Molecular and cellular aspects of endometrial receptivity

Henning M.Beier[1] and Karin Beier-Hellwig

Department of Anatomy and Reproductive Biology, RWTH University of Aachen, School of Medicine, D-52057 Aachen, Germany

TABLE OF CONTENTS

Endocrine and paracrine controls regulate the endometrium during the luteal phase of the cycle to permit implantation. Part of this differentiation process is the production of a specific secretion which fills the intrauterine cavity and glandular lumen. Its molecular composition originates from the gland secretion, from transudations from stroma, from the endometrial blood vessels, and last, but not least, from cellular components of apoptotic and exfoliated cells. We have studied the secretions of all phases during the menstrual cycle using patterns evaluated by SDS–PAGE, by laser densitometry or Western blots. Uterine secretion electrophoresis (USE) permits detailed analyses of the intrauterine micromilieu and allows clinical assessment of the receptive stage of endometrium during the luteal phase. Several individual protein bands have been defined as characteristic markers for such receptive pattern. We have isolated and identified the molecular structure of several of these proteins, e.g. histones, cyclophilin, transthyretin, haptoglobin and uteroglobin. Investigations on the endocrine regulation of these proteins, were carried out on the uterine secretions of patients treated with progesterone antagonists (mifepristone and onapristone). The results demonstrate how progesterone-dependent components produce a receptive pattern, which can serve as a useful and precise marker in the clinical diagnosis of the luteal phase. Essential progesterone-dependent components differentiating during the luteal phase may provide new targets for contraceptive interventions by preventing the physiological changes typical of receptivity.

Key words: endometrial contraception/endometrial proteins/ electrophoretic assessment/progesterone antagonism/ receptivity

Introduction

There are still no widely accepted criteria for evaluating endometrial receptivity. Novel methodological approaches include classical means such as light and electron microscopy, cell and tissue culture, and biochemical and physicochemical analysis. However, the most promising means are available using molecular biology and gene technology, to resolve cell physiology, including the switching-on and -off of specific genes. The final confirmation of receptivity will be indicated by the blastocyst itself rather than by the results of any polymerase chain reaction (PCR) investigations. It is this crucial phenomenon of receptivity which will be the decisive question for any clinical investigation on the newly envisaged 'endometrial contraception', which is based on the strategic concept of inhibiting or preventing a receptive stage of the endometrium. This, in turn, should prevent the early establishment of pregnancy and act as a locally restricted contraceptive mechanism.

The endometrium: a complex organ, not just a simple tissue

The endometrium represents the mucosa which lines the uterine cavity. The common description that this mucosa consists of two main cell populations, namely 'epithelial' and 'stromal' cells, is a simplification which is no longer

[1]To whom correspondence should be addressed. Fax: 49-241-888-8508; email: hmbeier@alpha.imib.rwth–aachen.de
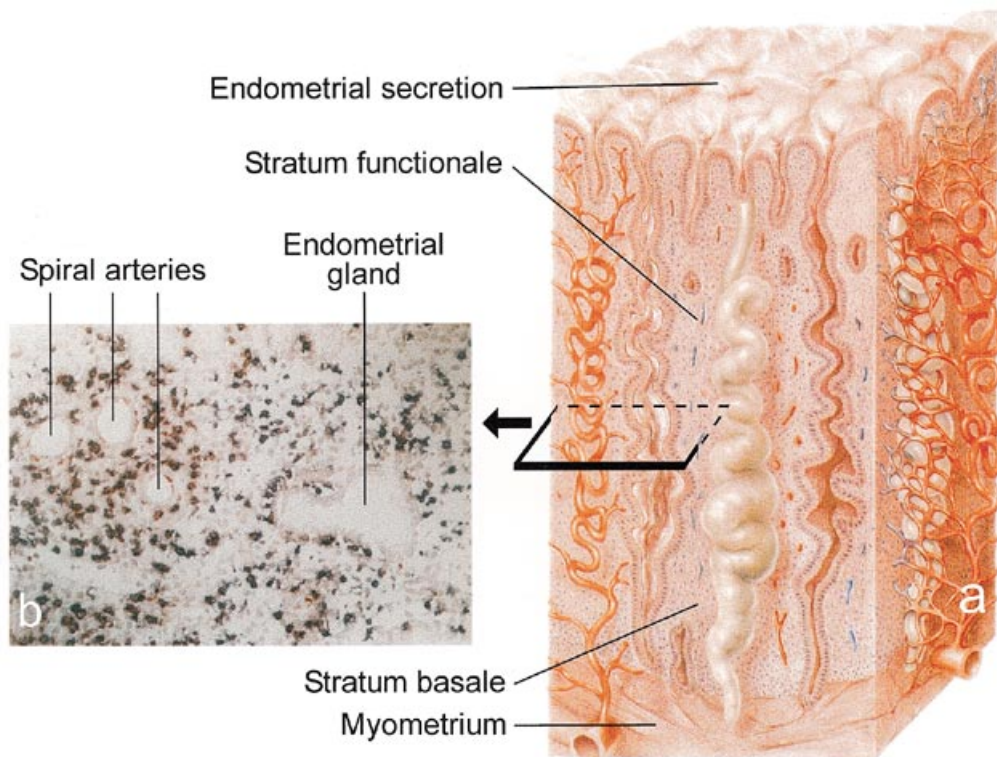
Figure 1. Morphological aspects of endometrial differentiation and maturation during the luteal phase of the human menstrual cycle. (**a**) Histological diagram of human endometrium during the luteal phase of the cycle. Stroma is filled with fibroblasts, predecidual cells and numerous blood vessels. Immunohistochemistry provides a more detailed view than textbook versions, allowing new insights into the differentiated cell populations of the stroma [see (**b**)]. (Reproduced with permission from Breckwoldt *et al*., 1994, Medical Service Munich.) (**b**) Histological cross-section from the luteal phase, day 24 of the cycle, showing abundant immunocompetent cells (blue staining). A large number of CD56-bright cells are evenly distributed, some in closer contact to spiral arteries and endometrial glands. Counterstaining (red) of many cells by MIB-1 antibodies against Ki-67 as proliferation marker. Cryosection; original magnification, ×400. (Original illustration by Joachim Alfer and Irmgard Classen-Linke, Dept of Anatomy and Reproductive Biology, Aachen.)

justified. The epithelial cell population is comprised of the surface epithelium and of the various glandular epithelium cells, which are found in the tubular glands of the stratum functionale and stratum basale (Figure 1a), while the 'stroma' contains the reticular connective tissue of the mucosa, filled abundantly with lymphocytes and granulocytes during the luteal phase of the menstrual cycle (Classen-Linke *et al.,* 1995). This reticular connective tissue is comprised of a very special population of fibroblasts which can be transformed rapidly into decidualized cells, if an embryo has begun to attach and to implant. These specialized cells are found in any menstrual cycle as predecidualized cells, which are located preferentially within the perivascular compartments of the stratum functionale. The abundantly proliferating blood vessels seem to play an extremely important role within the mucosa. Basal anastomosing arteries branch into the terminal 'spiral' arteries, which sprout into capillaries that drain the periglandular and subepithelial compartments into venous sinuses. An enormous number of lymphocytes, granulocytes and macrophages migrate from the blood vessels into the compartment of the 'stroma' (Figure 1b), and this is also the source of all endocrine signals for endometrial proliferation and transformation.

