MUC20	Membrane associated	3129	19	ND	Higuchi et al. 2004

[a] ft, Fallopian tube; ut, uterus; en, endocervix; ec, ectocervix; v, vagina; ov, ovary; –, negative in all above; ND, not determined.

cins identified to date along with several of their characteristics. Human mucins have been designated MUC followed by a number that designates chronologically their order of discovery. For example, MUC1 was the first of the human mucin genes to be cloned and characterized. Mouse homologues to human mucin genes are designated Muc and rat genes rMuc.

10.2.1 Secreted Mucins

Two types of secreted mucins have been identified – large gel-forming mucins (four of the five are encoded on chromosome 11p15.5), and two smaller soluble mucins (for reviews see Gendler and Spicer 1995; Moniaux et al. 2001; Chen et al. 2003). The gel-forming mucins are the largest glycoproteins known. They have common, cysteine-rich structural motifs that allow homomultimer formation, which is believed to give rise to the rheological properties of mucus (for a

review see Desseyn et al. 1998, 2000; Fig. 1). These mucins are expressed by the cells classically known to secrete mucus – the goblet and mucous cells of the respiratory, gastrointestinal, endocervical and ocular surface epithelia, and the submucosal glands associated with these epithelia. These epithelia have a tissue- and cell-specific expression pattern of the gel-forming mucin genes (Gum et al. 1994; Ho et al. 1995; Nielsen et al. 1997; Sharma et al. 1998).

The second category of secreted mucins, the soluble mucins, includes MUC7 (Fig. 1, p. 222) and MUC9 (also known as oviductin). Originally cloned from salivary gland and hamster oviduct, respectively, they are the smallest mucins known. Their apomucins are 39 kDa and 120 kDa, respectively (Arias et al. 1994; Bobek et al. 1993). MUC7 is secreted by serous cells rather than mucus cells of the submucosal glands of both the salivary glands (Nielsen et al. 1997) and the bronchial airways (Sharma et al. 1998), as well as by acinar cells of the lacrimal gland (see Jumblatt et al. 2003). MUC7 has also been found in urine of patients with bladder cancer and may be a tumor marker (Retz et al. 2003). To date, oviductin appears to be specific to the oviduct tube (Lapensee et al. 1997). This mucin is unique among mucins in that it has a chitinase domain in the amino portion of the molecule and tandem repeats in the carboxy portion (Malette et al. 1995). The enzymatic region binds to the ovulated ova or embryo, and it is hypothesized that the mucin domain prevents adhesion to oviductal epithelia (Malette et al. 1995). Synthesis of the glycoprotein is estrogen (E_2) dependent (Arias et al. 1994).

10.2.2 Membrane-Associated Mucins

Increasing numbers of mucins are being characterized as having hydrophobic membrane-associated domains near their carboxy termini (Fig. 1). These mucins appear to be major constituents of the glycocalyx of cells of all wet-surfaced epithelia, including both simple and stratified, where they have been estimated to extend 200 nm–500 nm from the cell surface (Bramwell et al. 1986). Mucins designated as membrane associated, to date, include MUCs 1, 3A, 3B, 4, 12, 13, 16, and 17 (Table 1). Genes of four of the group (MUCs 3A, 3B, 12, and 17) are clustered on chromosome 7q22 (Gum et al.

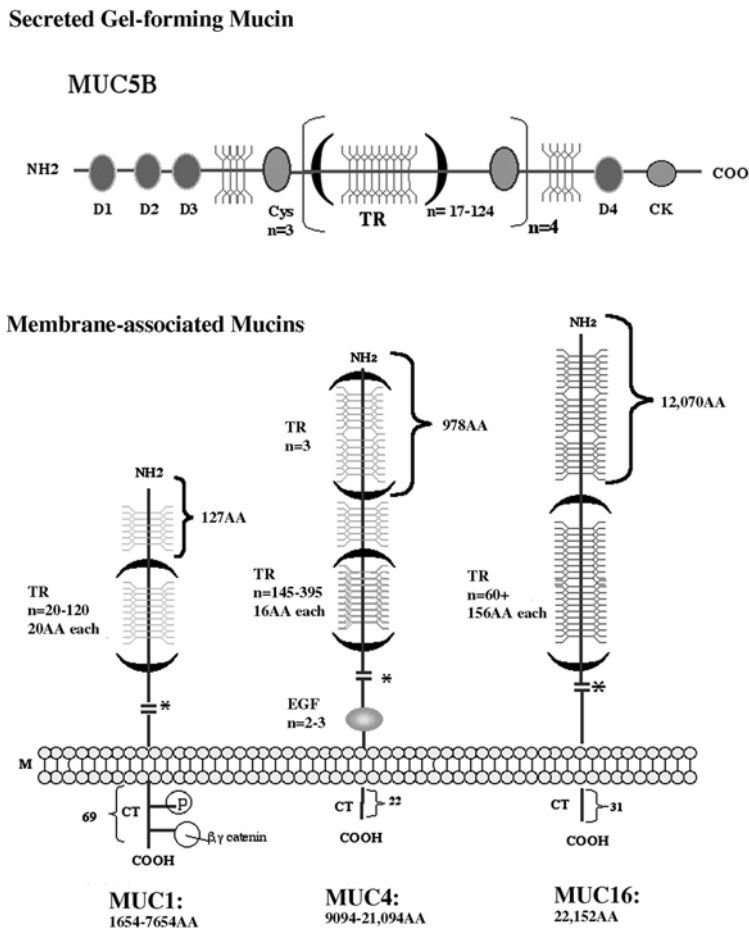

Fig. 1. Organization of structural motifs in secreted gel-forming mucins and membrane-associated mucins produced by reproductive tract epithelia. A common characteristic of all mucins is the presence of a variable number (*n*) of tandem repeat (*TR*) domains that are highly O-glycosylated (*YYY*). The secreted mucin MUC5B is produced by endocervical epithelium. MUC5B is a gel-forming mucin that contains cysteine-rich domains (*D1, D2, D3, D4*) similar to the D domains of von Willebrand Factor. These domains flank the central TR domains and are involved in disulfide cross-linking, which allows polymerization of the mucins to form the mucus network. Additional cysteines (*Cys*) and a cysteine knot (*CK*) flank the TR. The

2002; Pratt et al. 2000; Williams et al. 1999) (Table 1). Of the membrane-associated mucins, MUCs 1 and 4 have been the most extensively studied (for reviews see Carraway et al. 2000; Gendler 2001).

The tandem repeat domain of the membrane-associated mucins occupies the major part of the extracellular portion of the molecule and in some instances extends nearly to the amino terminus. These extracellular domains, or ectodomains, are envisioned as extending from the cell surface to form the glycocalyx. Experimental studies indicate that the extracellular, highly glycosylated tandem repeat domain functions as a 'disadhesive', preventing cell–cell and cell–matrix adhesions (Komatsu et al. 1997).

The membrane-associated mucins do not have cysteine-rich domains and are monomeric. They have short cytoplasmic domains, which in MUC1 is conserved between mammalian species and is reported to be associated directly with β- or γ-catenin and the actin cytoskeleton (Parry et al. 1990; Yamamoto et al. 1997). There is also growing evidence that the cytoplasmic domains interact with cytoplasmic proteins to facilitate signal transduction.

