EFFECTS OF MIFEPRISTONE ON THE HUMAN ENDOMETRIUM AND THE FALLOPIAN TUBE DURING THE LUTEAL PHASE

Xiaoxi Sun

Stockholm 2005
To my family
ABSTRACT

Mifepristone given postovulatory has been shown effective for contraception, but the precise mechanism of action is still poorly understood. A better understanding of the mechanisms of action of mifepristone, when used for contraception, is important for further development and optimizing the regimen of use.

To study the effects of mifepristone during the luteal phase, a single dose of 200mg mifepristone was administered on day LH+2. Endometrial biopsies were obtained on day LH+6 to LH+8 (the expected time for endometrial receptivity and implantation) and the fallopian tube on day LH+4 to LH+6 (approximately the time when the embryo is still in the fallopian tube). Immunohistochemistry, RT-PCR and Western blot were used for analysis of the endometrium and the fallopian tube samples.

After treatment with mifepristone, progesterone receptor isoform B (PR-B) concentrations increased in glandular cells of the endometrium and in epithelial and stromal cells in the fallopian tube. In the endometrium endothelial nitric oxide synthase (eNOS) expression was attenuated in the glandular epithelium, in contrast to endothelial eNOS, which was not changed. The expression of insulin-like growth factor binding protein-1 (IGFBP-1) was significantly increased in the glandular epithelial cells. The staining intensity of heparin-binding epidermal growth factor-like growth factor (HB-EGF) was not affected by mifepristone. Treatment with mifepristone increased the immunostaining of HB-EGF receptor HER1 in the epithelium and the stroma in the endometrium, while the opposite was seen in the luminal epithelium of the fallopian tube. The immunostaining of HB-EGF receptor HER4 increased in the epithelial cells of the endometrium, while a decrease was seen in the stroma of the fallopian tube.

In conclusion these results indicate that mifepristone administered immediately after ovulation has pronounced effects on the endometrium at the expected time of endometrial receptivity. These changes may contribute to cause defective endometrial receptivity and altered intraluminal milieu. The effect on the fallopian tube may also be of importance for the contraceptive effect of mifepristone.

Key words: mifepristone, endometrial receptivity, emergency contraception, PR-B, eNOS, IGFBP-1, PRL, HB-EGF, HB-EGF receptors HER1 and HER4
LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:


IV. Xiaoxi Sun, Kristina Gemzell-Danielsson, Hongzhen Li, Berit Ståbi, Anneli Stavreus-Evers. Expression of heparin-binding epidermal growth factor-like growth factor and its receptors in the human fallopian tube and endometrium after treatment with mifepristone. Submitted
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<td>androgen receptor</td>
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<td>ART</td>
<td>assisted reproductive technology</td>
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<td>COH</td>
<td>controlled ovarian hyperstimulation</td>
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<td>COX</td>
<td>cyclooxygenase</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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1 INTRODUCTION

Implantation is a sequence of events whereby the blastocyst becomes attached to the uterine wall. A synchronization between the developing embryo and the maturing endometrium seems to be essential for successful implantation (Navot, et al 1991). Implantation is composed of three distinct and consecutive phases: apposition, attachment and invasion. The human endometrium is receptive to blastocyst implantation only during a very narrow time in the luteal phase, the implantation window, which occurs approximately 7 days after the luteinizing hormone (LH) surge (Davis and Rosenwaks 1993). The implantation window can be defined as the period where the uterus is receptive for implantation.

Medical treatment of infertility has increased in recent years and it is estimated that 1.33% of live births in Europe are the result of assisted reproductive technologies (ART) (Nygren and Andersen 2001). Implantation failure is considered to be the major limiting factor of successful infertility treatment (Devroey, et al 2004). Since the endometrium is essential for blastocyst implantation it is an important target for fertility regulation.

1.1 THE HUMAN FEMALE REPRODUCTIVE ORGAN

The female reproductive organ consists of ovaries, fallopian tube, uterus, cervix and vagina. The reproductive functions in human females are cyclic. The shifting balance of hormones during the ovarian cycle is responsible for regulation of biological processes in the reproductive organ.

1.1.1 Uterus

The uterus is a pear-shaped muscular organ into which the fallopian tubes open. The uterine wall is divided into three major parts: an outer serosal layer (peritoneum), an intermediate layer of smooth muscle cells (myometrium), and an inner mucosal lining (endometrium) (Figure 1). The endometrium consists of two layers: a lower basal layer (basalis) at the junction with the myometrium, and an upper functional layer (functionalis) lining the lumen. The functionalis undergoes cycles of proliferation, secretion and degeneration. The purpose of the functionalis layer is to prepare the endometrium for implantation.
1.1.1.1 Composition of the endometrium

Figure 1. The human endometrium

1.1.1.1.1 Luminal epithelium
The luminal epithelium is the first maternal interface encountered by the implanting blastocyst. It contains both ciliated and non-ciliated cells. The proportion of non-ciliated to ciliated cells varies during the menstrual cycle; initially there is an increase from the early to the late proliferative phase. There is a subsequent decrease after day 20 (Ferenczy, et al 1972, Masterton, et al 1975). The changing proportions seem to be determined by fluctuations in the levels of ovarian steroids. Non-ciliated cells form pinopodes at the time of implantation (Martel, et al 1981). The ciliated cells might promote uterine fluid flow, thus influencing distribution of uterine secretions, movement of spermatozoa and migration of the ovum.

1.1.1.1.2 Glandular epithelium
The glandular epithelium consists of a single layer of columnar epithelial cells, whose height varies throughout the menstrual cycle. The proliferative glands enlarge and become more tortuous and the cytoplasmic content of the cells increases as RNA accumulates. Glandular secretion occurs in the progesterone dominant secretory stage. Vacuoles and granules appear in the cell cytoplasm and the position of nuclei within glandular cells alters during the cycle. Substances like glycogen and glycoproteins are secreted into the glandular lumen (Dockery, et al 1988a, Dockery, et al 1988b). The presence of giant mitochondrial profiles during early luteal phase might provide energy for the dynamic changes occurring within the cells at this time (Dockery, et al 1997).
1.1.1.3 **Stroma**
The stromal cells include fibroblasts, perivascular cells and leukocytes, i.e. granulated leukocytes, lymphocytes and macrophages. Stromal decidualization, essential for successful implantation, is initiated during the midsecretory stage of the menstrual cycle, when transformation of endometrial stromal cells into decidual cells occurs. The decidua is permissive for trophoblast invasion and at the same time impedes invasive trophoblasts by forming a physical barrier (Kearns and Lala 1983, Kliman 2000). The large accumulation of leukocytes in stroma during the receptive phase may aid in controlling trophoblast invasion and in immunosuppression (Pijnenborg 2002). It has been shown that women with unexplained infertility have alterations in the leukocyte populations (Klentzeris, et al 1994).

1.1.1.4 **Blood vessels**
The blood supply to the endometrium comes from the arcuate arteries in the myometrium. Radial arteries arise from the arcuate arteries towards the endometrium. After crossing the endo-myometrial junction, radial arteries branch into the small basal and spiral arteries. The small basal arteries supply the basal endometrium and these arterioles are not affected by cyclic hormonal changes. The spiral arterioles run towards the endometrial surface (functional layer). The endometrial spiral arterioles are acutely hormone sensitive (Ramsey 1982). Unlike other vascular beds, the endometrial vasculature undergoes cycles of growth and regression during the menstrual cycle (Rogers 1996). Although circulating sex steroids may exert some overall control over these vessels, local factors have been increasingly demonstrated to be of primary importance (Pepper, et al 1996, Smith 1995).

1.1.1.5 **Extracellular matrix (ECM)**
The ECM compartment consists predominantly of collagen and proteoglycans, which are produced mainly by stromal fibroblasts. There are two functions of the ECM. First, it acts as a structural framework for the tissue, and second, it interacts, via integrins and other receptors, with the resident cell populations of the uterus (Aplin 1997).