Surface specializations: the pinopodes

The initial step of blastocyst attachment to the endometrial surface is thought to involve extraction of fluid from the uterine lumen by specialized cellular apical protrusions (Figure 2). These specialized bulging surface membranes were described as 'pinopodes' (Parr and Parr, 1974; Psychoyos, 1994; Nikas *et al*., 1995). However, despite these descriptions, which postulate that pinopodes are progesterone-dependent cellular organelles appearing for only 2 or 3 days between days 19 and 21 of the normal menstrual cycle, there is no conclusive evidence available that these structures have any clear-cut predictive value as markers of the 'implantation window'. We have shown by scanning electron microscopy (SEM) that these apical protrusions are seen preferentially at the funnel-shaped openings of the glands and that they are membrane
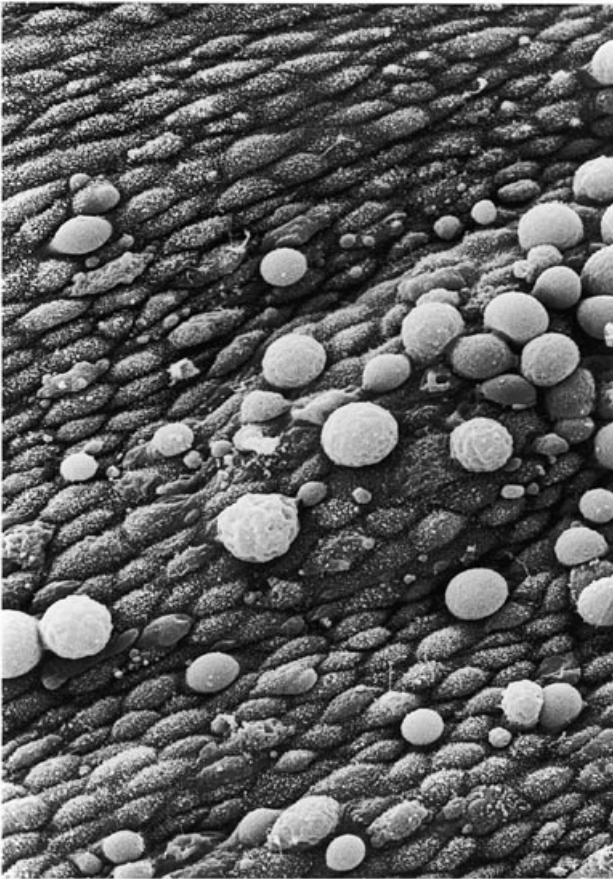
Figure 2. Scanning electron micrograph showing the apical surface of the luminal uterine epithelium in an endometrial sample obtained from a patient at day 11 of the cycle after clomiphene treatment (100 mg each day from days 5–9 of the cycle). Fully developed pinopodes appear at the edge of a funnel-shaped gland opening. Original magnification, ×3600.

specializations, possibly for easier transmembrane carrier processes, perhaps in both directions. Occasionally, these pinopodes may be pinched off (Figure 3a and b), probably releasing more fluid than they may have taken up.

Paracrine modulation of endometrial function

Modern cell biology has unravelled those molecules responsible for the fine-tuning of endocrine regulation. Many functions of endometrial cells are regulated by potent intercellular signals, the cytokines. These molecules act as paracrine or even autocrine factors from cell to cell, or from a cell via extracellular pathways back on the same cell. Although cytokines were initially recognized as factors involved in immune reactions, recent research clearly indicates that they represent the network of polypeptide signals which cell populations of complex tissues, such as the endometrium, require for local and for immediate cell–cell communication. There is ample evidence that
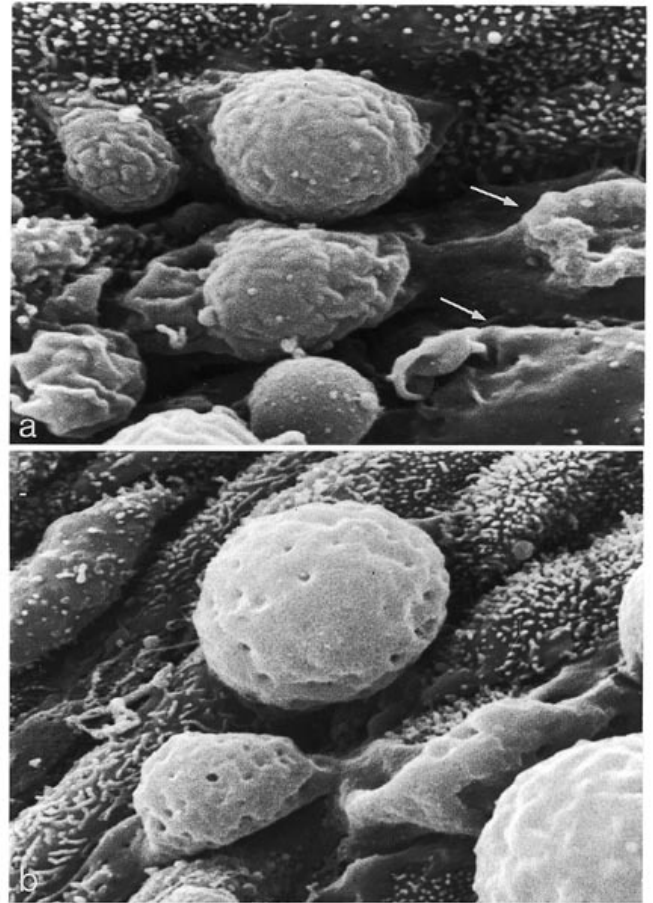

Figure 3. Scanning electron micrograph showing larger magnifications of pinopodes in various stages of their morphological development (same patient as in Figure 2). (**a**) Extended apical protrusions are shown in the centre, whereas at the left almost completely wrinkled structures are seen, possibly undergoing a process of shrinking. This indicates that pinopodes more frequently shrink than become pinched off (arrows at right). (**b**) A fully extended pinopode is shown in the centre of the illustration. Pinopodes on the left are at various stages of shrinking. (a, b) Original magnification, ×17 000.

cytokines participate in the modulation of oestrogen and progesterone control of endometrial differentiation and transformation, as well as in the stepwise achievement of receptivity that enables the embryo to implant.

Cytokines such as interleukin (IL)-1, tumour necrosis factor-α (TNF-α), transforming growth factor-α (TGF-α), endothelial growth factor (EGF) and colony stimulating factor-1 [CSF-1 (M-CSF)] are obviously involved in establishing regional specific distribution of various cell populations and their differential proliferative activity within the endometrium. Particular microenvironmental conditions may be created by the assembly of cytokine-responsive cells which are attracted around cells that produce membrane-bound cytokines. On the other hand, cytokines may be released by certain cells and attract other responsive cells. Consequently, local production and

paracrine effects of cytokines may be decisive modulatory events to polarize tissue compartments, such as the basalis and functionalis of the human endometrium. From these stromal compartments—and in particular from lymphoid cell aggregates—major modulations of glandular and luminal epithelium, on epithelial cell differentiation and function may be initiated by cytokines, e.g. as has been shown for interferon-γ (IFN-γ) and TGF-α. Further influences on epithelial cell cycles and on preparations for menstrual processes are shown by IL-1 and by IL-1-mediated effects through the elaboration of IL-6 (Tabibzadeh and Sun, 1992; Simon *et al.*, 1995).

Individual molecules of the cytokine family may be seen as characters of an alphabet or a code. In this way, information transmitted to target cells may be the product of several cytokines, or the net effects of a battery of regulatory peptides, rather than just being composed of one individual cytokine (Tabibzadeh, 1994).