There is evidence that several of the membrane-associated mucins are present in both a membrane-associated and a soluble form (Carraway et al. 2002; Ligtenberg et al. 1992; Yin and Lloyd 2001). The soluble form may be the result of splice variants in which the membrane-associated domain is post-transcriptionally removed (Gendler 2001; Moniaux et al. 2001) or the ectodomain of the mucin is shed from the surface of cells (Rossi et al. 1996). MUCs 1, 4,

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membrane-associated mucins, MUCs 1, 4 and 16, are produced by oviduct, uterus and endocervix; MUC1 and MUC4 are also expressed in ectocervix and vagina. They have a transmembrane (*TM*) domain in the carboxy terminus responsible for tethering the mucin in the apical cell membrane. MUC1 contains tyrosine residues in its cytoplasmic tail (*CT*) that can be phosphorylated (*P*) and thus may participate in signal transduction. The cytoplasmic tail of MUC1 associates with beta gamma catenins, MUC4 has EGF-like domains and may regulate epithelial cell growth. The *asterisk* indicates presence of a putative cleavage site associated with shedding of the mucin ectodomain from the apical surface of the cell. The cleavage site is proposed to be at G/SVVV within the SEA module for MUC1, at GDPH for MUC4, and in the SEA module for MUC16. (For review of mucin structure see Moniaux et al. 2000 and Yin and Lloyd 2001)

and 16 all appear to be shed from the epithelial surface (Auersperg et al. 1999; Ligtenberg et al. 1992; Sheng et al. 1990).

10.3 Localization of Mucin Gene Expression in Human Female Reproductive Tract Epithelia

Human female reproductive tract epithelia express members of both the secreted/gel-forming and soluble classes of mucins and the membrane-associated class, as well as several mucins that remain unclassified. Mucin gene expression has been analyzed by northern blot analysis and in situ hybridization assays, as well as immunohistochemistry. Table 1 summarizes the mucins known to date and provides information on those for which expression by the female reproductive tract epithelia has been determined.

10.3.1 Secreted Gel-Forming Mucins

Endocervical epithelia are the only epithelia of the reproductive tract shown to express members of the secreted gel-forming mucin class. Three of these mucins, MUCs 5AC, 5B, and 6, were found to be expressed in all samples assayed by both Northern blot and in situ hybridization (Gipson et al. 1999). Endocervical tissue has not, to our knowledge, been assayed for expression of a newly described gel-forming mucin MUC19.

By in situ hybridization, message for MUC5B was found localized uniformly over all endocervical epithelial cells (regardless of glandular or luminal position) and very intense labeling with a 984-bp cRNA antisense probe to the tandem repeat region of this mucin was evident (Fig. 2). By comparison, MUC5AC and MUC6 mRNA localization was not uniformly located over the cervical epithelial cells, nor was the labeling as intense – even using probes to the tandem repeat region that bind to multiple sites and thus have an amplified signal. No major variance with menstrual cycle was detected by these assays, but neither assay is quantitative, as tandem repeat probes were used. Immunohistochemical localization of the gel-forming mucins MUC5B, 5AC and 6, in human endocervix has been reported (Gipson et al. 1997).

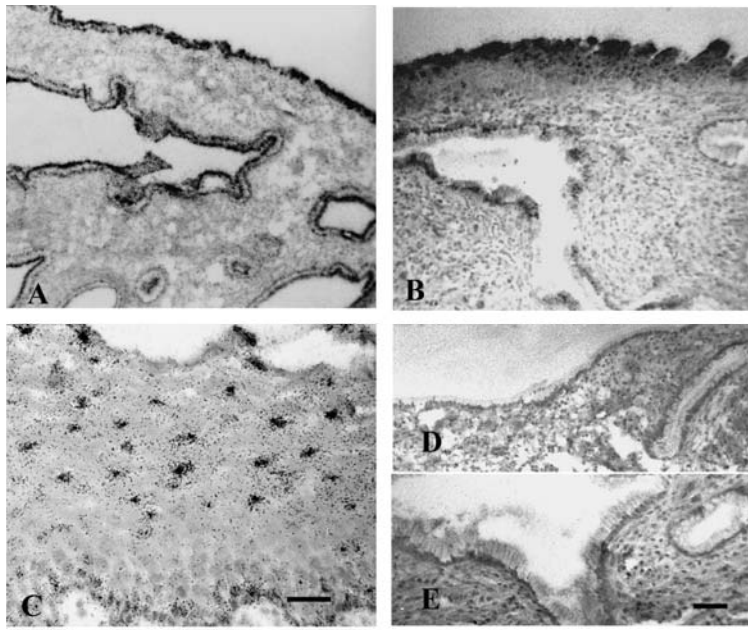

Fig. 2. Localization of MUC5B mRNA in human endocervical epithelia (**A**) and MUC4 mRNA in human endocervical and ectocervical epithelia (**B**, **C**) by in situ hybridization. **A** ^{35}S-labeled MUC5B RNA probe and a ^{35}S-labeled oligonucleotide probe for MUC4 tandem repeats were used. Note in **A** the intense binding of the MUC5B probe to luminal as well as glandular epithelia; the section is taken from tissue in the early secretory phase of the menstrual cycle. In the proliferative stage endocervical tissue, the MUC4 oligoprobe binding is especially intense at the luminal surface as compared to glandular epithelium (**B**, *top* of micrograph). Binding of the MUC4 probe to the ectocervical epithelium (**C**) shows particularly strong binding to apical cells of the stratified epithelium. There is no binding of sense control probes for MUC5B (**D**) and MUC4 (**E**) to sections of endocervical tissue. Bars, 100 μm

10.3.2 Membrane-Associated Mucins

Several of the membrane-associated mucins are expressed by reproductive tract epithelia. By Northern blot and in situ hybridization, MUC1 can be demonstrated in fallopian tube, uterus, cervix, ecto-

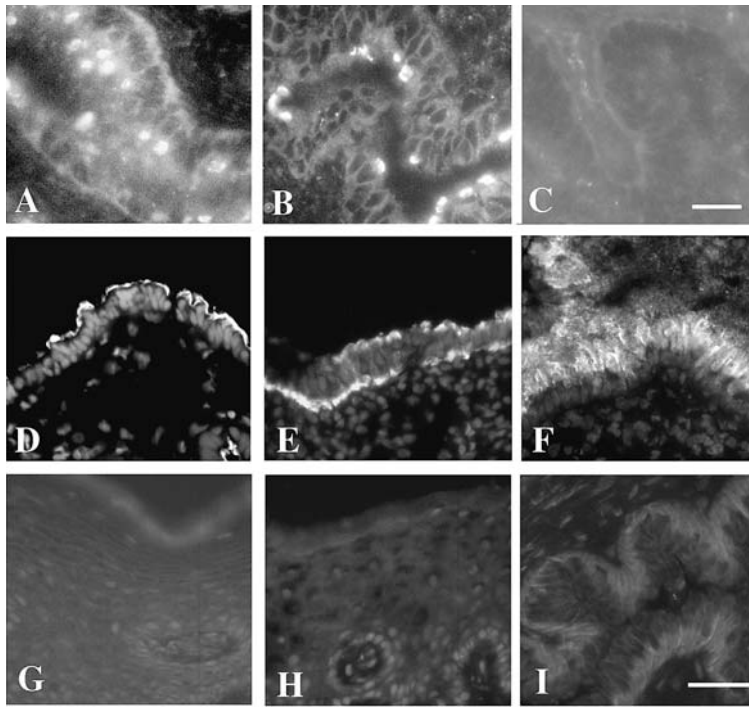

Fig. 3. Immunofluorescence microscopy demonstrating binding of MUC5B antibody to MUC5B mucin in endocervix tissue taken at the proliferative stage (**A**) and the secretory stage (**B**) of the menstrual cycle. Note the more apical localization of the binding in the secretory sample. The specificity of the binding of the antibody to MUC5B is confirmed by the lack of binding to conjunctival tissue (**C**), which expresses MUCs 1, 4, and 5AC but not MUC5B. **D–H** Binding of CA125 antibody to MUC16 in oviduct (**A**), uterus (**B**), endocervix (**C**), and lack of binding to ectocervix (**G**) and vaginal (**H**) epithelia. **I** The amount of binding to endocervix attributable to background from the secondary antibody. Bar, 10 μm