1.1.1.2 **The menstrual cycle**
The menstrual cycle is divided into a proliferative, a secretory and a menstrual phase. During the proliferative phase, when increased estrogen levels are induced by ovarian follicular growth, the endometrial glands grow from narrow tubular structures lined by
a single layer of columnar epithelium to complex branched structures, whereas the stroma becomes a loose syncytium-like structure. The spiral vessels grow in length during this stage. The endometrial thickness increases from a postmenstrual state of 0.5 mm to 3.5-10.0 mm. In the following secretory phase, development in the endometrium is influenced by combined actions of estrogen and progesterone, leading to further development and differentiation to assume specialized functions in order to facilitate implantation. The secretory phase is characterized by increased edema, coiling of the spiral arteries and decidualization of the stroma. The implantation occurs during the mid-secretory phase. In absence of implantation, the transient corpus luteum undergoes regression which results in an abrupt decrease in estrogen and progesterone levels with subsequent shedding of the functionalis layer.

1.1.1.3 The implantation window
The implantation window is defined as the limited period during which the uterus is receptive for implantation of a blastocyst. There are several ways to date the endometrium, especially in relation to the implantation window. The most common is dating according to Noyes et al 1950, where the cycle length is standardized to 28 days. The implantation window is assumed to coincide with cycle day 20-22 in a standardized cycle (Davis and Rosenwaks 1993, Noyes, et al 1950), around 7 days after the LH surge (Lessey 2000). This period corresponds well with the presence of endometrial pinopodes on the apical surface on endometrial epithelial cells (Martel, et al 1981). Morphometric analysis, a detail quantitative and histological technique, has been used to study the endometrial receptivity (Dockery, et al 1988b, Li, et al 1988). Despite historical importance and wide acceptance among clinicians and pathologists, the accuracy and utility of the traditional endometrial dating criteria have been repeatedly questioned (Coutifaris, et al 2004, Murray, et al 2004). Observed and expected histological dates correlate much better when the days of ovulation and tissue collection are defined prospectively, using the mid-cycle urinary LH peak or the collapse of the dominant follicle as observed by serial transvaginal ultrasound examinations as a reference point (Johannisson, et al 1987, Jordan, et al 1994, Shoupe, et al 1989).

1.1.2 Fallopian tube (Oviduct)
The fallopian tube is a tubular organ connecting the peri-ovarian space with the uterus. From the ovary to the uterus, fimbria, ampulla, isthmus and intramural segments are
distinguished (Figure 2). Moving from the periphery to the centre, a cross section shows mesosalpinx, myosalpinx, endosalpinx and finally the lumen. The myosalpinx is composed of smooth muscle which is very thin in the ampulla and thick in the isthmus. The endosalpinx is a mucosa lined by monolayered epithelium. In the ampulla, ciliated cells are the more abundant cells. The epithelial surface of the ampulla is huge, suggesting a great capacity for metabolic exchange between the luminal, cellular and vascular compartments. In the isthmus, the mucosa presents few folds and abundant secretory cells. When ovulation occurs, the fimbria catches the oocyte, which is then transported along the ampullary segment. From here, spermatozoa adhering to the epithelium are progressively released so that they will arrive at the fertilization site one by one until the oocytes are fertilized. Following fertilization, the zygote enters isthmus and starts its pre-implantation development, while it is carried to the uterus. The morphological and functional characteristics of the oviduct are controlled by ovarian steroids (Croxatto 2002).

Figure 2. The human fallopian tube. Modified from Lippincott Williams & Wilkins (2003)

1.2 THE EMBRYO

Fertilization of the ovum takes place in the ampullar part of the fallopian tube and the fertilized oocyte enters the uterine cavity as a morula approximately 96-120 hours after the LH surge, which is just before the expected window of implantation (Croxatto 2002). At this time, the 12- to 16-cell stage morula consists of a group of centrally located cells, the inner cell mass, and a surrounding layer, the outer cell mass. The inner
cell mass will give rise to the tissues of the embryo proper, while the outer cell mass forms the trophoblast, which contributes to the placenta (Edwards 1994).

1.3 THE IMPLANTATION PROCESS
The implantation process is defined as a sequence of events by which the embryo attaches to the uterine wall, penetrates the maternal epithelium and circulatory system to form a placenta. The first step in implantation is the initiation of a dialogue between the free floating blastocyst and the endometrium. This serves to synchronize endometrial maturation and embryo development, and is mediated by hormones, cytokines and growth factors (Carson, et al 2000). This is followed by apposition where the embryo loosely attaches to the endometrial epithelium by its abembryonic pole. Local paracrine signaling between embryo and endometrium occurs to trigger a stronger attachment, during which trophectoderm cells from the blastocyst migrate between the epithelial cells, displacing them and penetrating as far as into the basement membrane. The trophoblast then invades through the basement membrane, making contact with the underlying stroma. If the endometrium is not competent to participate in all these different stages, implantation will fail (Sharkey and Smith 2003).

1.4 FACTORS OF IMPORTANCE FOR ENDOMETRIAL RECEPTIVITY
Spatiotemporal elaboration of various cytokines, growth factors, adhesion molecules and other factors in the uterus by steroid hormones is thought to play an important role in uterine preparation for implantation (Beier and Beier-Hellwig 1998, Cavagna and Mantese 2003). Several biochemical factors of presumed importance to endometrial receptivity have recently been discovered.

1.4.1 Steroid hormones and receptors
1.4.1.1 Steroid hormones
Steroid hormones are important regulators of uterine receptivity. The endometrium undergoes pronounced morphological and physiological alterations in response to the steroid hormones. In the first part of the menstrual cycle, the proliferative phase, the level of estrogen is high. Estrogen initiates hypertrophy and hyperplasia of endometrial epithelia. Following ovulation, the second part of the cycle, progesterone is the dominant hormone and the main regulator of endometrium development. Progesterone
transforms the prepared endometrium into a secretory tissue and creates an environment within the uterine milieu that is conducive to embryo implantation. 

In the fallopian tube, estrogen induces hypertrophy, maturation and an increase in cell height of non-ciliated secretory epithelial cells, whereas progesterone causes atrophy and diminished secretory activity (Leese, et al 2001).

Steroid hormones act by way of their intracellular receptors to regulate gene expression of their downstream effectors, including peptide hormones, cytokines and growth factors (Bagchi, et al 2001).

1.4.1.2 Steroid hormone receptors

Tissue-specific responses to estrogen and progesterone and their analogs may be due to the differential expression of estrogen and progesterone receptor isotypes (ERα, ERβ, PR-A and PR-B) in target cells/tissues. Both ER and PR isoforms are seen in epithelial and stroma cells of human endometrium throughout the menstrual cycle (Noe, et al 1999). In general, the expression of ER and PR expression increases during the proliferative phase and decreases during the secretory phase of the menstrual cycle (Matsuzaki, et al 1999, Mote, et al 1999).

The human progesterone receptor (PR) exists as two functionally distinct isoforms, PR-A and PR-B, which arise from different promoter usage in a single gene. PR-B differs from PR-A by containing an additional 164 amino acids at the N-terminus. As ligand activated transcription factors, both PR-A and PR-B contain a centrally located DNA binding domain (DBD), which is flanked at the N terminus by an activation function (AF-1) and at the C-terminus by a hinge region containing nuclear localization signals as well as a ligand binding domain (LBD) containing a second activation function (AF-2). A third activation function (AF-3) is located within the N-terminal region specific to PR-B. This part encodes a transactivation function to PR-B that is crucial for specifying target genes activated by PR-B and not by PR-A (Figure 3).