The expression of genes for cytokines and their receptors has gained particular attention in research on early mammalian development, which appears in synchrony with endometrial differentiation to achieve receptivity. Various cytokines (growth factors) including insulin-like growth factor (IGF)-I and -II, EGF, TGF-α, platelet derived growth factor (PDGF)-A, CSF-1 and leukaemia inhibitory factor (LIF) (Figure 4) are expressed in temporal and spatial variables to serve a most sophisticated pattern of events, which finally accomplishes embryonic and trophoblastic development. Thus, paracrine modulations may enable a molecular cross-talk between endometrial cells and blastocyst cells, contributing locally to the endocrine embryo–maternal dialogue. The molecular network of cytokines involved in cellular control of this complex tissue does not require any departure from the traditional view of steroids being the main forces that 'pull the strings' of endometrial function. Cytokines broaden our understanding of the intricate cellular operations which establish receptivity and immunological tolerance in the endometrium.

Composition of endometrial secretion

In the uterus, the fluid layer on the inner surface of the lumen contains a considerable amount of protein. These proteins are transudates of serum origin, leakage products of apoptotic and sloughed-off cells, and products of the release of local glandular secretion (Beier, 1974). All components of transudate, leakage and secretion material vary in composition and amount during the menstrual cycle, depending on ovarian hormonal control. Due to steroid hormone influence, the viscosity and biochemical composition in terms of electrolyte concentration, and glucosaminoglycan, glycogen, peptide and protein contents
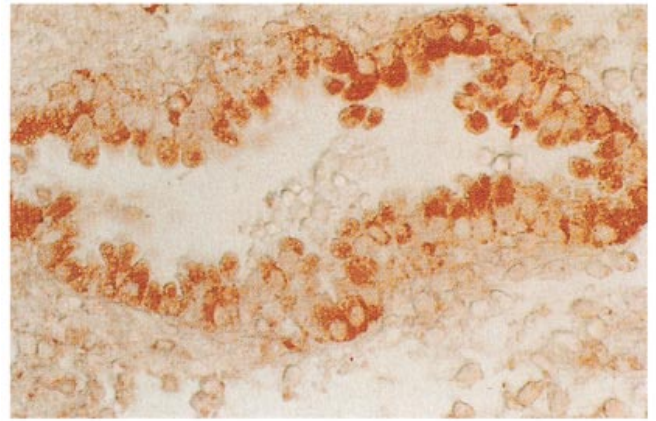

Figure 4. Endometrial section from the luteal phase on day 24 of the cycle, showing the immunoreaction of leukaemia inhibitory factor (LIF) (red staining). This section demonstrates a strong response for LIF located in the apical cytoplasm and apical protrusions of glandular epithelial cells. Paraffin section, original magnification, ×500. (Original illustration by Joachim Alfer and Irmgard Classen-Linke, Dept of Anatomy and Reproductive Biology, Aachen.)

change. Oestrogens control the permeability of capillary endothelia and thus transudation; progesterone is responsible for the secretory activity and the control of apoptosis of endometrial epithelia (Terada *et al.*, 1989; Rotello *et al.*, 1991; von Rango *et al.*, 1998). Physicochemically, oestrogen decreases the viscosity of uterine secretion, while progesterone stimulates an increase in viscosity.

More than two decades ago, we presented the first biochemical analyses of proteins in human uterine secretions (Beier *et al.*, 1970; Beier and Beier-Hellwig, 1973). At that time, uterine fluid containing distinct amounts of protein in amounts sufficient for electrophoretic resolution was obtained by gently flushing the uterine lumen after hysterectomy. Today, methodological improvements in the analysis of minute fluid volumes have made it possible to investigate samples of protein of as little as 60–80 μg. SDS treatment of proteins further permits useful resolution of protein patterns. Consequently, among patients from infertility clinics, systematic and comparative analysis has revealed numerous characteristic variations and changes in the protein patterns of uterine secretion, the definition of which has led to a greater accuracy in diagnosing either normal or deficient endometrial performance.

Biochemical analysis of the uterine secretion proteins

After SDS treatment of the protein samples, a considerable number of protein bands appear in polyacrylamide gel electrophoresis, particularly among the lower-molecular
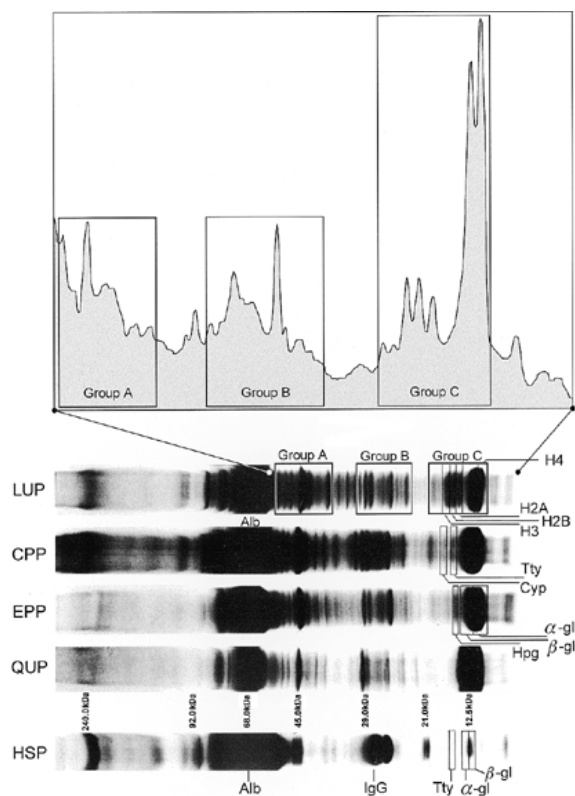
Figure 5. Protein patterns of human uterine secretion at various stages of the menstrual cycle. For comparison, a protein pattern of human blood serum (HSP) is shown. The patterns demonstrated are from the quiescent phase (QUP) and luteal phase (LUP). Molecular weight ranges are indicated between HSP and QUP. Particular families of protein bands are indicated by the areas of groups A, B and C. Albumin (Alb), immunoglobulin (IgG), histones (H2A, H2B, H3 and H4), cyclophilin (Cyp), transthyretin (Tty), haptoglobin (Hpg) and both chains of α-globin (α-gl) and β-globin (β-gl) are indicated. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed in a polyacrylamide gradient of 8.3–16.6%, with Coomassie blue staining. The upper part of the figure shows the laser densitometric tracing of the LUP protein pattern at the electrophoretic segment from 60.0 to 6.5 kDa, and particular profiles of groups A, B and C are indicated within boxes.

weight fractions (Figure 5). This area is represented by the bands between M_r 68 kDa (marker: albumin) and M_r 6.5 kDa (marker: trypsin inhibitor from the lung). The totally expressed electrophoretic pattern under these conditions comprises some 60–70 protein bands, the most pronounced and heavy staining of which are the albumin fraction at 68 kDa and those of the α- and β-chains of haemoglobin, close to a position of 12.5 kDa. Bands below 68 kDa which form three groups of very similarly sized, partly faintly staining bands are the focus of our investigation. Group A is represented by bands between 45 and 34 kDa, group B between 29 and 25 kDa, and group C between 18 and 12 kDa.

The protein patterns, as demonstrated in Figure 5, represent a dynamic spectrum of bands which appear and disappear in the course of endometrial differentiation, reaching a maximum of individual bands, together with the most intensely staining fractions, between days 15 and 24 of the ideal 28-day cycle. All protein patterns analysed for the normal cycle were obtained from patients without hormonal stimulation (Beier-Hellwig *et al.*, 1988, 1989).