cervix and vagina; and MUC4 has been demonstrated in endocervical, ectocervical and vaginal epithelial cells (Fig. 2) (Gipson et al. 1999). MUC4 is expressed uniformly by endocervical and ectocervical epithelia, and in a patchy distribution by vaginal epithelia. Data from rats demonstrate rMuc4 in the uterus of the female reproductive tract (McNeer et al. 1998). In more recent studies, assays by

Northern blot analysis demonstrated that uterus/endometrium expresses MUC12 (Williams et al. 1999). The CA125 antigen (now designated MUC16) is present in many regions of the reproductive tract epithelia, including fallopian tube, uterus, endocervix, but not in ectocervix or vagina (Fig. 3) (Gipson et al., unpublished results).

10.4 Variation of Mucins with Cycle

Having determined the expression profile of mucin genes known at that time in the human female endocervical epithelia, semiquantitative RT–PCR was used to determine which of the genes predominate and whether their expression levels vary with the menstrual cycle (Gipson et al. 1999). RNA isolated from cytobrush samples were taken from cycling women at 2-week intervals over two menstrual cycles. Blood samples taken at the time of cervical cytobrush sampling were analyzed for E_2 and progesterone levels, and luteinizing hormone (LH) surge was detected by urinalysis. mRNA levels of the three secreted gel-forming mucins, MUCs 5AC, 5B and 6, and the membrane-associated mucin MUC4 were determined relative to β2-microglobulin mRNA levels. In order to assay MUC4, which at the time of the study was relatively uncharacterized – with only the tandem repeat reported (Porchet et al. 1991), we sequenced a 2.7-kb region 5′ to the tandem repeat (Gipson et al. 1999). This allowed the design of primer sets for the quantitative PCR.

MUC5B consistently amplified at fewer cycles than MUC5AC and MUC6, indicating that, of the four gel-forming mucins described at that time, MUC5B predominated. Of the two membrane-associated mucins known to be present in the cervical epithelia at that time, MUC4 was expressed at higher levels. These data demonstrated that MUCs 5B and 4 were the major gel-forming and membrane-associated mucin species of the endocervix, respectively. Furthermore, the relative levels of mRNA for the two prevalent mucins were correlated to cycle day as well as levels of E_2 and progesterone in the blood (Gipson et al. 1999). Pooling the data for the relative levels of MUC5B mRNA from nine subjects demonstrates that MUC5B mRNA peaks prior to midcycle and drops dramatically after midcycle (Fig. 4 A). Relative levels of MUC4 mRNA from six

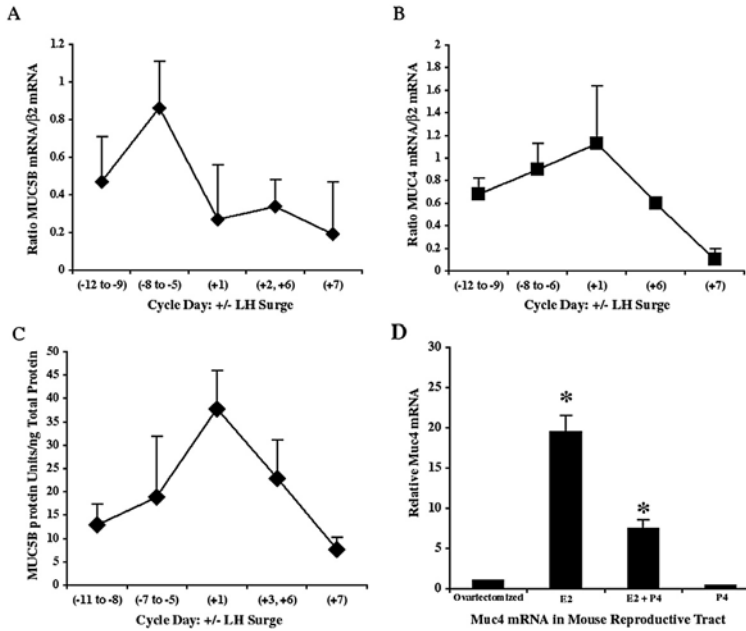

Fig. 4. Levels of MUC5B mRNA (**A**) and MUC4 mRNA (**B**) relative to that of β^2-microglobulin in endocervical epithelial RNA samples taken through the cycle, as determined by date of LH surge. RNA was isolated from cytobrush samples of human endocervical epithelia. Values represent the mean ± SEM for 33 samples from nine individuals (MUC5B, *diamonds*) and 17 samples from six individuals (MUC4, *squares*). MUC5B mRNA levels peak prior to midcycle, while MUC4 mRNA levels peak at midcycle. Message for both mucins declines late in the cycle when blood progesterone levels rise. MUC5B mucin protein content (**C**) in samples of cervical mucus harvested from three individuals at various times in the menstrual cycle, as determined by date of LH surge. Values represent the mean ± SEM for 25 samples from three individuals. The amount of MUC5B protein in cervical mucus peaks at midcycle when the mucus character changes to facilitate sperm penetration. **D** Influence of 7-day treatments of estrogen (*E2*), estrogen plus progesterone (*E2 + P4*), and progesterone (*P4*) on mucin mRNA levels in ovariectomized mouse tissues, as measured by real-time RT-PCR. Expression by ovariectomized nonhormone-treated animals was used as the baseline data point

subjects peaked at midcycle, followed by a drop after midcycle (Fig. 4B). Although there was no correlation of mucin gene expression to E_2 levels, mucin mRNA levels were highest during the stage of the cycle when E_2 was unopposed. There was a consistent inverse relationship between both MUC5B and MUC4 mRNA levels and blood progesterone (P_4) levels (Gipson et al. 1999). Perhaps P_4 antagonizes E_2 upregulation of mucin gene expression. Prevalence of these mucin genes needs to be reevaluated in light of identification of additional mucin genes.

10.4.1 Mucin Protein Levels in Cervical Mucus with Cycle

Since the gel-forming mucin MUC5B was identified as a major mucin mRNA of the endocervical epithelium, we sought to determine if the quantity of MUC5B in cervical mucus changed through the menstrual cycle.

In order to assay mucin glycoproteins in very small sample sizes, a direct assay that could be used on native mucus was required. An antibody was made to a synthetic peptide, a 19-amino acid segment of a region within the cysteine-rich region of the D4 domain located C terminal to the tandem repeat region of the MUC5B glycoprotein (Fig. 1). The peptide mimicking the 19-mer in the D4 region is located between cysteines and lacks glycosylation sites. Development of antibodies specific to individual mucin gene products has been difficult, and skepticism regarding specificity of mucin antibodies is widespread. Thus, we used several methods to demonstrate the specificity of the Chicken IgY antibody to MUC5B. Firstly, the antibody is preadsorbable with its peptide. Secondly, the antibody binds by immunohistochemistry only to mucin-secreting epithelia that express the MUC5B mRNA (Fig. 3). Third, the antibody binds by ELISA only to secretions known to contain MUC5B, and by immunoblot, the antibody binds a very high molecular weight protein.