In the human endometrium the concentrations and ratio of PR-A to PR-B vary during the menstrual cycle (Mangal, et al 1997, Mote, et al 2000). Their relative concentrations are crucial for the tissue response to progesterone (Tung, et al 1993, Wen, et al 1994). In tissue culture, PR-A and PR-B exhibit different transactivation properties that are specific to particular cell types and promoter contexts. PR-B has been shown to function as a stronger activator of transcription of several PR target genes regulated by both receptors (Richer, et al 2002). When the A and B receptors are coexpressed in cells where the endogenous PR-A gene is inactive, the expressed PR-A
can transrepress PR-B as well as the activity of other nuclear receptors (Vegeto, et al 1993). In addition, PR isoforms display a differential response to progestin antagonists. Although antagonist-bound PR-A is inactive, antagonist-bound PR-B can be converted into a strongly active transcription factor (Conneely and Lydon 2000).

Studies in PR-A and PR-B knockout mice (PRAKO and PRBKO, respectively) have revealed that PR-A and PR-B have distinct functions in the endometrium. In PRAKO mice, the decidualization process in response to progesterone is inhibited (Mulac-Jericevic, et al 2000), suggesting that PR-A plays an important role in decidualization of the stroma prior to implantation. 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 anti-proliferative and implantation associated responses to progesterone (Mulac-Jericevic, et al 2003). 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).

**Figure 3.** Structure organization of the human PR-A and PR-B isoforms.

### 1.4.2 Pinopodes

Pinopodes are progesterone associated organelles, appearing as apical cellular protrusions that become visible approximately on days LH+6 to LH+10 during the secretory phase, as shown by scanning electron microscopy in endometrial biopsies (Stavreus-Evers, et al 2001). The mechanisms of action of pinopodes are still unclear, but they may extract fluid from the uterus, and/or facilitate adhesion of a blastocyst to the endometrium (Stavreus-Evers, et al 2002a, Stavreus-Evers, et al 2002b). The volume of uterine fluid is decreased during the window of implantation. The

### 1.4.3 Cytokines and growth factors

Several cytokines and growth factors have been implicated to be involved in endometrial receptivity. They include the interleukin-1 (IL-1) system, LIF, colony-stimulating factor–1, heparin-binding epidermal growth factor-like growth factor (HB-EGF), insulin-like growth factor (IGF) family, vascular endothelial growth factor (VEGF) and transforming growth factor-β (Ghosh and Sengupta 2004, Lindhard, et al 2002).

#### 1.4.3.1 Interleukin-1 (IL-1) system

There is evidence for the IL-1 system being involved in the endometrial and embryonic crosstalk during human implantation (Simon, et al 1997). Human endometrial epithelial cells produce IL-1, mainly interleukin-1β (IL-1β), and interleukin-1 receptor antagonist (IL-1ra) (Fukuda, et al 1995). The expression of mRNA for the type 1 receptor of IL-1 occurs throughout the menstrual cycle, but its expression is maximal in the mid-luteal phase (Simon, et al 1997).

IL-1 knock-out mice have successful implantation, but when their IL-1 receptor is blocked, implantation does not occur. A possible pathway is the down-regulation of the expression of $\alpha_4$, $\alpha_v$, $\beta_3$ integrins on endometrial epithelium (Abbodanzo, et al 1996, Simon, et al 1998). High concentrations of embryonic IL-1 in the culture media seem to be associated with implantation after ART procedures. IL-1 is believed to be the first cytokine active in maternal-embryonic cross-talk, which results in a second wave of cytokines (Lindhard, et al 2002). Recently, a study investigating the effect of leptin on the IL-1 system expression in human endometrium found that the expression may be regulated by leptin (Gonzalez, et al 2003).

#### 1.4.3.2 Leukaemia inhibitory factor (LIF)

LIF is a pleiotropic glycoprotein of the interleukin-6 family, associated with normal implantation in murine models. LIF is expressed in human endometrium predominantly

1.4.3.3 **Heparin-binding epidermal growth factor-like growth factor (HB-EGF)**


1.4.3.4 **Insulin-like growth factor binding protein-1 (IGFBP-1)**

The insulin-like growth factor family consists of the structurally related peptides insulin-like growth factor-I (IGF-I) and II (IGF-II), their receptors (Type I and Type II) and a group of seven structurally homologous binding proteins (IGFBP-1-7) (Rutanen 2000). The IGF family and their binding proteins are supposed to participate in endometrial growth, differentiation, angiogenesis and apoptosis (Giudice and Irwin 1999). The precise role of IGF-II in embryo implantation is not known but its abundance at the invading front of the trophoblast and the proximity of decidual cells expressing IGFBP-1 are suggesting a role in regulation of trophoblast invasion.
IGFBP-1, secreted by the secretory endometrium and decidua, serves as restraint on trophoblast invasion by binding IGF-I and IGF-II, thereby blocking their actions. It has been suggested that the appropriate expression of IGFBP-1 at the maternal-fetal interface is critical for successful implantation. Insufficient invasion characteristic of pre-eclampsia may be due to elevated decidual levels of IGFBP-1 (Giudice, et al 1997). Increased expression of IGFBP-1 has been associated with decreased implantation rates in mice (Huang, et al 1997). IGFBP-1 has been implicated in embryo recognition and the events that are associated with early implantation because of its binding to \( \alpha_5\beta_1 \) integrins on the cytotrophoblast cells (Irwin and Giudice 1998).

### 1.4.3.5 Vascular endothelial growth factor (VEGF)

Angiogenesis is the formation of new blood vessels from existing ones. VEGF is a key modulator of vascular growth and remodeling and it increases vascular permeability in the endometrium. VEGF knock-out mice do not produce viable offspring, although they can conceive (Carmeliet, et al 1996). Expression of VEGF in human endometrium reaches a maximum in secretory phase endometrium, during the window of implantation (Shifren, et al 1996). High serum concentrations of VEGF on the day of oocyte retrieval in an IVF cycle were predictive of subsequent pregnancy (Dorn, et al 2003). VEGF as an important angiogenesis factor possibly plays an important role in the process of implantation (Smith 2000).

### 1.4.4 Prolactin (PRL)

Prolactin (PRL) is a peptide hormone essentially secreted by the anterior pituitary and, to a lesser extent, by extrapituitary tissues (Ben-Jonathan, et al 1996). The endometrium is one of the first extrapituitary sites that has been reported to synthesize and secrete PRL (Brosens, et al 1999, Tseng and Mazella 1999). Prolactin synthesis is detected between the mid-secretory phase and menses, and coincides with the first histological signs of decidualization (Maslar and Riddick 1979). In the event of pregnancy, decidual prolactin secretion increases steadily after implantation. Prolactin, synthesized de novo by decidual cells, is indistinguishable from pituitary prolactin by chemical, immunological and biological criteria (Tomita, et al 1982). Moreover, the amino acid encoding sequence is identical for pituitary and decidual prolactin (Takahashi, et al 1984). Biological functions of PRL and its receptor are critical to implantation and maintenance of pregnancy suggested by the impaired fertility of PRL.
and PRL-R knockout mice (Ormandy, et al 1997). Lack of expression of endometrial PRL during the implantation window in some patients affected by unexplained infertility and repeated miscarriages were reported (Garzia, et al 2004). The temporal and spatial expression of prolactin in the human endometrium suggests a role in the preparation for implantation and subsequent placentation (Jabbour and Critchley 2001).

### 1.4.5 Nitric oxide synthase (NOS)

Nitric oxide, a short-lived free radical gas, is synthesized by conversion of L-arginine to L-citrulline by a family of enzymes known as nitric oxide synthases (NOS). Three distinct isoforms of NOS have been characterized and cloned: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). eNOS and nNOS are constitutively expressed, whereas iNOS is typically expressed at the site of an inflammation. Nitric oxide has diverse physiologic roles, including regulation of vascular homeostasis, smooth muscle relaxation, and neurotransmission. Nitric oxide has also been shown to inhibit platelet aggregation, stimulate angiogenesis, and alter the function of the immune system. Nitric oxide was recently implicated as an important regulatory agent in various female reproductive processes, such as ovulation, implantation, pregnancy maintenance, labor, and delivery (Chwalisz and Garfield 2000). Studies in mice have shown that both inducible NOS and eNOS are up-regulated at the implantation site (Purcell, et al 1999). Administration of N-omega-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor during the preimplantation phase, reduces the implantation rate in rats (Biswas, et al 1998). Several studies have identified both eNOS and iNOS in the human endometrium (Maul, et al 2003).