It is obvious that a number of bands are missing, while others are only very weakly expressed (except albumin and the α-/β-globin fractions) during the beginning of the menstrual cycle, i.e. days 1–5, and also at the end of the cycle, at days 25–28. In particular, groups A and C lack various bands during the phase of quiescence, which represents a type of intermediate phase between the end of the secretory phase and the start of the next proliferative phase. This reinforces the interpretation that, for diagnostic reasons, the physiological menstrual cycle may be divided into three functional states rather than two. During the period of endometrial proliferation, several more intensely staining bands appear. At days 12–14, the pattern of proliferation is completed. Groups A and B are now strongly expressed, whereas group C awaits its completion at 24–48 h post ovulation. Particular attention must be paid to the three intensely staining protein bands within group C in the M_r range 15–18 kDa. The 12.5-kDa protein fraction decreases in width and staining intensity during the periovulatory period and remains less prominent for the entire luteal phase. As early as 24–48 h post ovulation, physiological cycles reveal a stable pattern over a period of up to 9–10 days; this is defined as the luteal phase pattern. It appears as though during these 9–10 days of an ideal menstrual cycle, there is no decrease or disappearance of components of protein patterns. Such a pattern reflects adequate endometrial performance, and may be considered as the 'receptive' uterine milieu (Beier-Hellwig *et al.*, 1989, 1994, 1995).

Identification of significant protein bands

The most obvious changes occurring during the luteal phase involve individual protein bands in group C, with an M_r range of 12.0–18.0 kDa. After SDS–PAGE, using 15% polyacrylamide gels, the resolved proteins were transferred to a Millipore Immobilon-P membrane using the discontinuous semidry blotting method for 45 min at 5 mA/cm² at 15°C. After staining with Coomassie brilliant blue G250, the three bands between 14.0–18.0 kDa were excised and frozen at –20°C. These samples were processed for amino acid sequencing using the Applied Biosystems 477A pulsed liquid protein sequencer. The sequence P-E-P-A-K-(X)-A-P-A-P, clearly identified

histone H2B. The identity was 90% in an overlap of the 10 amino acids.

Subsequent investigations using co-migration of histones H2A, H2B, H3 and H4 (purchased from Boehringer Mannheim, Germany) in SDS–PAGE provided convincing evidence that all of these histones had corresponding protein bands in samples of the uterine secretion (Hilmes *et al.*, 1993). Finally, we could present definitive proof of molecular identity by immunological identification using polyclonal rabbit antibodies directed specifically to histones H2A, H2B, H3 and H4. There were no cross-reactions with human blood serum samples, nor with uterine secretion samples from the follicular phase. Clear evidence was obtained by Northern blot and in-situ hybridization that histones H2A, H2B and H3 are controlled by ovarian steroid hormones during the menstrual cycle. By contrast, histone H4 is not regulated by steroids, but represents rather a constitutive component of uterine secretion (Hilmes *et al.*, 1995, 1996; von Rango *et al.*, 1998).

Further identification of lower-molecular size protein bands of USE patterns revealed that another interesting cell protein migrates in two isoforms of approximately 17 and 18 kDa. The first 10 amino acids of the isolated protein were sequenced as V-N-P-T-V-F-F-D-I-A, which represents a 100% identity in the overlap of these 10 amino acids and identifies this protein as cyclophilin A, the synonym of which is peptidyl-prolyl-*cis-trans*-isomerase A (EC 5.2.1.8). This protein is composed of a total of 164 amino acids (Harding, 1991). Cyclophilins are receiving increasing attention because they are involved in the conformation of steroid hormone receptors bound to non-activated receptor molecules, together with the heat shock proteins hsp70 and hsp90. These proteins belong to the important components of the receptor complex that accomplish their specific functions only after protein folding. There is evidence that this cyclophilin is involved in the biochemical activity of the oestrogen receptor (Ratajczak *et al.*, 1993) and the progesterone receptor (Lebeau and Baulieu, 1994; Fischer, 1994).

In another identification, we defined two serum proteins as being part of the USE pattern, where subunits migrate as bands in the group C region. The sequence S-P-T-G-T-G-E-S-K, which represents an 88.9% identity in the overlap of these nine amino acids, identified the protein as transthyretin (prealbumin). This serum protein comprises 147 amino acids, of which 21 form the signal peptide. Transthyretin is a binding protein for thyroxine, its subunits being arranged as a homotetramer. Two pairs of loops form an internal molecular channel where two binding sites for thyroxine are located. Less than 1% of these protein molecules are normally involved in thyroxine transport. About 40% of the transthyretin circulates in a tight protein–protein complex with the plasma retinol binding protein (RBP), the specific transport protein for vitamin A. Stoichiometry and polarization data suggest four binding sites and a molecular mass of 54 980 Da; the monomeric subunit migrates in PAGE at approximately the 15 kDa position.

The sequence V-D-S-G-N-D-V-T-D-I-A-D-D-G led to the identification of haptoglobin 1. This analysis represents a 100% identity in the overlap of these 14 amino acids. The protein has a signal peptide of 18 amino acids and a total of 347 amino acids. The peptide content of the native molecule is 84%; consequently, 16% of the carbohydrates form part of the molecule. Haptoglobin normally binds free plasma haemoglobin, normally as a dimer of one α-chain and one β-globin chain. In all body tissues, haptoglobin is found in extracellular compartments, the molecule forming a tetramer of two α_1- and two β-chains. Although the haptoglobin β-chain is clearly related to serine proteases, haptoglobin has no enzymatic activity. A common haptoglobin allele codes for a variation with different amino acid positions in the α-chain, resulting in an α_2-chain.

Further, from the USE protein pattern, we identified the sequence V-L-S-P-A-D-K-T-N-V-K-A as the α-chain of haemoglobin (α-globin). There was a 100% identity in the overlap of the 12 amino acids. Our minisequence represents the first 12 amino acids from a total of 141 in this molecule. Another sequence from a USE protein band resulted in 12 amino acids, V-H-L-T-P-E-E-K-S-A-V-T, which was clearly identified as the β-chain of haemoglobin (β-globin). There was a 100% identity in the overlap of these 12 amino acids. This molecule comprises a total of 146 amino acids, with an approximate molecular size of 15 000 Da, very similar to that of α-globin. Both chains, α-globin and β-globin, migrate in PAGE separation close to the 12.5 kDa position.

Among these proteins, we detected the human equivalent of the rabbit uteroglobin. The isolation and molecular identification of human uteroglobin was described recently (Aoki *et al.*, 1996). This unique protein was also detected as part of the human uterine secretion by ELISA (Table I) and was found in only small amounts within the intrauterine microenvironment, suggesting that its significance in humans may differ from that in rabbit or rat (Figure 6).

Further identification of USE pattern protein bands is currently under investigation in our laboratory. Protein bands identified to date by isolation, sequencing and immunological reactions are listed in Table I.
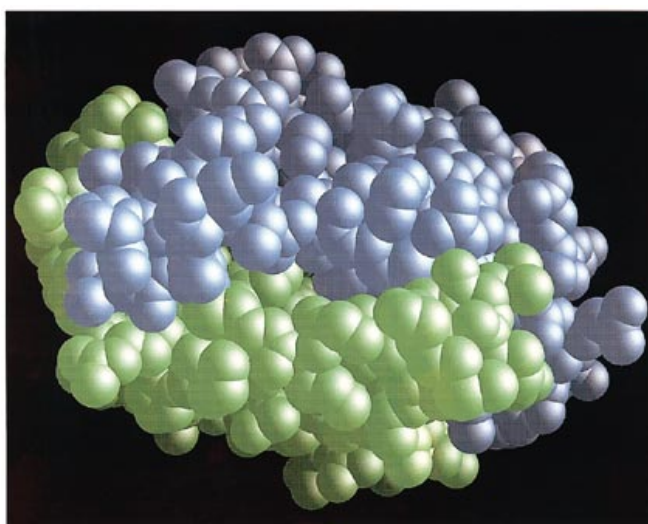
```
                5      10     15     20     25     30     35
human UGL    eICPs  FqrVI  EtLLm  dTPSS  YEAAm  elFsP  DqDMr

rabbit UGL   gICPr  FahVI  EnLLL  GTPSS  YEtsL  KeFeP  DdtMk

rat UGL      ssdICPg FlqVl  KaLLL  GseSn  YEAAL  KpFnP  asDlq


                40     45     50     55     60     65     70
human UGL    eAGAQ  LKKLV  DTLPQ  kpREs  IiKLm  EKIaq  SsLCn

rabbit UGL   dAGmQ  mKKvl  DsLPQ  tTREN  ImKLT  EKIvk  SPLCm

rat UGL      nAGtQ  LKrLV  DTLPQ  eTRiN  IvKLT  EKIlt  SPLCe  qdlrv
```