It is of interest that the MUC5B antibody recognizes both native and denatured MUC5B protein. The binding to native protein may relate not only to lack of glycosylation sites in the antigenic region but also to its position within an intercysteine region of the D4 domain of MUC5B. Intercysteine sequences typically loop out from as-

sociated cysteines, thus providing ready access to antibody binding (Barlow et al. 1986). Besides surface exposure, the relative flexibility of the looped sequences may more readily form shapes assumed by small, flexible peptide antigens (Tainer et al. 1984). Examples of looped epitopes among secreted proteins include the gonadotrophins LH/human choriogonadotropin (Keutmann et al. 1987; Troalen et al. 1990), follicle stimulation hormone (FSH) (Weiner et al. 1990), and human growth hormone (Cunningham et al. 1989).

Using the antibody to MUC5B, a quantitative ELISA was developed using a cervical mucin standard prepared by conventional mucin isolation techniques from mucus obtained from patients at an intrauterine insemination clinic. The amount of the mucin per nanogram total protein was determined for cervical mucus from three subjects, each sampled over four hormone cycles (Gipson et al. 2001). LH surge and blood E_2, and progesterone levels were monitored. For each subject, cumulatively, two or three samples were taken before LH surge, one around the LH surge, and one or two after the LH surge. ELISAs were performed on a range of concentrations of the cervical mucus samples in order to be certain that the full range of detectability was assayed.

At all concentrations of cervical mucus tested, there was an obvious peak in MUC5B antibody binding per unit total protein in the midcycle samples compared with those from early or late in the cycle. As indicated in Fig. 4C, which shows the average of all the data points from the three subjects sampled through several cycles, there was a dramatic three- to fivefold increase in the amount of MUC5B protein at midcycle. The amount of MUC5B in the cervical mucus dropped precipitously during the luteal phase, as mRNA levels dropped (compare Fig. 4A with Fig. 4B) and blood progesterone levels increased (Gipson et al. 2001).

10.4.2 Glycosylation of Mucins of the Endocervix

The hydrophilic character of mucins is ascribed to the O-linked carbohydrates on the apomucin. Assay of cervical mucin carbohydrates indicates a microheterogeneous population of neutral, sialylated, and sulfated oligosaccharides that varies from 2 to 9 residues in length

(Carlstedt et al. 1983; Yurewicz et al. 1987). Other structural studies of cervical mucins have been performed that identify core structures of mucins (Yurewicz et al. 1987), but little information is available on mucin O-glycosylation during the menstrual cycle.

Using lectins as carbohydrate probes, the samples of cervical mucus taken during the cycle for which MUC5B was quantified (Fig. 4) were assayed for two common carbohydrate structures, T antigen and *N*-acetyllactosamine (Argueso et al. 2002). Choice of these two carbohydrate structures was based on binding of the two lectins that recognize these structures (peanut agglutinin and *Erythrina cristagalli* agglutinin) to secretory granules in human endocervical cells from the proliferatory phase of the menstrual cycle. The amounts of T antigen and *N*-acetyllactosamine oligosaccharides on MUC5B increased during the first half of the cycle, peaked at midcycle, then dropped during the second half of the cycle. As MUC5B protein levels dropped, it appeared that its glycosylation is altered. The peak in O-glycans on mucins at midcycle supports the hypothesis of their role in holding water within the endocervical canal during ovulation, to facilitate sperm migration.

10.5 Role of Mucins in Reproduction

While there is indication of hormone influence on levels of mucin mRNA and protein, there is little information demonstrating how mucin genes are regulated by E_2 or progesterone (P_4). As is the case in our own studies of MUC5B and MUC4 (Gipson et al. 1999, 2001), information regarding regulation of expression of the membrane-associated mucin MUC1 (Aplin et al. 1996; Hey et al. 1994, 1995) and the rat homologue to MUC4 are limited to correlation of mucin message and protein levels to circulating E_2 or P_4 levels (Idris and Carraway 1999; McNeer et al. 1998). In a review of the MUC1 data, DeSouza et al. (1998) point out that the different regions of the reproductive tract respond differently or distinctively to the steroid hormones. Thus, regulation of MUC1 in the uterus may not relate to either the endocervical or ectocervical/vaginal epithelia, and furthermore, regulatory regions of the different mucin species will be unique. Human endometrial cells appear to upregulate

MUC1 in receptive compared to unreceptive phases, and in vitro, progesterone combined with estradiol priming induces an upregulation of MUC1 in the receptive endometrium (Meseguer et al. 2001).

Carraway and coworkers (McNeer et al. 1998) have demonstrated that sialomucin complex (SMC) mRNA (the rat MUC4 homologue) is expressed in the uterus of the rat. They studied SMC protein level in ovarectomized rats supplemented with E_2 or P_4 alone, or a combination of the two, and they found high levels of SMC protein in E_2-supplemented rats, a diminution in E_2- and P_4-supplemented rats, and no SMC protein with P_4 supplement alone. These data suggest that P_4 downregulated the SMC message. Experiments with ovarectomized mice show similar results (Fig. 4D) (Lange et al. 2003). The rat and mouse data are in agreement with our data from humans in which we show that MUC4 and MUC5B mRNA and MUC5B protein are at their maximum during the E_2, follicular phase, and decrease in the luteal phase (Fig. 4) (Gipson et al. 1999, 2001). These data indicate that there is similarity between human endocervix, rat uterus, and mouse reproductive tract epithelia, in that E_2 upregulates and P_4 downregulates mucin expression.

As reviewed by Cooke et al. (1998), there is evidence that the estradiol effect on secretory activity of uterine epithelium is a direct rather than indirect effect (via subepithelial cell intermediates) through E_2 receptor-α. An E_2 receptor-α knockout mouse (ERKO) (Cooke et al. 1998) that has an intact β receptor, does not respond to E_2 to effect the uterine and vaginal changes associated with the estrus cycle. Thus, if MUC5B and MUC4 gene expression is directly regulated by E_2, it can be assumed that it is through E_2 receptor-α.

P_4 antagonism of E_2 action has long been recognized and can be accomplished by several mechanisms (as reviewed by Alexander et al. 1990; Katzenellenbogen 1996; and papers referenced therein). These included induction of estradiol metabolism, downregulation of E_2 receptors, and competition between steroid hormone receptors for factors that mediate enhancer function. Thus, there are multiple possibilities for direct or indirect mechanisms of regulation of mucin gene expression by E_2 and P_4.