### 1.4.6 Other factors of importance for endometrial receptivity

Chemoattractant cytokines (chemokines) for neutrophils and lymphocytes, such as interleukin (IL)-8 and monocyte chemoattractant protein-1 (MCP-1), seem to mediate the steroid dependent recruitment of leukocytes to the endometrium (Jones, et al 2004). Chemokines are also responsible for the secondary induction of other cytokines and growth factors, including LIF, IL-1 and HB-EGF (Cross, et al 1994). IL-8 and MCP-1 are expressed in endometrial glandular and luminal epithelial cells where they are down-regulated by progesterone during the window of implantation (Caballero-Campo, et al 2002).

**Prostaglandins** (PGs), which are generated by cyclooxygenases (COX1 and COX2), facilitate increased vascular permeability in the endometrium during implantation.

COX-1 deficient female mice are fertile with specific parturition defects while COX-2 deficient females have multiple reproductive failures including defects in ovulation, fertilization, and implantation. The uterine expression of COX-2 in an implantation-specific manner and defective implantation and decidualization in COX-2 deficient mice establish that uterine COX-2 is essential for these processes (Dinckuk, et al 1995, Lim, et al 1997).

The initial attachment of an embryo may involve temporary adhesion between exposed surface receptors and ligands on the embryonic and endometrial epithelium. **Integrins** are glycoproteins that serve as receptors for ECM ligands and act as modulators of cellular function. The expression of $\alpha_4\beta_1$ and $\alpha_v\beta_3$ integrins during the window of implantation has been documented (Lessey, et al 1994). An insufficient expression of integrin $\alpha_v\beta_3$ is associated with infertility (Lessey and Castelbaum 1995, Lessey, et al 1995).

1.5 **MIFEPRISTONE**

Mifepristone is a 19-nor steroid that binds with high affinity to the progesterone receptor, thereby inhibiting the effect of endogenous progesterone. It has both antagonist and agonist activities on the action of progesterone in regulating gene transcription and also exhibits anti-glucocorticoid, anti-estrogenic and anti-androgenic effects. The essential clinical application for mifepristone involves its anti-progesterone effect, currently used on several indications including medical termination of early pregnancy, emergency contraception, cervical ripening, labor induction, menstrual regulation, uterine myoma, and endometriosis. Other indications include Cushing's syndrome, steroid receptor containing tumors (breast, ovary, prostate and endometrium) and psychotic depression (Spitz 2003).

1.5.1 **Mifepristone for contraception**

Emergency contraception is defined as the use of any drug or device after an unprotected intercourse to prevent an unwanted pregnancy. One of the main barriers for the wide spread use is the concern about the mechanisms of action, such as impairment of implantation, or dislodgement of an implanted fetus. In many developing countries lack of access to emergency contraception may subject women to unsafe abortions,
which contribute significantly to maternal mortality and morbidity. It has been estimated that millions of unwanted pregnancies could be avoided if emergency contraception was widely accessible (Heimburger, et al 2003).

To show that the effect of mifepristone on endometrial receptivity is sufficient to prevent pregnancy, a single dose of 200 mg mifepristone was given on LH+2 as the only contraceptive method. In 124 ovulatory cycles, only one pregnancy occurred (Gemzell-Danielsson, et al 1993). When 600 mg of mifepristone was administered within 72h of an unprotected intercourse, no pregnancy occurred among 597 women treated (Glasier, et al 1992, Webb, et al 1992). A single dose of 10 mg mifepristone within 120h after an unprotected intercourse has been shown to be as effective as doses of 50 or 600 mg but with significantly less side-effects (WHO 1999).

The contraceptive efficacy of low daily or intermittent doses of mifepristone was also investigated. When 5mg was administered once weekly during 60 cycles, three pregnancies occurred (Marions, et al 1998a). Treatment with 0.5 mg mifepristone daily administered during 141 cycles resulted in five pregnancies (Marions, et al 1999). In a more recent study, there were no pregnancies in 200 months of exposure in 50 women who used daily doses between 2 and 5mg mifepristone as their sole method of contraception.

1.5.2 The action of mifepristone in endometrium

1.5.2.1 Preovulatory treatment

The effect of mifepristone during the menstrual cycle is well known to be dependent on the dose given and the time of treatment. Administration of a single dose of 10 mg of mifepristone 2 days prior to ovulation inhibited or delayed the LH surge in all subjects (Marions, et al 2002). The luteal phase levels of estrone and pregnanediol-glucuronide were slightly decreased compared to control levels (Marions, et al 2004). Two subjects showed a slight inhibition of endometrial development on cycle days LH+6 to+8. The expression of COX-1 and COX -2, integrin α4 and β3 subunits, progesterone receptors and DBA-lectin binding as well as pinopode development were the same as observed in control cycles. In contrast, a higher dose of mifepristone given in the periovulatory phase, even when too late to inhibit ovulation, affected endometrial development during the luteal phase. Administration of a single dose of 200 mg mifepristone at the onset of LH surge inhibited or delayed the development of a normal secretory
endometrium without preventing ovulation or formation of luteinized follicle (Brown, et al 2003).

1.5.2.2 Postovulatory treatment

Treatment with a single dose of 200 mg mifepristone on day LH+2 has been shown to be an effective contraceptive method (Gemzell-Danielsson, et al 1994, Hapangama, et al 2001). The normal menstrual rhythm remains undisturbed and serum levels of estradiol and progesterone remain essentially unchanged (Swahn, et al 1990). However it has profound effects on endometrial development; these effects include decreased glandular diameter, increased glandular apoptosis, decreased number of vacuolated cells and increased number of stromal mitoses (Critchley, et al 1999, Swahn, et al 1988). Expression of 17β-hydroxysteroid dehydrogenase in endometrial glandular and luminal epithelium is prevented, as well as endometrial secretory activity measured by DBA lectin binding and serum levels of glycodelin. However the number of pinopodes does not seem to be decreased. Additionally, ER, PR and AR show intense staining in the nuclei of both glandular and stromal cells. Treatment with mifepristone reduces endometrial glandular LIF, COX-1, integrin α4 and β3 subunit expression and COX-2 in luminal epithelium as well (Cameron, et al 1996, Danielsson, et al 1997, Gemzell-Danielsson, et al 1994, Maentausta, et al 1993, Marions, et al 1998b, Marions and Danielsson 1999, Slayden, et al 2001).

When a single dose of 10 mg 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. Following 10 mg, the endometrium was slightly out of phase, while no obvious effect was seen on LIF and integrin expression or on pinopode development. DBA lectin binding, reflecting endometrial secretory activity, was reduced in four out of six subjects and down-regulation of progesterone receptors was inhibited in five out of six subjects (Marions, et al 2002).


1.5.3 The action of mifepristone in the fallopian tube

The tubal microenvironment is probably of great importance to ensure normal embryo development, and stage-specific expression of receptors for various growth factors has
been found on human embryos (Smotrich, et al 1996). Too rapid or too slow tubal transport could also be expected to cause desynchronization between the embryo and the tube, and/or the blastocyst and the endometrium. Progesterone regulates tubal transport in vitro, as confirmed in a study (Mahmood, et al 1998). Cilia from the human fallopian tube beat significantly slower after treatment with high doses of progesterone, an effect that could be reversed by mifepristone. Furthermore, animal studies have previously shown accelerated tubal egg transport after mifepristone treatment (Psychoyos and Prapas 1987).

A spatially dependent expression of progesterone receptors IL-8, TNF₆, TGFβ, and LIF has been shown in the human fallopian tube during mid-cycle. Down-regulation of progesterone was found to be inhibited after treatment with the antiprogestin mifepristone. Mifepristone affected the distribution and expression of TNF₆ and IL-8 as well (Christow, et al 2002, Li, et al 2004).