Figure 6. Uteroglobin (UGL) in human endometrium and human uterine secretion. The dimeric structure shows the design of an extremely globular molecule, displaying the centrally located binding pouch for progesterone and other lipophilic molecules. This computer design was drawn using the coordinates published by Morize *et al*. (1987). The lower part of the figure shows the alignment of amino acids of human, rabbit and rat UGL. Capital letters indicate residues which are conserved within the UGL family. The homology of human to rabbit UGL reaches 57.4%. The sequence homology of rabbit and rat UGL is very similar, with an overlap of 39 within 68 amino acids. Rat UGL shows slightly lower homology to human UGL by an overlap of 37 within 68 residues (Aoki *et al*., 1996).

Assessment of USE patterns

To date, monitoring of the menstrual cycle in the clinical procedure lacks any reliable and significant assessment of the so-called 'endometrial factor' that might serve as a useful predictive parameter for a receptive endometrium or the implantation window. The endometrium regularly reacts as the target tissue of the ovarian hormones. Consequently, histological dating proved to be a sensitive indicator of ovarian function, reflecting perturbations of the physiological balances of ovarian steroids. Conversely, however, the assumption that normal or rather sufficient steroid hormone concentrations measured in blood plasma would guarantee a normal endometrial development is false.

There are many reports in the literature which indicate that the histological transformation of the endometrium

can be inadequate, despite a normal progesterone output during the luteal phase. Rarely, an atrophic endometrium can occur, together with normal ovarian function. Jones (1949, 1976) was the first to discuss a defective endometrial response to hormonal stimulation as a cause of luteal inadequacy. As mentioned previously, endometrial transformation is, as a rule, absolutely dependent on steroid hormone control of the ovary. The dynamic process of transformation is paralleled by a remarkable and characteristic secretory activity. Circumstantially, the endometrial reaction can be dissociated from hormonal control. Under such conditions, the physiological dependency is broken down, and the endometrium turns out to be completely refractory or provides only a partial response in that proliferation and/or transformation are started, but not completed. Thus, the endometrium loses its capacity of building up the necessary full composition of protein patterns in uterine secretion that seems to be a prerequisite for the support of implantation. The physiological cycle appears as a dynamic sequence of a continuously changing protein release that in turn can be analysed by the sequentially changing protein patterns seen in PAGE.

We have evidence that densitometric tracings of SDS–PAGE samples of USE protein patterns will serve as a promising means for clinical evaluation and eventually may lead to new approaches for luteal phase management. Even within a cycle of conception, assessment of USE protein patterns is possible. Diagnostic analyses of samples obtained on day 2 after the luteinizing hormone (LH) peak express patterns of 'adequate' luteal phases. Moreover, these samples were taken from patients in conception cycles that went on to normal clinical pregnancies (Figure 7).

Progesterone regulates endometrial receptivity and the expression of proteins

Initially, progesterone antagonists were studied in our rabbit model, utilizing uteroglobin synthesis and release as markers (Beier, *et al*., 1987, 1991, 1994). Based on these investigations, we used competitive receptor antagonists to progesterone for more detailed studies of the effects of progesterone on human endometrial proteins. Since mifepristone (RU 486) is the only registered progesterone antagonist permitted for clinical application (e.g. in France, UK and Sweden), this compound was the first to be studied for its effects on the human luteal phase. Earlier investigations at the Karolinska Hospital (Swahn *et al*., 1990, 1991) on dose ranging and classical clinical parameters had shown that a single dose of 200 mg of mifepristone given orally on day 2 after the LH peak inhibited the establish-

ment of pregnancy and allowed an undisturbed menstrual bleeding. Also, with this protocol, mifepristone was found to be ineffective in decreasing epithelial progesterone receptor concentrations in the endometrium and, in turn, retarding endometrial maturation; however, it did not alter the serum concentration of follicle stimulating hormone (FSH), oestradiol and progesterone.

Following the same protocol, we investigated the effect of single administration of 200 mg mifepristone on the uterine secretion protein pattern assessed by USE. In the first four patients, each uterine secretion sample was obtained at day 6 after LH peak in a non-treated control cycle. The following cycle served as the treatment cycle, when 200 mg mifepristone was given at day LH +2, and again the uterine secretion sample was obtained at day LH +6. The most striking changes following mifepristone treatment occurred among the histone bands in group C of the USE pattern. In two patients, there were significant reductions of the H2A and H3 peaks, whereas the peak in the position of H2B was only partly reduced compared with the control. Some additional protein bands of group A and group B appeared markedly changed after progesterone antagonist application (Figure 8). Detailed information will be available only after more patients have been investigated. Two of the four patients with a history of abnormal cycles showed a relatively weak response to mifepristone, beginning with an unusually extended control cycle; consequently, more individuals must be included in this study. However, preliminary evidence already shows that progesterone-dependent events in the luteal phase of the human cycle can be altered significantly by progesterone antagonists, particularly in the partial or total inhibition of the appearance of several single endometrial proteins.

Fertility-regulating approaches using progesterone antagonists have been numerous, although only the post-ovulatory or early luteal phase applications have yet proved effective in preventing pregnancies (Glasier *et al.*, 1992; Gemzell-Danielsson *et al.*, 1993). Such treatments during the early luteal phase have offered a successful contraceptive strategy, and we could demonstrate significant changes in protein patterns within the uterine secretions in these patients. We may therefore deduce that the proteins under investigation can serve as markers for subtle changes of endometrial function, and which may in fact prevent normal receptivity. An alternative approach to this post-ovulatory contraception is to administer progesterone antagonists during the whole cycle, either daily or once weekly. This strategy had emerged from primate studies, where pregnancies were totally abolished (Ishwad *et al.*, 1993; Chwalisz *et al.*, 1995; Katkam *et al.*, 1995). Within this context, an extremely interesting report from Katkam *et al.* (1995) showed how low-dose onapristone treatment (2.5 mg or 5.0 mg every third day) for four to seven consecutive cycles prevented pregnancy without disturbing the menstrual cycle and without inhibiting ovulation in the majority of cycles. However, anovulation and luteal insufficiency occurred in some animals during prolonged treatment. The contraceptive effect in the ovulatory cycles seems primarily related to the retardation of endometrial development, resulting in the inhibition of endometrial receptivity.