10.6 Summary

The changes that occur in the physical and biochemical nature of cervical mucus during the menstrual cycle have been studied extensively (for reviews see Carlstedt and Sheehan 1989; Katz et al. 1997; Vigil et al. 1991), and the importance of the mucus in mucosal protection and sperm penetrance is widely acknowledged. Until recently, however, there has been little information on the molecular character of the major structural components of cervical mucus – the mucins. As summarized above, data demonstrate that a major gel-forming mucin of the endocervical epithelium is MUC5B. Of the gel-forming mucins, MUC5B mRNA predominates over that of MUCs 5AC and 6, which are also expressed but at lower levels (Gipson et al. 1999). The amount of MUC5B mRNA is high during preovulatory stages of the menstrual cycle, and there is a dramatic peak in MUC5B glycoprotein/unit total protein in cervical mucus at the time of ovulation. What role does this specific mucin play in reproduction and why, of the four gel-forming mucins, does 5B predominate? At least two roles can be proposed. Firstly, mucins are extraordinarily hydrophilic, and the ability of their surfaces to bind water accounts for the mucins' space-filling ability (Gerken 1993). Increased gel-forming mucin in the endocervical canal at ovulation may function to hold water in place at the canal surface, thus, keeping the canal patent for sperm motility. A second potential role is that increased mucin is required for protection of the cervix and uterus at the time when increased water is secreted into the cervical canal to facilitate sperm penetrance. Pathogens and other seminal fluid components may be excluded from entering the uterus by mucin trapping.

The question of why MUC5B predominates is an interesting one. Perhaps MUC5B is more hydrophilic than other mucins; it has an extraordinarily large central hydrophilic domain. Another possibility is the stability of MUC5B. Of the gel-forming mucins, MUC5B is the only one that is not polymorphic (Vinall et al. 1998). If MUC5B is a vital part of the reproductive process, perhaps a consistent size and character is required within the cervical canal to allow sperm to penetrate. Perhaps molecule deletion studies will provide information on the specific function(s) of MUC5B.

With regard to membrane-associated mucins of the reproductive tract epithelia, there has been intense interest in the role of MUC1 in implantation (for reviews see DeSouza et al. 1998; Lagow et al. 1999). Studies of MUC1 mRNA and protein expression in mice, rat, baboon, and rabbit have led to the hypothesis that reduction of MUC1 expression on the surface epithelium of the uterus is necessary for embryo implantation (DeSouza et al. 1998; Hild-Petito et al. 1996; Hoffman et al. 1998; Surveyor et al. 1995). Muc1 null mice have been generated, but they are healthy, normal and fertile, with the only phenotypes described being: delayed mammary tumor progression (not metastases) (Spicer et al. 1995), impaired maturation of T-lymphocytes (Gendler 2001), and increased ocular surface infection in some but not all strains bearing the null mutation (Danjo et al. 2000; Kardon et al. 1999). The lack of impairment of fertility in MUC1 null mice may indicate that the mucin has no vital role in the reproductive process in mice, and perhaps its removal in mice actually facilitates or enhances blastocyst adherence. Studies of endometrial samples in mid-proliferative (nonreceptive) and mid-luteal (receptive) phases of the menstrual cycle demonstrate that MUC1 expression is maintained in the luminal epithelium throughout the cycle, but there may be regional specialization in the pattern of expression at the level of carbohydrate structure (DeLoia et al. 1998). Recent work with in vitro models of human endometrial cells suggests that MUC1 acts as an endometrial anti-adhesive molecule that is locally removed by the human blastocyst at the implantation site (Meseguer et al. 2001). There is little direct in vivo information on the function of MUC1 in the various regions of epithelia of the human reproductive tract.

MUC4 has also been the subject of recent interest among those studying the reproductive tract. MUC4 has a widespread tissue distribution, being present in the simple epithelium of the bronchus, stomach, jejunum, ileum, colon, and prostate (Porchet et al. 1995), endocervix (Gipson et al. 1997) and trachea (Reid et al. 1997), and the stratified epithelium of the conjunctiva (Gipson and Inatomi 1997), ectocervix and vagina (Gipson et al. 1997). The role of MUC4 in cervical mucus is unclear. It is not known whether the extracellular domain is shed into the cervical mucus or whether it is retained on the endocervical epithelial cell surface to act as lubricant

and anti-adhesive. In rats, rMuc4 protein disappears from uterine epithelium at the time of blastocyst receptivity but does not appear to vary in protein content in cervical tissue (Idris and Carraway 1999; McNeer et al. 1998). Use of human-specific probes/antibodies to MUC4 will facilitate analysis of MUC4 in human uterine and cervical epithelia. In our hands, MUC4 mRNA levels were considerably lower in the uterus than in endocervix, at all stages of the menstrual cycle (Gipson et al. 1997). Similarly in rats, cervical and vaginal epithelia express higher levels of rMuc4 protein than does the uterus (Idris and Carraway 1999).

The repertoire of membrane-associated mucins in female reproductive tract epithelia is growing, and, additionally, studies in other systems suggest a role for the cytoplasmic domain of membrane-associated mucins in signal transduction. There is much to be learned about the function of the individual membrane-associated mucins in the reproductive tract epithelia.

10.7 Perspective

Studies of cervical mucin gene and protein expression with the hormone cycle have established the groundwork to determine the structural basis for physical changes in mucins in response to hormone status. The baselines on MUC5B and MUC4 expression will also facilitate future testing of functional roles of each mucin in the reproductive process and allow studies of potential roles of mucin gene expression in unexplained infertility.

Little is known regarding the regulation of mucin genes at the biosynthetic level via genomic hormone response elements. Development and characterization of an appropriate endocervical cell line that expresses MUC5B and MUC4 will be necessary for study of regulation of mucin gene expression by this epithelium. In addition, studies of the effects of hormones on post-translational glycosylation of mucins may yield important information regarding mucin character and function during the cycle.

From a clinical and practical standpoint, availability of cDNA probes and protein core antibodies, as well as carbohydrate epitope probes to cervical mucins, will allow direct assay of alterations in

cellular and secreted-mucin content with disease, age, and infertility. Finally, knowledge of mucin character/regulation and its functional role may yield clues to more effective methods of vaginal contraception.

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11 Fallopian Tube Physiology: Preliminaries to Monospermic Fertilization and Cellular Events Post-fertilization

R. H. F. Hunter

11.1 Introduction

The evolution of systems of internal fertilization has been accompanied by diverse specializations of the genital tract in eutherian mammals. Placing the focus on females, progressive elaboration of the Müllerian duct system has generated various forms of cervix, uterus and utero-tubal junction whereas the cranial region of the Müllerian ducts remains distinguishable as paired Fallopian tubes. These structures can be divided into isthmus and ampullary portions, with the ampulla merging into the fimbriated extremity of the infundibulum that is applied to or envelops the gonad. Fertilization takes place at the ampullary-isthmic junction of the Fallopian tube (Fig. 1), to

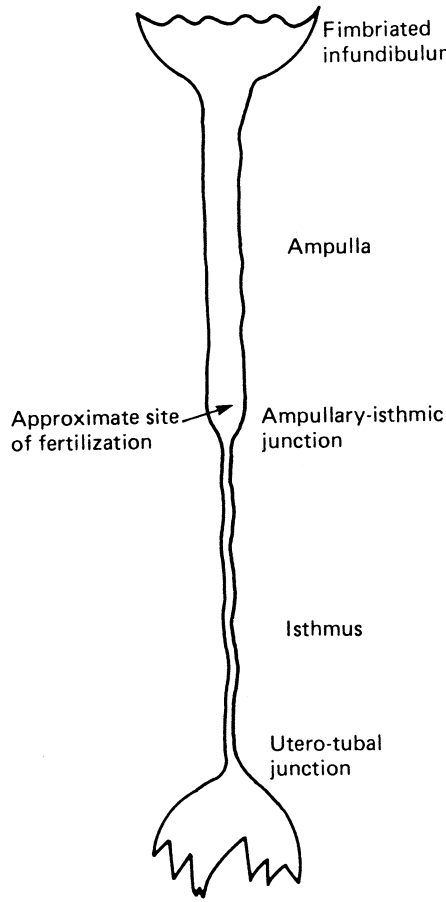

Fig. 1. Linear representation of a Fallopian tube to depict the thin-walled ampulla leading into the strongly muscular isthmus. The site of fertilization – that is of successful gamete fusion – is the region where the ampulla merges into the isthmus

which region the newly-ovulated oocyte(s) is transported within 10–12 min in rabbits (Harper 1961 a, b), rather more slowly in sheep (Wintenberger-Torres 1961) and 30–45 min in pigs (Hunter 1974). Such relatively rapid timing nonetheless permits a further phase of maturation in the oocyte(s) after its dramatic change of milieu from

the gonad – from a Graafian follicle – to the genital duct (for a review see Hunter 2003 a). Molecular modifications in the oocyte surface would be anticipated.