### 1.5.4 The effect of mifepristone on the oocyte and embryo

There are no data on the direct exposure of human embryos to mifepristone. Exposure of mifepristone to monkey embryos did not affect embryo development or their ability to implant (Wolf et al., 1989).

To investigate if mifepristone interferes with gonadotrophininduced oocyte maturation and fertilization, clomiphene was given to 40 volunteers for 5 days in order to stimulate follicular growth (Messinis and Templeton 1988). On day 16, the women received 100 mg mifepristone 1 h before induction of ovulation by injection of hCG. Laparoscopy was performed 34 h after hCG and all follicles with a diameter of 15mm were aspirated, and collected oocytes submitted to IVF. The number of retrieved oocytes, the rate of fertilization, and the cleavage rate did not differ between the mifepristone group and the controls.

In rhesus monkey, administration of mifepristone on day 2 after ovulation depressed preimplantation stage embryo development at the morula to blastocyst transition characterized by loss of cell polarity of mitochondrial maturity, and lack of differentiation in trophoblast cells (Ghosh, et al 2000).

Although mifepristone given postovulatory has been shown to be effective for contraception, the precise mechanism of action is still poorly understood. A better understanding of the mechanisms of action of mifepristone, when used for
contraception, is important for further development and for optimizing the regimen of use.
2 AIMS OF THE STUDY

✧ To investigate the localization and evaluate the expression of progesterone receptor isoform B in the human endometrium and fallopian tube after treatment with mifepristone during the implantation phase.

✧ To study the effect of mifepristone on some factors suggested as important for endometrial receptivity such as IGFBP-1, PRL and eNOS in the endometrium at the time of implantation.

✧ To evaluate the effects of mifepristone on expression of HB-EGF and its receptors in the human endometrium and fallopian tube during the implantation phase.

✧ To compare the effects of mifepristone on the human endometrium and fallopian tube.
3 MATERIAL AND METHODS

3.1 SUBJECTS
Healthy women, 22 to 45 years of age who had regular menstrual cycles (25 to 35 days) and proven fertility were included. None of them had used a steroid hormone contraceptive or an intrauterine device for a minimum of 3 months prior to the study. The women were non-smoking and a gynecological examination was performed on admission. The volunteers were advised to use a barrier method for contraception. Informed written consent was obtained from each volunteer or patient before inclusion in this study. In the treatment group, women were given a single oral dose of 200 mg mifepristone immediately after ovulation (day LH+2).

For the studies of the endometrium, 7 (paper I), 9 (paper II), 8 (paper III) women were recruited for endometrial biopsies. The women served as their own controls and were observed for one control and one treatment cycles. In addition 29 women (paper IV) were randomly allocated into one control- and one treatment group.

For the studies on the fallopian tube, 14 volunteers were recruited among women admitted to the hospital for sterilization by laparoscopic technique and using a plastic ring (Lay; Instrumenta AB, Stockholm, Sweden). With this technique, an elastic plastic ring is put over a loop of the tube which in this way gets blocked. The women were randomly allocated into one control- and one treatment group.

3.2 HORMONE ANALYSIS
All subjects determined the LH peak in urine samples collected twice daily from approximately cycle day 10 to LH+2 by using a rapid self-test (Clearplan, Searle Unipath Ltd, Bedford, UK). Daily morning urine samples were analyzed for estrone and pregnanediol-glucuronides and LH using enzyme immunoassay (EIA) (Cekan, et al 1986). The hormones were expressed in nmol per mmol creatinine for estrone- and pregnanediol-glucuronide and per mmol creatinine for LH (Metcalf and Hunt 1976). For creatinine analysis, a commercial kit (Sigma Diagnostics, St Louis, MO, USA) was used.

3.3 TISSUE BIOPSIES
Endometrial biopsies were obtained from the uterine fundus, using a Randall curette, in the mid-luteal phase (LH+6 to +8), i.e., the expected time for endometrial receptivity
and implantation. Fallopian tube surgery was performed on day LH+4 to LH+6, i.e., approximately the time when the embryo is still in the fallopian tube, reaching the morula to blastocyst transitional stage. The biopsy from the right side was taken from the isthmalic part of the tube, while on the left side the biopsy was taken from the distal, ampullar part of the tube.

3.4 IMMUNOHISTOCHEMISTRY (I-IV)

For PR-B, eNOS, HB-EGF, HER1 and HER4, cryosections were fixed with acetone. For IGFBP-1 and PRL the sections were fixed with formaldehyde. Endogenous peroxidases were quenched by incubation with H$_2$O$_2$ in methanol. Unspecific binding of antibodies was blocked with 1.5% normal horse serum in a humidified chamber for 30 minutes at room temperature. The primary antibody was placed on the slides and incubated at 4°C overnight. The samples were rinsed in PBS buffer and incubated with the biotinylated secondary antibody for 30 minutes at room temperature. After rinsing, staining procedures were performed by a standard-biotin peroxidase technique. Thereafter, the sections were counterstained with hematoxylin and mounted.

A similar protocol was used for IGFBP-1 and PRL immunolocalization, with the exception that EBSS supplemented with 1% HEPES buffer was used instead of PBS. Negative controls were incubated similarly, but phosphate-buffered saline replaced the primary antibody. To check for primary antibody specificity, the primary antibody was replaced with nonimmunoserum of the equivalent concentration from the same species. The immunostaining intensity was evaluated using a semi-quantitative method. A scoring system was used to determine the intensity of the staining. The staining was graded on a scale of 0 = absent (–), 1 = weak (+), 2 = moderate (++) and 3 = strong (+++).

Considerations

The advantages of immunohistochemistry are that the localization of the protein is shown and the relative amounts can be determined by semi-quantitative measurements. A disadvantage is that, though Comparisons can be made between different cell types and/or different groups, the absolute concentration can not be determined.

The advantages of monoclonal antibodies compared to polyclonal antibodies are high homogeneity, absence of non-specific antibodies and less batch-to-batch variability. Unfortunately, monoclonal antibodies increase the risk of false negative results, since there is only one epitope that could be found. On the other hand, polyclonal antibodies
increase the risk of false positive signals because there is a possibility that several proteins share epitopes. To eliminate false positive or negative results, positive and negative control samples were analyzed at the same time as the rest of the samples.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antibody class</th>
<th>Manufacturer</th>
<th>Papers</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS</td>
<td>Monoclonal Mouse</td>
<td>N30020 Transduction Laboratories, USA</td>
<td>II</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>Polyclonal Goat</td>
<td>AF-259-NA R&amp;D Systems Inc. UK</td>
<td>IV</td>
</tr>
<tr>
<td>HER1</td>
<td>Monoclonal Mouse</td>
<td>SC-120 Santa Cruz Biotechnology, Inc. USA</td>
<td>IV</td>
</tr>
<tr>
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<td>Monoclonal Mouse</td>
<td>SC-8050 Santa Cruz Biotechnology, Inc. USA</td>
<td>IV</td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>Polyclonal Goat</td>
<td>SC-6072 Santa Cruz Biotechnology, Inc. USA</td>
<td>III</td>
</tr>
<tr>
<td>PR A+B</td>
<td>Monoclonal Mouse</td>
<td>NCL-PGR-312 Novocastra Laboratories Ltd, UK</td>
<td>III</td>
</tr>
<tr>
<td>PR-B</td>
<td>Monoclonal Mouse</td>
<td>MA1-411 Affinity Bioreagents Inc., USA</td>
<td>I</td>
</tr>
<tr>
<td>PRL</td>
<td>Polyclonal Goat</td>
<td>SC-7805 Santa Cruz Biotechnology, Inc. USA</td>
<td>III</td>
</tr>
</tbody>
</table>

3.5 RT-PCR (I-IV)

Total RNA was extracted using TRIZOL reagent. RNA was reverse transcribed using the First-Strand complementary DNA synthesis kit. PCR was performed with gene specific primers. The PCR products were electrophoresed through a 1.5% agarose gel and visualized with ethidium bromide.