Table I. Proteins of human uterine secretion: identification by isolation, sequencing, Western Blot and enzyme-linked immuno-sorbent assay (ELISA)

| Protein	Molecular weight (Da)	No. of amino acid residues	SDS–PAGE localization	Origin and function of the protein
Uteroglobin (monomer)	7913	70	6.5 kDa	Secretory protein (identical with Clara-cell protein CC 16)(Beier, 1982; Akoi *et al.,* 1996)
α-Globin	15000	141	12.0 kDa	Haemoglobin α-chain, serum protein
Histone H4	11236	102	12.0 kDa	Cellular protein, basic nuclear protein
β-Globin	16000	146	12.5 kDa	Haemoglobin β-chain, serum protein
Histone H2A	13960	129	13.0 kDa	Cellular protein, basic nuclear protein
Histone H2B	13775	125	15.0 kDa	Cellular protein, basic nuclear protein
Transthyretin (one subunit of homotetramer)	13745	147	15.0 kDa	Serum protein, hormone binding protein (synonymous with prealbumin)
Histone H3	15273	135	16.0 kDa	Cellular protein, basic nuclear protein
Haptoglobin 1 (α-chain dimer)	17686	166	17.0 kDa	Serum protein, haemoglobin binding protein
Cyclophilin A	17881	164	17.0 kDa	Cellular protein, protein folding enzyme (peptidyl-prolyl-*cis/trans*-isomerase)
Glycodelin A	20624	180	26.0 kDa	Secretory protein of endometrial glands (synonymous with PP 14) (Huhtala *et al.,* 1987)
IGF-BP1	27903	259	28.0/56.0 kDa	Protein of fibroblasts (predecidual and decidual cells) (synonymous with PP12) (Koistenen *et al.,* 1986)
Albumin	66290	585	66.0 kDa	Serum protein, hormone binding protein
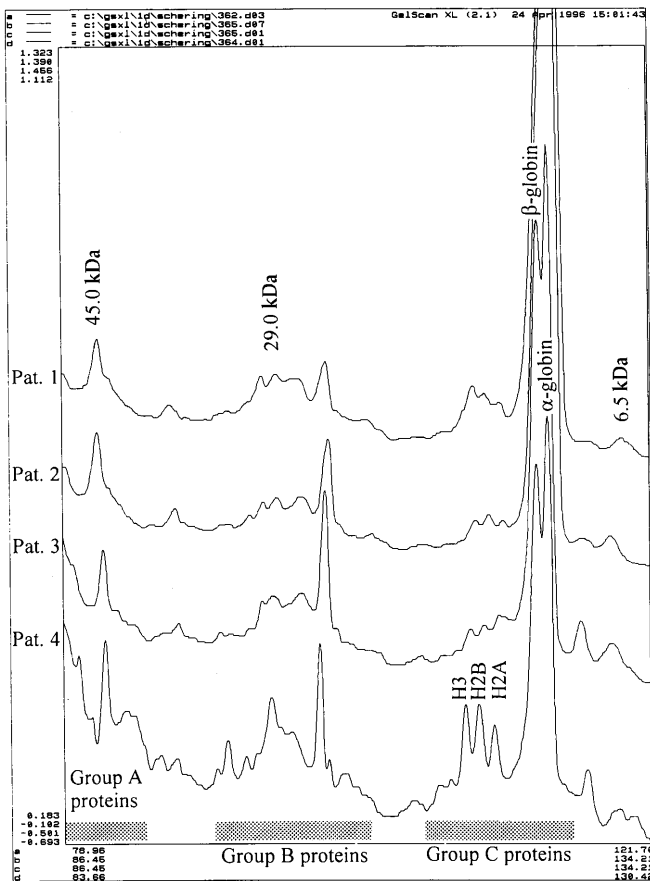
Figure 7. Laser densitometric assessment of human uterine secretion electrophoretic (USE) samples from the early luteal phase (day 2 after luteinizing hormone peak). This figure demonstrates four USE resolutions within segments of the molecular weight ranges from 45.0 to 6.5 kDa. The samples express the typical adequate luteal phase patterns within the cycle of conception. Each patient experienced a normal clinical pregnancy which began in this cycle. SDS–PAGE was performed in a polyacrylamide gel gradient of 8.3–16.6%. Laser densitometry was performed using a helium–neon laser at 633 nm. Histones H2A, H2B and H3, and α- and β-globins are indicated.

Figure 8. Laser densitometric assessment of human uterine secretion electrophoretic (USE) samples from day 6 after luteinizing hormone (LH) peak. Treatment with the progesterone antagonist, mifepristone (RU 486, 200 mg) was performed at day 2 after the LH peak. The control sample was assessed equally after collection of the USE sample at day 6 after the LH peak of the preceding non-treated control cycle. Significant changes appear in the histone bands H2A, H2B and H3. Further changes can be recognized in higher molecular weight ranges. SDS–PAGE was performed in a high-density gel (15% polyacrylamide). A segment of the laser densitometric scan is shown between 40.0 and 6.5 kDa.

Based on several detailed studies in animal models (Beier, 1986; Hegele-Hartung and Beier, 1986; Niemann *et al.*, 1987), it has become evident that numerous cellular and molecular steps of endometrial transformation were inhibited or delayed when progesterone was cut off from its normal triggering and regulation of luteal phase events. This inhibition of normal endometrial transformation and luteal function prevented the establishment of pregnancy.

We have also investigated endometrial secretory proteins in one of the first clinical studies on continuous low-dose progesterone antagonist treatment to achieve endometrial contraception. In this study, onapristone was administered either daily (1, 3 or 10 mg) or once weekly (10, 30 or 100 mg). Onapristone an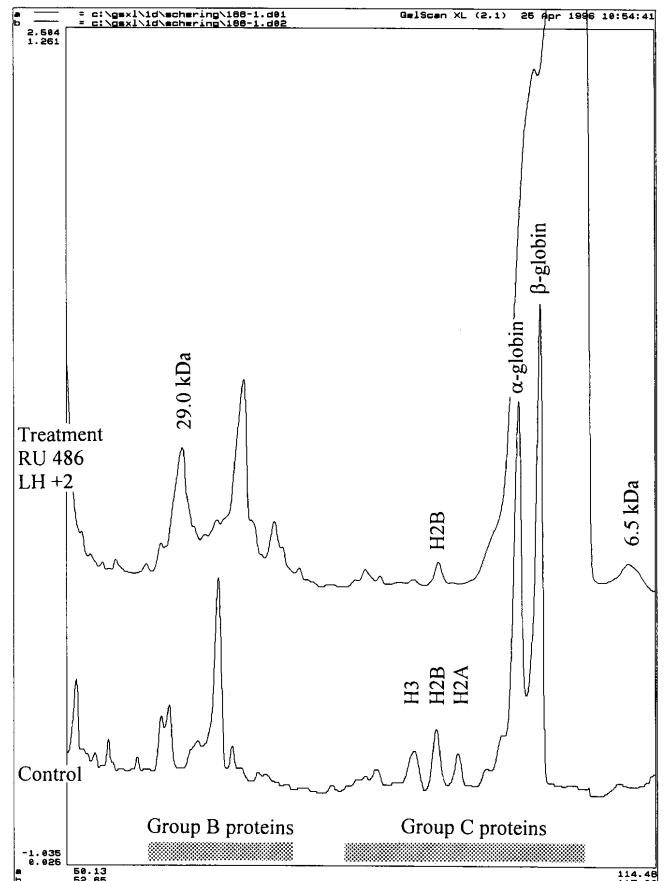d mifepristone are both selective progesterone antagonists, but with clearly different actions at the molecular level. Unlike mifepristone, onapristone does not induce stable receptor dimers and inhibits the binding of the hormone–hormone receptor complex at the hormone-responsive element of the DNA. This results in different pharmacodynamics, the half-life of onapristone being only 2–3 h compared with 24–48 h for mifepristone. The assessment of USE by laser densitometry reveals marked changes in luteal phase patterns (obtained around day 5 after the LH peak) as compared with controls of the same patients and under the same clinical study conditions. Considerable reductions occurred in several protein peaks, most obviously among the low-molecular weight proteins (group C of the USE pattern). Within the 30 patients of each

treatment group (daily and once weekly), two typical response categories were identified, one exhibiting pronounced reductions of particular peaks of the pattern, and the other showing a general quantitative diminution of distinct parts of the pattern. Nevertheless, the results of changes in the uterine protein patterns have demonstrated that endometrial function under continuous onapristone administration is significantly impaired (Figure 9). This preliminary observation is strikingly in accord with a recently published investigation by Gemzell-Danielsson *et al.* (1996) who used mifepristone at low weekly doses (2.5 and 5 mg/week). In summary, there are definite beneficial effects of low-dose applications of progesterone antagonists on endometrial function, in particular the delay of cellular and tissue transformation and inhibition of secretory activity. This treatment does not inhibit ovulation, so whether such effects will prove to be sufficient to prevent pregnancy under the daily circumstances of life awaits further clinical studies in unprotected cycles.