Physiological aspects of Fallopian tube function have received various reviews in recent years. Because the present treatment will be relatively narrow, interested readers are referred to the essays of Harper (1994), Yanagimachi (1994), Croxatto (1996), Buhi et al. (1997), Hunter (1997, 2001, 2002 a), Leese et al. (2001), Rodriguez-Martinez et al. (2001), Suarez (2001) and Hunter and Rodriguez-Martinez (2004).

11.2 Sperm Interactions with Müllerian Ducts

Turning to the male gamete, spermatozoa encounter differing epithelial surfaces as they progress towards the Fallopian tubes although, for a proportion of spermatozoa ejaculated and suspended in a mixture of male and female fluids, the first contact with solid tissues may be at the utero–tubal junction. Beyond this region of morphological constriction, sperm cells encounter anatomical and physiological specializations *en route* to the site of fertilization. The foremost of these constitutes a principal topic of this essay. In modern literature, it is usually referred to as the caudal isthmus, and this region is now recognized as the functional sperm reservoir, the site from which fertilizing spermatozoa are activated and released close to the time of ovulation (Hunter et al. 1980; Hunter 1981). After the normal sequence of a preovulatory mating, viable spermatozoa entering the caudal isthmus exhibit suppressed motility and a specialized form of attachment to the epithelium (for reviews see Overstreet and Katz 1977; Drobnis and Overstreet 1992). Because spermatozoa are highly active when in suspension in the uterus, the significant reduction in motility when they enter the isthmus early in estrus can be interpreted as an adaptation for preovulatory storage in a cell with little residual cytoplasm. Suppression of motility appears to be brought about by a combination of factors which include:

a Tight constriction in the layers of the myosalpinx
b Accumulation of viscous glycoprotein secretions in this region of the duct

c A specific ionic environment within the luminal fluid
d A reduced temperature before ovulation (for reviews see Hunter 1995 a, 2002 a).

Adhesive contacts with the epithelium may explain a dramatic reduction in motility, but the reciprocal situation needs to be considered also. The viscous glycoprotein secretions that accumulate in the caudal isthmus serve to isolate the arrested spermatozoa from metabolic stimulation that would be caused by further contact with uterine or ampullary fluid (Hunter 2001, 2002 a).

Over and above stabilizing the male gamete and conserving its limited reserves of energy, the preovulatory phase of binding may be part of a mechanism to suppress completion of capacitation and provide direct communication with underlying changes in the endosalpinx. Stored spermatozoa need some means of recognizing the status of the ovaries and imminence of ovulation in order that a controlled activation and release are closely coordinated with shedding of an oocyte from a Graafian follicle. Changes in endocrine activity of such preovulatory follicles are thought to be the key, secreted hormones acting locally on tissues of the Fallopian tube. Membranous contact between a sperm cell and epithelium would facilitate such coordination. During estrus and close to the time of ovulation, a counter-current exchange between the ovarian vein and tubal branch of the ovarian artery enables concentrations of estradiol 15 times higher than in the systemic circulation and those of progesterone 20 times higher to reach tissues of the isthmus (Table 1; Hunter et al. 1983; Hunter 1995 a, 1997).

11.3 Contractile Activity of Myosalpinx

What appears to have been overlooked in the earlier discussions concerned with sperm binding is a need – indeed an absolute requirement – to counteract the potential influence of waves of contractile activity in the smooth muscle layers of the Fallopian tube. Such intrinsic contractility could act to displace a population of spermatozoa already established in the caudal isthmus, thereby compromising or removing the steep preovulatory sperm gradient that is essential

Table 1. Evidence for a counter-current mechanism within the ovarian pedicle of estrous animals for relatively high concentrations of gonadal hormones

Time of collection[a]	Sample details		Estradiol (pmol/l)	Testosterone (nmol/l)	Andro-stenedione	Proges-terone[b]	PGF-2α	PGE-2
	Site of collection	Adjacent ovary						
1st day of estrus (12–16 h)	Right isthmus	11 follicles of 8 mm	>1836	8.7	132	36.2	17.5	0.5
	Left isthmus	4 follicles of 8 mm	460	1.5	13	2.7	24.3	1.3
	Peripheral		109	<0.7	1	1.2	3.1	0.4
2nd day of estrus (20–24 h)	Right isthmus	5 follicles of 10 mm	229	0.7	5.3	9.7	7.3	0.7
	Left isthmus	7 follicles of 10 mm	1691	1.2	17.4	10.1	15.5	1.1
	Peripheral		136	<0.7	<0.7	3.3	2.3	0.4
2nd day of estrus (36–40 h)	Right isthmus	9 recent ovulations	74	<0.7	1.4	267	9.0	0.9
	Left isthmus	5 recent ovulations	47	<0.7	1.2	149	6.5	0.2
	Peripheral		30	<0.7	<1.0	9.1	2.5	0.7

The measurements are of steroids and prostaglandins in blood plasma sampled from the arterial arcade bordering the isthmus of each Fallopian tube and from the systemic circulation in three pigs at different stages of estrus. Indicates below the figure given, which is the sensitivity of the assay. [a] Approximate time after onset of estrus. [b] The sensitivity of this assay was 1 nmol/l. If Graafian follicles are the source of most of the androstenedione in the isthmus blood samples, then it is of interest that relatively high quantities of androstenedione can pass from the ovaries into the circulation rather than passing inwards from thecal to granulosa cells for aromatization to estradiol.

up to the site of fertilization. Alternatively, cells might be displaced in a retrograde manner through the utero-tubal junction. Contractile activity of the myosalpinx is heightened under the influence of ovarian estradiol secretion, for elevated titers of this steroid potentiate α-adrenergic activity in the myosalpinx (for reviews see Hunter 1977, 1988). In terms of frequency and amplitude, contractions reach a peak during the period of estrus and close to the time of ovulation (Seckinger 1923; Wislocki and Guttmacher 1924). Both peristaltic and anti-peristaltic waves have been recorded. Thus, the endocrine stage is set to facilitate displacement of particulate matter within the lumen of the tube by means of contractile movements.