The RNA preparation and RT-PCR experiments were described detail in the material and method sections in the papers I-IV.

Considerations

RT-PCR provides a valuable tool for detection and analysis of mRNA transcripts. The advantages of RT-PCR are sensitivity, specificity and efficiency. mRNA relative amounts in all cells of a tissue can be determined by semi-quantitative measurements.
However localization of mRNA cannot be determined. To directly compare mRNA levels between PR-A and PR-B is inconclusive since PR-A and PR-B arise from different promoter usage in a single gene. PR-B differs from PR-A by containing an additional 164 amino acids at the N-terminus. In paper I, we therefore chose to look exclusively at expression and regulation of PR-B.

The pairs of primers used in the experiment:

<table>
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<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Product size (bp)</th>
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<td>eNOS</td>
<td>GGACTTCATCAACCAGTAC ATGTAGGTTGAAACATTCCC</td>
<td>250</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>GGCTGAGTGAAGCAAGACAAGAC TTGTGGCTTGGAGGATAAAGTG</td>
<td>439</td>
</tr>
<tr>
<td>HER1</td>
<td>CAGCGCTACCTTGTCATTCAG TCATATATCCCTCCGTGTC</td>
<td>727</td>
</tr>
<tr>
<td>HER4</td>
<td>AAGTACAGTGTGACCCACC GTTTGCAAAGGTGTTGAGGT</td>
<td>264</td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>GAGAGCACGGAGATAACTGAGG TTTGTTGACATGGAGAGCCTTCG</td>
<td>131</td>
</tr>
<tr>
<td>PR A+B</td>
<td>GTGGGCGTTCCAAATGAAAGCCAAG AATTCAACACTCACTAGTGCCGGACT</td>
<td>737</td>
</tr>
<tr>
<td>PR-B</td>
<td>GAGGGGCGTGGAAACTCAG AAGGGGAACCTGCTGCTGCTG</td>
<td>293</td>
</tr>
<tr>
<td>PRL</td>
<td>GCCCCCTTGGCCCATCTGTCC AGAAGCCGTTTGGCTTCG</td>
<td>386</td>
</tr>
<tr>
<td>28S</td>
<td>GTGCAGATTTTGGTGGTAGTAGC AGAGCCAATCCTTTCCTCCGAAGTT</td>
<td>552</td>
</tr>
<tr>
<td>GADPH</td>
<td>CACACCACGGCAGATCAGTGCCAGTCCACC</td>
<td>598</td>
</tr>
</tbody>
</table>

3.6 WESTERN BLOT ASSAY (II)

Electrophoresis was performed in an 8% SDS-PAGE solution. Proteins were transferred to nitrocellulose membranes. The same primary antibody against eNOS and secondary antibody were used as for immunohistochemistry. Detection was carried out with ECL western blotting detection reagents. Human umbilical vein endothelial cells (HUVEC) were used as a positive control for eNOS.

Considerations

Western blot is a reliable way of validating immunohistochemistry result. Comparisons can be made between absolute protein concentrations in different tissues, but the
localization of protein in the cells is not shown. The sensitivity and specificity of this method depends on the monoclonal or polyclonal antibody used.

3.7 STATISTICAL ANALYSIS
Differences in immunohistochemical staining between control and treatment cycles were analyzed using the Mann-Whitney U or Wilcoxon signed ranks test. Specific mRNA levels are presented as means (±SE). Data were analyzed by Student’s t-test or Wilcoxon signed ranks test. P< 0.05 was considered statistically significant.
4 RESULTS AND DISCUSSIONS

4.1 EFFECTS OF MIFEPRISTONE ON THE ENDOMETRIUM

4.1.1 PR-B

Progesterone receptor concentration could be expected to be highly associated with successful implantation since many of the relevant local factors such as cytokines and growth factors are progesterone-regulated. Down-regulation of the progesterone receptor has been shown to be highly associated with the development of endometrial receptivity (Garcia, et al 1988).

However, reports on variation in the expression of PR-B in human endometrium during the menstrual cycle are not conclusive. Using immunofluorescent histochemistry, Mote and coworkers reported that PR-B expression increased during the proliferative phase and reached the highest expression during the late proliferative phase. In the mid-secretory phase, PR-B was said to be the predominant isoform expressed in most glands (Mote, et al 1999). Wang and colleagues demonstrated using immunohistochemistry that PR-B was present in glands and stroma in the proliferative phase, and dramatically reduced in the glands during the secretory phase. The scores for PR-B immunostaining in stromal cells were slightly higher than the ones in glandular cells (Wang, et al 1998). Our results (paper I) on PR-B expression in the endometrium of the control group are consistent with the latter findings. The conflicting reports on PR-B expression may be explained in part by different PR isoform antibodies, which have different affinity capability and characteristics, and especially by the fact that it is not possible to raise antibodies specific to PR-A. Thus, all immunohistochemical analysis of PR-A is by subtractive inference. In our study we therefore chose to look exclusively at expression of PR-B.

Previous studies showed that progesterone dependent down-regulation of PR in endometrial glandular cells in mid-luteal phase was inhibited by mifepristone (Gemzell-Danielsson, et al 1994).

In paper I it was shown that following treatment with 200 mg mifepristone on cycle day LH+2, the expression of PR-B was significantly increased in the endometrium, especially located to the glandular cells. This suggests that PR-B expression may serve as a reliable predictor of success or failure of endometrial receptivity. Recently, it was shown that down-regulation of PR-B occurs at the onset of pinopode formation in the
human endometrium (Stavreus-Evers, et al 2001). Following treatment with 10 mg mifepristone on LH+2, the number of pinopodes did not seem to be decreased. However, the amount of uterine fluid was significantly increased compared to controls (Danielsson, et al 2003, Gemzell-Danielsson and Hamberg 1994). The up-regulated expression of PR-B at this time suggests that these changes of activity in the human uterus are mediated primarily by PR-B. Further, the PR-A and PR-B proteins also respond differently to progesterone antagonists. While antagonist bound PR-A is inactive, antagonist bound PR-B can be converted into a strongly active transcription factor (Giangrande and McDonnell 1999). The following increase of transcription mediated by PR-B treatment with mifepristone could involve changes in the amount of uterine fluid and affect endometrial receptivity consequently.

4.1.2 eNOS

In paper II it was shown that eNOS protein is expressed in the human endometrium during the implantation stage of the menstrual cycle. Our results agree with those of previous investigations of the human endometrium (Khorram, et al 1999, Taguchi, et al 2000, Telfer, et al 1997). These studies report that eNOS was localized to blood vessels and that it was present in glandular epithelial cells.

eNOS expression was decreased in the glandular epithelium after treatment with mifepristone. A similar decrease in eNOS has been observed both in vitro and in vivo studies, in the absence of an influence of progesterone on the endometrium (Khorram, et al 1999, Zervou, et al 1999). Our results support a role of progesterone in glandular eNOS regulation in the human endometrium, whereas endothelial eNOS seems to be regulated in a different manner.

NO has been found to induce COX-2 production and elevate PGF\(_2\) concentrations in inflammatory sites (Salvemini, et al 1995). In vitro experiments reveal that IL-1\(\beta\) can induce NO production and hereby increase PGF\(_2\) concentration (Lin, et al 1998). Since human embryos have been found to produce IL-1\(\beta\) this could be a signal from the embryo to promote implantation (Fazleabas, et al 2004). Furthermore treatment with mifepristone significantly decreases the expression of COX-1 and COX-2 in glandular and luminal epithelium (Marions and Danielsson 1999). Thus decreasing eNOS expression in the glandular epithelium after treatment with mifepristone could inhibit PGF\(_2\) concentrations, and thereby interfere with implantation.