Finally, a comparative aspect of the rabbit preimplantation model must be recalled in which a single uterine protein alone has never been used successfully as a marker of receptivity. Several components, forming a pattern, have always provided the most reliable information on the diagnosis of receptivity (Beier, 1982, 1986; Hegele-Hartung and Beier, 1986; Hegele-Hartung *et al.*, 1992).

Based on our research in animal models, we now rely on techniques that permit protein analyses of the human uterine milieu to be achieved because this is the site where attachment and implantation begin. Only by biochemical analyses of uterine proteins have we been able to diagnose and define the numerous changes in the endocrine system. For instance, the phenomenon of delayed secretion in the rabbit, initiated by postcoital injection of oestrogens, showed that the assessment of protein patterns in uterine secretion was a reliable tool (Beier, 1974; Beier and Mootz, 1979). Indeed, this tool has now made it possible to predict the correct time when implantation will be successful following the transfer of a viable embryo.
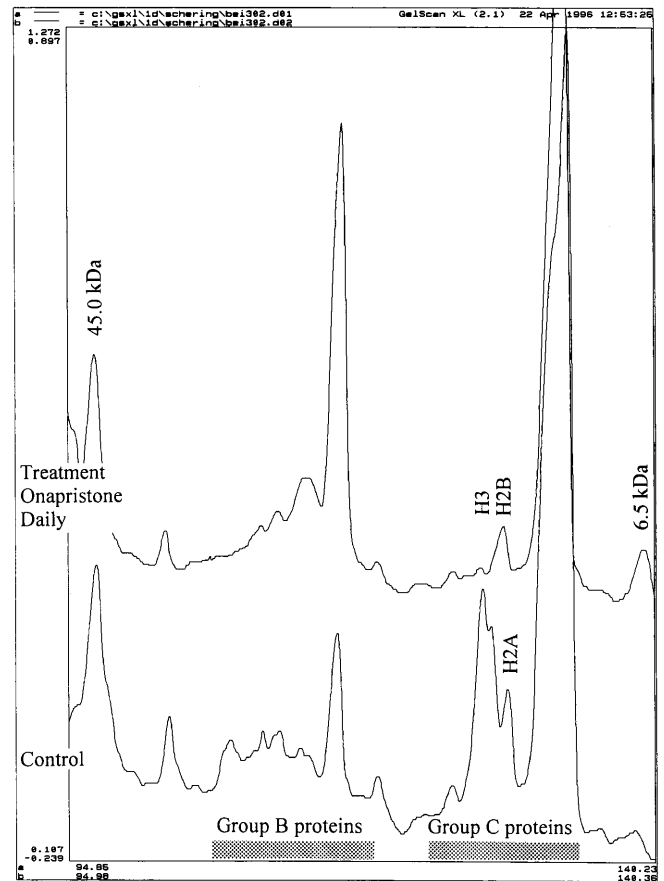
Acknowledgements

Figure 9. Laser densitometric assessment of human uterine secretion electrophoretic (USE) samples from day 5 after luteinizing hormone (LH) peak. Treatment with the progesterone antagonist, onapristone (ZK 98.299, 10 mg daily) was performed throughout the whole cycle. The control sample was assessed equally after collection of the USE sample at day 5 after the LH peak of the preceding non-treated control cycle. Significant changes appear in the histone bands H2A, H2B and H3. Further changes can be recognized in higher molecular weight ranges. SDS–PAGE was performed in a polyacrylamide gel gradient of 8.3–16.6%. A segment of the laser densitometric scan is shown between 45.0 and 6.5 kDa.

References

Aoki, A., Pasolli, H.A., Raida, M. *et al.* (1996) Isolation of human uteroglobin from blood filtrate. *Mol. Hum. Reprod.*, **2**, 489–497.

Beier, H.M. (1974) Oviductal and uterine fluids. *J. Reprod. Fertil.*, **37**, 221–237.

Beier, H.M. (1982) Uteroglobin and other endometrial proteins: biochemistry and biological significance in beginning pregnancy. In Beier, H.M. and Karlson, P. (eds), *Proteins and Steroids in Early Pregnancy.* Springer-Verlag, Berlin, pp. 38–71.

Beier, H.M. (1986) Hormone und Hormonantagonisten in der Implantationsforschung. *Fortschr. Fertilitätsforsch.*, **13**, 2–15.

Beier, H.M. and Beier-Hellwig, K. (1973) Specific secretory protein of the female genital tract. *Acta Endocrinol. Suppl. (Copenh.)*, **180**, 404–425.

Beier, H.M. and Mootz, U. (1979) Significance of maternal uterine proteins in the establishment of pregnancy. In *Maternal Recognition of Pregnancy.* Ciba Foundation Symposium Series 64. Excerpta Medica, Amsterdam, pp. 111–140.

Beier, H.M., Petry, G. and Kühnel, W. (1970) Endometrial secretion and early mammalian development. In Gibian, H. and Plotz, E.J. (eds), *Mammalian Reproduction*. Kolloquium der Gesellschaft für Biologische Chemie, Mosbach. Springer-Verlag, Berlin, pp. 264–285.

Beier, H.M., Elger, W. and Hegele-Hartung, C. (1987) Effects of antiprogestins on the endometrium during the luteal phase after postovulatory treatment. In Naftolin, F. and De Cherney, A. (eds) *The Control of Follicle Development, Ovulation and Luteal Function: Lessons from* In Vitro *Fertilization*. Raven Press, New York, pp. 331–343.

Beier, H.M., Elger, W., Hegele-Hartung, C. *et al.* (1991) Dissociation of corpus luteum, endometrium and blastocyst in human implantation research. *J. Reprod. Fertil.*, **92**, 511–523.

Beier, H.M., Hegele-Hartung, C., Mootz, U. and Beier-Hellwig, K. (1994) Modification of endometrial cell biology using progesterone antagonists to manipulate the implantation window. *Hum. Reprod.*, **9**(suppl.1), 98–115.

Beier-Hellwig, K., Sterzik, K. and Beier, H.M. (1988) Zur Rezeptivität des Endometriums: die Diagnostik der Proteinmuster des menschlichen Uterussekretes. *Fertilität*, **4**, 128–134.

Beier-Hellwig, K., Sterzik, K., Bonn, B. and Beier, H.M. (1989) Contribution to the physiology and pathology of endometrial receptivity: the determination protein patterns in human uterine secretions. *Hum. Reprod.*, **4**(suppl.1), 115–120.

Beier-Hellwig, K., Sterzik, K., Bonn, B. *et al.* (1994) Hormone regulation and hormone antagonist effects on protein patterns of human endometrial secretion during receptivity. *Ann. N. Y. Acad. Sci.*, **734**, 143–156.

Beier-Hellwig, K., Bonn, B., Sterzik, K. *et al.* (1995) Uterine receptivity and endometrial secretory protein patterns. In Dey, S.K. (ed.), *Molecular and Cellular Aspects of Periimplantation Processes*. Serono Symposia, USA. Springer-Verlag, New York, pp. 87–102.