Demonstrations of an ability of the Fallopian tubes to displace substances or particles in an ad-ovarian direction exist in several species, including our own. For example, a classical test of patency in putatively infertile women is instillation of a radio-opaque material in the uterine fundus and observation of its passage through one or both Fallopian tubes with spillage into the abdominal cavity. In laboratory species, there was the elegant demonstration of periovulatory displacement of microdroplets of oil or India ink from the caudal isthmus to the ampullary-isthmic junction in golden hamsters (Battalia and Yanagimachi 1979, 1980). In rabbits and pigs, pre- and periovulatory waves of contraction in the isthmus proceed principally towards the ampullary-isthmic junction, as does the beat of endosalpingeal cilia (Blandau and Gaddum-Rosse 1974). Working with an in vitro system using portions of the isthmus taken just before ovulation, *Lycopodium* spores or microspheres of approximately 15 μm diameter were transported to the ampullary-isthmic junction in a current of fluid (Blandau and Gaddum-Rosse 1974). In the light of such observations, it seems reasonable to presume that spermatozoa suspended in the lumen of the caudal isthmus would – if unattached – be propelled onwards by contractile activity towards the site of fertilization with the ensuing risk of polyspermic penetration of the vitellus.

11.4 Sperm Binding in Relation to Multiple Mating

There are further considerations of physiological significance. As demonstrated most clearly in domestic farm animals, the presence of

a mature male in the vicinity of estrous females leads to increased contractile activity in their tubular genitalia. Mounting, mating and ejaculation further enhance such contractile activity. This is primarily by means of a neuro-endocrine loop expressed as a release of oxytocin, but it is also due to the influence of seminal prostaglandins (Kelly et al. 1976; Poyser 1981). Despite these influences of mating and ejaculation on the contractile activity of the Fallopian tubes, double mating of pigs (Hunter 1984) or multiple mating of sheep (Hunter and Nichol 1986) after establishment of a functional sperm reservoir in the first part of estrus failed to displace viable spermatozoa from the caudal isthmus towards the site of fertilization. Alternatively, if there was dislodgement of spermatozoa, such cells rapidly became moribund or dead. In other words, the presence of mature males in the close vicinity of estrous females and, more specifically, the occurrence of multiple mating before ovulation do not act to increase the risk of polyspermic fertilization. The highly controlled periovulatory mechanisms of sperm release continue to take their normal course, preovulatory coital stimulation notwithstanding, and it is essential that this should be so for maintenance of a successful breeding population.

Avid binding of spermatozoa is thereby emphasized to be of special significance in facilitating normal fertilization and avoiding the pathological condition of polyspermy. Various experiments have shown that artificially increasing the numbers of spermatozoa at the site of fertilization shortly before ovulation leads to polyspermic penetration of the egg cytoplasm (Hunter and Léglise 1971; Hunter 1972, 1973, 1976), even if not necessarily to the formation of polyploid embryos (Beatty 1957; Bomsel-Helmreich 1965, 1971). Molecular events underlying specific binding of viable spermatozoa to organelles of the epithelium – to cilia or microvilli or both – have begun to be examined in detail. Cell adhesion molecules on the plasmalemma overlying the sperm head may be critical, and thus the integrity of this membrane could assume a vital role in selection of competent sperm cells destined to undergo a phase of storage. In the case of bull spermatozoa, carbohydrate moieties are a principal feature of the cell–cell adhesive interactions and involve fucose and a fucose-containing ligand on the epithelium (Lefebvre et al. 1997; Suarez 1998, 2001; Revah et al. 2000).

11.5 Controlled Release of Spermatozoa From Caudal Isthmus

Whereas the previous paragraph sheds some light on the significance and means of binding, the manner of periovulatory release of viable spermatozoa from temporary attachment to the epithelium remains an outstanding question (Fig. 2). One view is that spermatozoa become fully capacitated in the caudal isthmus and, as a consequence,

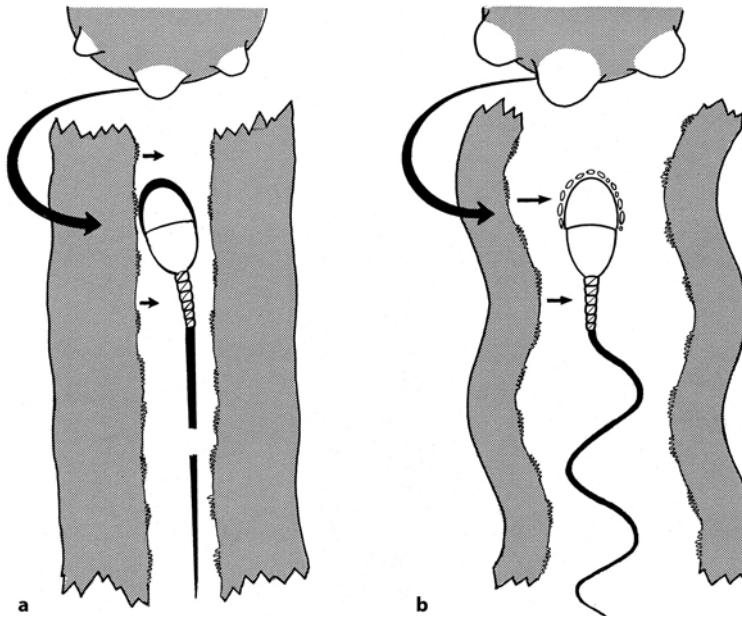

Fig. 2. Model to illustrate the way in which the endocrine activity of pre- or periovulatory Graafian follicles acts locally via a vascular counter-current system to program the membrane configuration and motility of spermatozoa in the lumen of the Fallopian tube isthmus. Gonadal hormones (from the follicles) act on the tubal epithelium whose transudates and secretions in turn influence the nature of the luminal fluids. Completion of capacitation is reasoned to be a periovulatory event, at least in the large farm species with a protracted interval between the gonadotrophin surge and ovulation. By contrast, a preovulatory strategy in the Fallopian tube would necessarily have been to suppress completion of capacitation. **a** Intact, relatively quiescent

express hyperactivated motility and break free from epithelial binding (see Smith 1997; Suarez 1998). The present author does not subscribe to such a scenario. Even if spermatozoa were able to express whiplash motility within the confines of the caudal isthmus and its viscous secretions, it would be tactically disastrous to complete the process of capacitation in that region of the duct. Full capacitation finds expression in both hyperactivated motility and a coordinated acrosome reaction. The proposed completion of capacitation in the caudal isthmus sits uneasily with claims that the acrosome reaction is induced by subsequent sperm head contact with the zona pellucida (see Yanagimachi 1994; Suarez 2001). If full capacitation were to be achieved in the caudal isthmus, it seems highly improbable that the destabilized sperm cell could progress up the length of the isthmus and penetrate through the cumulus oophorus with a vesiculated acrosome still *in situ* on the anterior portion of the sperm head. More probably, displacement would have occurred, rendering the sperm cell non-functional.

An alternative explanation for liberation from epithelial binding has been put forward. Under the influence of elevated but changing titers of ovarian follicular hormones transferred to the mucosa of the caudal isthmus by a vascular counter-current exchange (Hunter et al.