The lack of effect of mifepristone on eNOS expression in the vascular endothelium may also indicate different regulations by local factors. It has been shown that the
endometrial spiral arteries are a main target of mifepristone (Johannisson, et al 1989). This effect does not seem to be mediated through an effect on eNOS. Whether addition of a specific eNOS inhibitor to treatment with mifepristone could further disrupt endometrial receptivity and increase the contraceptive efficacy remains to be studied. A synergistic effect between NOS inhibitors and antiprogestins to prevent implantation has been found in animal studies (Chwalisz, et al 1999).

![Figure 4. Immunohistochemical location of eNOS in the human endometrium during the implantation phase. A: eNOS immunoreactivity in control cycles. B: Endometrium after mifepristone treatment.](image)

### 4.1.3 IGFBP-1 and PRL

In paper III, it was shown that mifepristone increased the expression of endogenous IGFBP-1, PRL mRNA and protein during the implantation phase in human endometrium in vivo (Figure 5). The observed effect on IGFBP-1 and PRL proteins is more surprising. Both proteins seemed to increase, particularly in the glands, although this was significant only for IGFBP-1. A stimulating effect of an antiprogestosterone is unexpected since accumulating evidence has suggested that progesterone is responsible for the production of these two proteins in the human endometrium (Rutanen, et al 1986). However, agonist-like proliferative effects have been reported with mifepristone in cultured breast cancer cell lines and in postmenopausal women under conditions in which inhibition would be expected (Bowden, et al 1989, Gravanis, et al 1985). Furthermore, an in-vitro study has shown that mifepristone causes a transient super-induction of IGFBP-1 and PRL secretion in human endometrial stromal cells (Tseng, et al 1992). As agonist, functional analysis of mifepristone-bound PR is believed to activate transcription by associating with coactivators, which act as bridging factors between the receptor and the general transcription machinery (Figure 6) (Spitz
2003). This is followed by an increase in the rate of transcription of IGFBP-1. A subsequent study has demonstrated that ligand-activated PR-A is a stronger transactivator than PR-B to increase the promoter activity as well as the induction of the endogenous IGFBP-1 gene in endometrial stromal cells (Gao, et al 2000).

The function of IGFBP-1:IGF-II at the feto-maternal interface may involve a delicate balance between invasion and its suppression to achieve normal implantation (Fowler, et al 2000, Giudice, et al 2002). IGFBP-1 may serve as one of several “maternal restraints” to curb trophoblast invasion into maternal endometrium (Giudice and Irwin 1999). IGFBP-1 has been shown to bind to α5β1 integrin on the cytotrophoblast cell membrane to inhibit cell invasion into decidualized human endometrial stromal cells (Irwin and Giudice 1998). It is interesting to note that over-expression of IGFBP-1 has been associated with decreased implantation rates (Huang, et al 1997). Increased serum levels of IGFBP-1 have been found in severe pre-eclampsia, a disorder that may be associated with inadequate placentation, probably due to high IGFBP-1 expression at the feto-maternal interface (Giudice, et al 1997). Our studies support the hypothesis that over-expression of IGFBP-1 in glandular cells after treatment with mifepristone may lead to suppression of normal implantation by destruction of the balance between IGFBP-1:IGF-II and binding to α5β1 integrin on the cytotrophoblast cell membrane.
Figure 5. Box & Whisker plot of immunostaining for IGFBP-1 in the endometrium after treatment with mifepristone. A: Immunostaining of the glandular epithelium. B: Immunostaining of the stroma. * significant difference compared to control group.

Figure 6. Schematic illustration of mifepristone as progesterone agonist: Binding of mifepristone results in a loss of heat shock proteins (HSP) and dimerization of the PR. The activated PR dimer binds to the progesterone-responsive elements (PREs). The agonist-bound PR then activates transcription by associating with coactivators.
4.1.4 HB-EGF and its receptors

The highest levels of HB-EGF, HER1 and HER4 are seen during the window of implantation, when the progesterone levels are high (Moller, et al 2001, Stavreus-Evers, et al 2002a). In paper IV, it was shown that neither HB-EGF nor HB-EGF mRNA in the endometrium were affected by mifepristone treatment. Data from cell culture of human endometrial epithelial and stromal cells show that a combination of estrogen and progesterone stimulate HB-EGF while estrogen and progesterone alone or in combination stimulated the secretion of HB-EGF endometrial stromal cells (Lessey, et al 2002). This shows that although a single oral dose of 200 mg mifepristone is enough to alter endometrial development and inhibit pregnancy (Gemzell-Danielsson, et al 2003), it does not alter the expression of HB-EGF in the endometrium.

In contrast to the effect of mifepristone on HB-EGF, there was an increase in the immunostaining of HER1 and HER4 in the endometrium after mifepristone treatment. This contradicts the finding that these receptors show the highest expression during the secretory phase endometrium, when serum progesterone levels are high (Moller, et al 2001, Srinivasan, et al 1999). The endometrial maturation is mediated by paracrine action of HB-EGF on HER1 and HER4 (Chobotova, et al 2002, Lessey, et al 2002). The increase in the receptors for HB-EGF after mifepristone treatment might lead to a defect endometrial development. Increased receptor concentration might favour paracrine action of HB-EGF on the endometrium rather than on the embryo.

4.2 EFFECTS OF MIFEPRISTONE ON THE FALLOPIAN TUBE

4.2.1 PR-B

The tubal microenvironment is probably of great importance to ensure normal embryo development (Smotrich, et al 1996). Progesterone-regulated secretory factors are of importance in determining the reproductive tract milieu supporting embryo development and differentiation. In paper I, it was shown that a spatial-dependent expression of PR-B occurs in the human fallopian tube. Following 200 mg mifepristone on LH+2, PR-B concentration increased in epithelial and stromal cells. Too rapid or too slow tubal transport could also be expected to cause desynchronization between the embryo and the tube, and/or blastocyst and endometrium. It has been shown in an in vitro study that progesterone regulates tubal function (Mahmood, et al 1998). Cilia from the human fallopian tube beat significantly slower after treatment with high doses of progesterone, an effect that could be reversed by mifepristone.
Our previous study has shown expression of IL-8, TNFα, TGFβ, LIF estrogen and progesterone receptors are present in the human fallopian tube during mid-cycle (Christow, et al 2002, Li, et al 2004). Down-regulation of progesterone was found to be inhibited after treatment with the antiprogestin mifepristone. Mifepristone affected the distribution and expression of TNFα and IL-8 as well (Christow, et al 2002, Li, et al 2004). Progesterone has previously been shown to down-regulate the expression of IL-8 and TNFα, which is potent chemotactic cytokines for neutrophils and lymphocytes (Ito, et al 1994, von Wolff, et al 1999). The anti-inflammatory role in the uterus is likely to be essential for the generation of an immunologically privileged tissue to facilitate development of the embryo and prevent embryonic rejection. Thus up-regulation of IL-8 and TNFα expression in the fallopian tube after treatment with mifepristone may be harmful to preimplanting embryos.

4.2.2 HB-EGF and its receptors
The presence of HB-EGF and its receptors in the embryo, fallopian tube and endometrium (Adachi, et al 1995, Chobotova, et al 2002) suggests that this molecule is important for the initial stages of human pregnancy. HER1 is present on the cumulus cells of the human embryo (el-Danasouri, et al 1993), and it is likely that there is a paracrine communication between the developing embryo and factors that belong to the EGF family, such as HB-EGF and TGF-α, secreted from the fallopian tube. In paper IV, we did not see any changes in the expression of HB-EGF in the fallopian tube after mifepristone treatment, which suggests that the early communication between the embryo and fallopian tube through HB-EGF and HER1 in the cumulus cells is not directly regulated by progesterone. There was a decrease in HER1 in the luminal epithelium and a decrease of HER4 in the stroma of the fallopian tube. This decrease in the receptors for HB-EGF and members of the EGF family suggests that mifepristone alters the function of the fallopian tube.