Breckwoldt, M., Beier, H.M., Neumann, F. and Bräuer, H. (1994) Exempla endokrinologica. Band 2, Bildatlas zu Aspekten der Pathophysiologie des endokrinen Systems. Medical Services, München.

Chwalisz, K., Stöckemann, K., Fuhrmann, U. *et al.* (1995) Mechanism of action of the antiprogestins in the pregnant uterus. *Ann. N. Y. Acad. Sci.*, **761**, 202–224.

Classen-Linke, I., Alfer, J., Hegele-Hartung, C. *et al.* (1995) Hormonal regulation of β-integrin expression and its relation to immunocompetent cells in human endometrium throughout the menstrual cycle. *Exp. Clin. Endocrinol.*, **103**(suppl.1), 113.

Fischer, G. (1994) Über Peptidyl-prolyl-cis/trans-isomerasen und ihre Effektoren. *Angew. Chem.*, **106**, 1479–1501.

Gemzell-Danielsson, K., Swahn, M.L., Svalander, P. and Bygdeman, M. (1993) Early luteal phase treatment with RU 486 for fertility regulation. *Hum. Reprod.*, **8**, 870–873.

Gemzell-Danielsson, K., Westlund, P., Johannisson, E. *et al.* (1996) Effect of low weekly doses of mifepristone on ovarian function and endometrial development. *Hum. Reprod.*, **11**, 256–264.

Glasier, A.F., Thong, K.J., Dewar, M. *et al.* (1992) Mifepristone (RU 486) compared with high-dose estrogen and progesterone emergency postcoital contraception. *N. Engl. J. Med.*, **327**, 1041–1044.

Harding, M.W. (1991) Structural and functional features of the peptidyl prolyl *cis-trans* isomerase, cyclophilin. *Pharmacotherapy*, **11**, 142S–148S.

Hegele-Hartung, C. and Beier, H.M. (1986) Distribution of uteroglobin in the rabbit endometrium after treatment with anti-progesterone (ZK 98.734): an immunocytochemical study. *Hum. Reprod.*, **1**, 497–505.

Hegele-Hartung, C., Mootz, U. and Beier, H.M. (1992) Luteal control of endometrial receptivity and its modification by progesterone antagonists. *Endocrinology*, **131**, 2446–2460.

Hilmes, U., Beier-Hellwig, K., Sterzik, K. *et al.* (1993) Identification of histones in human uterine secretion samples. *J. Reprod. Fertil. Abstr. Ser.*, **12**, 43.

Hilmes, U., Krusche, C., Classen-Linke, I. *et al.* (1995) Histonexpression in Korrelation zu Proliferationsaktivität und apoptotischen Vorgängen im menschlichen Endometrium. *J. Fertil. Reprod.*, **5**, 33–34.

Hilmes, U., Krusche, C., Classen-Linke, I. *et al.* (1996) Histonexpression in Korrelation zu Proliferation und Apoptose im menschlichen Endometrium. *Ann. Anat.*, **178**(suppl.), 84–85.

Huhtala, M.-L., Seppälä, M., Närvänen, A. *et al.* (1987) Amino acid sequence homology between human placental protein 14 and β-lactoglobulins from various species. *Endocrinology*, **120**, 2620–2622.

Ishwad, P.C., Katkam, R.R., Hinduja, I.N. *et al.* (1993) Treatment with a progesterone antagonist ZK 98.299 delays endometrial development without blocking ovulation in bonnet monkeys. *Contraception*, **48**, 57–70.

Jones, G.S. (1949) Some never aspects of the management of infertility. *JAMA*, **141**, 1123.

Jones, G.S. (1976) The luteal phase defect. *Fertil. Steril.*, **27**, 351–356.

Katkam, R.R., Gopalkrishnan, K., Chwalisz, K. *et al.* (1995) Onapristone (ZK 98.299): a potential antiprogestin for endometrial contraception. *Am. J. Obstet. Gynecol.*, **173**, 779–787.

Koistinen, R., Kalkkinen, N., Huhtala, M.-L. *et al.* (1986) Placental protein 12 is a decidual protein that binds somatomedin and has an identical N-terminal amino acid sequence with somatomedin-binding protein from human amniotic fluid. *Endocrinology*, **118**, 1375–1378.

Lebeau, M.-C. and Baulieu, E.E. (1994) Steroid antagonists and receptor-associated proteins. *Hum. Reprod.*, **9**(suppl. 2), 11–21.

Morize, I., Surcouf, E., Vaney, M.C. *et al.* (1987) Refinement of the c222$_1$ crystal form of oxidized uteroglobin at 1.34Å resolution. *J. Mol. Biol.,* **194,** 725–739

Niemann, L.K., Choate, T.M., Chrousos, G.P. *et al.* (1987) The progesterone antagonist RU 486. A potential new contraceptive agent. *N. Engl. J. Med.*, **316**, 187–191.

Nikas, G., Drakakis, P., Loutradis, D. *et al.* (1995) Uterine pinopodes as markers of the 'nidation window' in cycling women receiving exogenous oestradiol and progesterone. *Hum. Reprod.*, **10**, 1208–1213.

Parr, M.B. and Parr, E.L. (1974) Uterine luminal epithelium: protrusions mediate endocytosis, not apocrine secretion, in the rat. *Biol. Reprod.*, **11**, 220–233.

Psychoyos, A. (1994) The implantation window: basic and clinical aspects. In Mori, T., Aono, T., Tominaga, T. and Hiroi, M. (eds), *Perspectives on Assisted Reproduction*. Ares-Serono Symposium Series. Frontiers in Endocrinology, Vol. 4, p. 57.

Ratajczak, T., Carrello, A., Mark, P.H. *et al.* (1993) The cyclophilin component of the unactivated estrogen receptor contains a tetratricopeptide repeat domain and shares identity with p59 (FKBP59). *J. Biol. Chem.*, **268**, 13187–13192.

Rotello, R.J., Lieberman, R.C., Purchio, A.F. and Gerschenson, L.E. (1991) Coordinated regulation of apoptosis and cell proliferation by transforming growth factor-β1 in cultured uterine epithelial cells. *Proc. Natl Acad. Sci. USA*, **88**, 3412–3415.

Simon, C., Pellicier, A. and Polan, M.L. (1995) Interleukin-1 system crosstalk between embryo and endometrium in implantation. *Hum. Reprod.*, **10**(suppl. 2), 43–54.

Swahn, M.L., Bygdeman, M., Cekan, S. *et al.* (1990) The effect of RU 486 administered during the early luteal phase on bleeding pattern, hormonal parameters and endometrium. *Hum. Reprod.*, **5**, 402–408.

Swahn, M.L., Gemzell, K. and Bygdeman, M. (1991) Contraception with mifepristone. *Lancet*, **338**, 942–943.

Tabibzadeh, S. (1994) Cytokines and the hypothalamic-pituitary-ovarian-endometrial axis. *Hum. Reprod.*, **9**, 947–967.

Tabibzadeh, S. and Sun, X.Z. (1992) Cytokine expression in human endometrium throughout the menstrual cycle. *Hum. Reprod.*, **7**, 1214–1221.

Terada, N., Yamamoto, R., Tadada, T. *et al.* (1989) Inhibitory effect of progesterone on cell death of mouse uterine epithelium. *J. Steroid Biochem.*, **33**, 1091–1096.

von Rango, U., Classen-Linke, I., Krusche, C.A. and Beier, H.M. (1998) The receptive endometrium is characterized by apoptosis in the glands. *Hum. Reprod.* **13,** 3177–3189.