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spermatozoon under the overall influence of preovulatory follicles. Membrane vesiculation on the anterior part of the sperm head is suppressed, as is the development of whiplash activity in the flagellum, presumably due to local molecular control mechanisms. The lumen of the isthmus is extremely narrow and contains viscous secretions, and myosalpingeal contractions are reduced. The heads of viable spermatozoa are bound to the epithelium. **b** An acrosome-reacted, hyperactive spermatozoon under the influence of Graafian follicles on the point of ovulation. The patency of the isthmus has begun to increase, allowing a more powerful pattern of flagellar beat. Progression of such spermatozoa to the site of fertilization may also be aided by enhanced contractile activity of the myosalpinx, and yet involves a strict numerical regulation. In fact, hyperactivation and an acrosome reaction in a fertilizing spermatozoon may develop only in the vicinity of the egg. A premature acrosome reaction would risk displacement of the vesiculated membranes before the site of fertilization. The model accords with the observation that high proportions of spermatozoa undergoing the acrosome reaction in ruminants are found predominantly in the ampulla adjoining the ovulatory ovary and only at or following ovulation

1983; Hunter 1995 a), ionic conversations between an attached sperm head and the endosalpinx result in Ca^{2+} ion traffic into the sperm cell. As a consequence of increased intracellular levels of Ca^{2+} ions, there would be a controlled downgrading in the expression of binding molecules on the plasmalemma and corresponding modifications would be anticipated in the endosalpingeal surface (Hunter 1997; Hunter et al. 1999). Threshold titers of progesterone reaching the isthmus mucosa may be the key in this interpretation. Progressive sperm motility would be restored, thereby coordinating release of an oocyte from its Graafian follicle with release of spermatozoa from the caudal isthmus (Hunter 2001, 2003 b).

Suppression of full capacitation is seen as an essential feature of preovulatory sperm storage in the caudal isthmus (Hunter 1996, 1997). The process of capacitation in a fertilizing sperm cell would be completed in the vicinity of an oocyte within the unique fluid environment at the ampullary-isthmic junction. This region would itself contain high concentrations of progesterone, in part due to the steroid content of follicular fluid, in part as a reflection of postovulatory steroid synthesis by the suspension of cumulus (granulosa) cells. Furthermore, progesterone might be acting directly on the sperm surface to precipitate an acrosome reaction, a so-called nongenomic influence (Austin et al. 1973). It could also be acting locally on the epithelium of the ampullary-isthmic junction to program selective transudation and specific secretion. The zygote remains in this region of the duct for a relatively prolonged period. Activation of genes in the endosalpinx as a response to differing numbers of embryos and differing populations of cumulus cells is currently being examined (Hunter and Einer-Jensen 2003; Hunter et al. 2004). Amplification of early embryonic signals by the suspension of cumulus cells is of particular interest.

11.6 Postovulatory Attenuation of Sperm Storage

As a postscript to these vital events, oocytes would undergo monospermic penetration with almost coincident activation of a cortical reaction leading to prompt establishment of a block to polyspermy (Austin 1956; Szöllösi 1962, 1967; Fléchon 1970; Guraya 1983).

The zygote would no longer be vulnerable to polyspermy, and control of sperm numbers passing from the isthmus reservoir to the site of fertilization would undergo progressive relaxation, commencing within 1–2 h of ovulation. In species such as the pig, relaxation of the myosalpinx and reduction of mucosal edema are reflected in increasing numbers of accessory spermatozoa on or in the substance of the zona pellucida. After natural mating, the numbers of accessory spermatozoa per individual embryo can reach 200–400 by the four-cell stage of development, sperm heads being trapped within the outer portion of the zona (Hunter 1974). The ploidy of the embryo is not compromised and, by the time of embryo passage through the isthmus into the uterus, competent spermatozoa are no longer bound to the epithelium of this preovulatory reservoir.

The suspension of cumulus (follicular granulosa) cells is similarly displaced to the uterus close to the time of embryo passage, although frequently somewhat in advance, as observed in domestic animals (Hunter 1978). By no means all of these ovarian somatic cells are yet moribund or dead, so it is of special interest to consider their possible influence on the establishment of pregnancy (Hunter et al. 2004). Their secretions could inform the uterus of the imminent arrival of an embryo and they might prompt changes in the endometrium to prepare for the onset of implantation. These proposals do not, of course, argue that such programming is essential for the establishment of pregnancy.

11.7 Considerations in Primates

Features of the functional sperm reservoir as described above for laboratory and domestic farm species may or may not be applicable to primates. The caudal isthmus of the Fallopian tube is represented by the intramural portion and, in our own species, contains a prominent mucus in the late follicular phase (Patek 1974; Jansen 1978). Arguments have been presented in two publications as to why the intramural portion of the human Fallopian tube could act as a functional sperm reservoir (Hunter 1987, 1995b) but, as of writing this review, no overwhelmingly persuasive evidence is available to this author. There is, however, a related suggestion that – during the fol-

licular phase – transport of human spermatozoa may be preferentially towards the Fallopian tube adjoining the ovary with an ovulatory follicle. Such a bias could reflect local endocrine programming of the duct system by the ovulatory ovary.

There is one other area of equivocation. Whereas residence in the Fallopian tubes can be shown to be of importance in laboratory and farm species for promoting normal development of an embryo, this appears not to be so in primates. Classical experiments in infertile women involving transplantation of an ovary to the uterus in instances of blocked Fallopian tubes – Estes' operation – suggest that pregnancies have been generated by this approach (Estes and Heitmeyer 1934; Preston 1953; von Ikle 1961). Neither oocyte nor spermatozoa would have been exposed to the Fallopian tubes, although secretions from these structures may have entered the uterus to influence both the gametes and an embryo. More persuasive still is the body of evidence accumulating from infertility clinics. Introduction of secondary oocytes directly into the uterine cavity either after bouts of coitus or in combination with insemination of a suitable sperm suspension can generate full-term pregnancies. The author has attempted to interpret such results in terms of the morphology of the utero-tubal junction in women and a substantial overlap in the composition of tubal and uterine luminal fluids, even if not specific mixing (Hunter 1998).

As an extension of such proposals concerning the absence of distinguishable fluid compartments, and in conjunction with fragments of endometrium developing within the Fallopian tube as one form of endometriosis, there is a further consideration of clinical relevance. Overlap in the composition of tubal and uterine fluids together with proliferating portions of endometrial tissue may be an important explanation for instances of tubal ectopic pregnancy (Hunter 2002b).

11.8 Concluding Remarks

The previous section of this essay touches on the approach of in vitro fertilization and culture of embryos. As noted in the Preface to a monograph some years ago, the ever-increasing extent of in vitro studies has tended to divert attention away from a closer physiologi-

cal examination of more subtle contributions of the Fallopian tubes (Hunter 1988). Now that so much experimentation has at least one foot on the molecular stage, it could be opportune once again to take note of that remark. The embryonic genome commences to be expressed during passage of the embryo down the Fallopian tubes, and components of the fluid milieu may have a specific impact on gene expression. Diverse proteins and glycoproteins, not least those resembling oviductin (Robitaille et al. 1988) could be influencing the pattern and/or extent of early gene expression. Whilst embryonic contact with fluid components of the Fallopian tube is clearly not essential for generating a pregnancy, the overall efficiency of reproduction and the normality – both anatomical and physiological – of the offspring so produced may well be superior with in vivo exposure throughout. There is accumulating evidence that in vitro procedures can alter the nature of gene expression in developing embryos (Wrenzycki et al. 1999; Lonergan et al. 2003).

One explanation for the surprisingly successful outcome of various in vitro techniques is that there is invariably selection of gametes before the in vitro fertilization procedures are embarked upon and invariably selection of embryos and indeed of recipients before the final step of transplantation into the uterus. Overall results therefore need to be interpreted with caution, especially should a ranking of individual fertility clinics be involved or should there be a commercial aspect.

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