4.3 DIFFERENT EFFECTS ON THE ENDOMETRIUM AND THE FALLOPIAN TUBE
In paper I, after treatment with mifepristone a significant increase in PR-B was found in the glandular cells of the endometrium and in the epithelial cells of the fallopian tube as well. There was a significantly increased immunoreactivity of PR-B in stromal cells of the fallopian tube but not in the endometrium.
In paper IV, treatment with mifepristone decreased the immunostaining on HER1 in the luminal epithelium of the fallopian tube, while the opposite was seen in the epithelium and the stroma of the endometrium. The immunostaining of HER4 decreased in the stroma of the fallopian tube, while an increase was seen in the epithelial cells of the endometrium (Table 1).

<table>
<thead>
<tr>
<th>Factors</th>
<th>Endometrium</th>
<th>Fallopian tube</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Luminal cells</td>
<td>Glandular cells</td>
</tr>
<tr>
<td>PR-B</td>
<td>—</td>
<td>↑*</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>HER1</td>
<td>↑*</td>
<td>↑*</td>
</tr>
<tr>
<td>HER4</td>
<td>↑*</td>
<td>↑*</td>
</tr>
</tbody>
</table>

Table 1. The immunostaining for PR-B, HB-EGF, HER1 and HER4 in the endometrium and the fallopian tube after treatment with mifepristone. * significant difference compared to control group. No change (-), a tendency of increase (↑) or decrease (↓).

The fallopian tube and the endometrium provide an optimal microenvironment for fertilization and early embryo development, and is therefore important for successful implantation (Croxatto 2002). The morphological and functional characteristics of the oviduct and endometrium are controlled by the ovarian steroid hormones (Jansen 1984, Punyadeera, et al 2003). Mifepristone has been shown to exert differential effects on endometrial epithelial and stromal cells and tissue-dependent differences between the endometrium and the fallopian tube in primates has been demonstrated (Slayden and Brenner 1994). Our studies showed that differential expression of PR-B and HB-EGF receptors in the endometrium and fallopian tube may explain tissue-dependent differences in the effects of mifepristone as well. There is a possibility that the viability of the preimplantation embryo could be compromised by the endometrial or fallopian tube desynchronization induced by mifepristone. The effects of mifepristone on PR-B and HB-EGF receptors expression in the endometrium and fallopian tube have been suggested to play crucial roles in establishing synchronous development and differentiation of embryo and endometrium during the mid-luteal phase. These changes induced by mifepristone are consistent with the high efficacy of this compound to prevent pregnancy when used as a postovulatory contraceptive method.
5 GENERAL SUMMARY AND CONCLUSIONS

Effects on the endometrial and the fallopian tube after treatment with mifepristone during receptivity phase were studied.

- Treatment with mifepristone increased progesterone receptor isoform B concentration in the glandular cells of the endometrium and also increased progesterone receptor isoform B concentration in epithelial and stromal cells in the fallopian tube.

- Mifepristone had differential effects on eNOS expression in the epithelium and endothelium of the human endometrium at the time of implantation. The results support a role of epithelial eNOS in human endometrial receptivity.

- Postovulatory treatment with mifepristone caused a significant increase in IGFBP-1 protein and mRNA. Prolactin expression increased only slightly after treatment. But did not reach statistical significance. The results indicate that the administration of mifepristone in the early luteal phase does not simply retard endometrial development. Furthermore, mifepristone, as progesterone agonist, seems to have diverse actions on glandular and stromal cells.

- Mifepristone had only limited effect on the regulation of HB-EGF and its receptors in the fallopian tube, while the effect on the receptors for HB-EGF in the endometrium was substantial. The increase in HER1 and HER4 in the endometrium, and thereby defect maturation of the endometrium after mifepristone treatment, is maybe one of the mechanisms of the contraceptive effect of mifepristone. Decrease in the receptors for HB-EGF in the fallopian tube suggests that mifepristone also alters the function of the fallopian tube.

- Differential expression of PR-B and HB-EGF receptors in the endometrium and fallopian tube may explain tissue-dependent differences in the effects of mifepristone.
For the endometrium, mifepristone up-regulates PR-B expression. It could, at least in part, induce eNOS down-regulation in the glandular epithelium. The decreasing eNOS expression could inhibit PGE$_2$ concentrations, and thereby interfere with implantation. Furthermore treatment with mifepristone significantly decreases the expression of COX-1 and COX-2 in glandular and luminal epithelium (Marions and Danielsson 1999). Over-expression of IGFBP-1 in the glandular cells may lead to suppression of normal implantation by destroying the balance between IGFBP-1:IGF-II and binding to $\alpha_5\beta_1$ integrin on the cytotrophoblast cell membrane. The increase in the receptors for HB-EGF after mifepristone treatment might lead to a defect endometrial development (Figure 7).

**Figure 7.** Schematic illustration of hypothetic roles of mifepristone in the endometrium during implantation phase.

In the fallopian tube, treatment with mifepristone leads to a PR-B concentration increase in epithelial and stromal cells. Blocking progesterone action could be expected to lead to an increased cilia beat frequency and possibly increased tubal contractions,
which could cause desynchronization between the embryo and the tube and/or blastocyte and endometrium. On the other hand, mifepristone affects epithelial cells to secrete cytokines (IL-8, TNF-α) and growth factors (HB-EGF receptors). Thus it could influence the development of the embryo negatively. This could be an additional contraceptive effect of mifepristone, besides the negative influence on ovulation and endometrial development (Figure 8).

**Figure 8.** Schematic illustration of hypothetic roles of mifepristone in the fallopian tube during luteal phase.

**Clinical relevance**

Early luteal phase administration of mifepristone has a variety of morphological, physiological and biochemical effects on the endometrium that are likely to interfere with embryo–endometrium and/or embryo-tube interactions. But the mechanism of action of emergency contraception is incompletely understood. Increased knowledge of the mechanism of action of mifepristone is essential for the development of new methods as well as for optimizing mifepristone regimens of use.

It is important to keep in mind that mifepristone is not a “pure antiprogestin”. Under certain physiologic conditions it behaves as a progesterone agonist, in some experimental models it opposes the proliferative action of estradiol on the endometrium. This pharmacological diversity can be explained to a great extent by
several levels of complexity built in the progesterone receptor system, progestins actions on target cells, dose given and time administered.

It remains to be seen whether some of the newly synthesized antiprogestins with higher selectivity will be clinically superior to mifepristone.

It was hypothesized that delayed endometrial maturation resulting from inadequate corpus luteum progesterone production might be a cause of infertility and early pregnancy loss (Jones 1976). A prevalence of between 5% and 10% in infertile women and approximately 10% to 25% in those with a history of recurrent early pregnancy loss is generally accepted (Balasch and Vanrell 1987, Phung Thi, et al 1979). Understanding the effects of mifepristone on the endometrium will aid in developing infertility treatment.

In IVF treatment, controlled ovarian hyperstimulation (COH) is associated with advanced endometrial histology and relatively high progesterone levels in the late follicular phase occurring in a relatively large proportion of IVF cycles despite GnRH analogue treatment, which is associated with impaired implantation and lower pregnancy rates (Bourgain and Devroey 2003, Schoolcraft, et al 1991). Low dosages of mifepristone have been shown to delay endometrial maturation (Batista, et al 1992). A progesterone antagonistic effect during the follicular phase can be hypothesized to counteract any premature progesterone activity, if any, during COH overcoming the histological advancement demonstrated.
6 FUTURE PERSPECTIVES:

In the future, I would like to study:

✧ The effect of mifepristone on other markers and mediators of implantation i.e. VEGF.

✧ The effect of mifepristone on endometrial spiral arteries.

✧ Low-dose regimens of mifepristone for contraception.

✧ The role of progesterone and progesterone withdrawal in normal and dysfunctional bleeding.

✧ Low dose mifepristone in preventing premature LH surges in women undergoing COH for IVF and the effect of this antiprogestin co-treatment on endometrial receptivity.
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REFERENCES


