Human Reproduction Update 1998, Vol. 4, No. 5 pp. 465–471

Molecular control of the implantation window

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Human endometrium is the end organ of the hypothalamic–pituitary–ovarian axis. Therefore, endometrium is susceptible to changes in the cases of infertility that originate from disturbances in the normal functioning of this axis. In addition, some cases of unexplained infertility may be due to altered endometrial function. This disturbed endometrial function may originate from lesions in the molecular repertoire that are crucial to implantation. Human endometrium becomes receptive to implantation by the blastocyst within a defined period during the menstrual cycle. The duration of this so-called 'endometrial receptivity' or 'implantation' period seems to span from few days after ovulation to several days prior to menstruation. Successful implantation results from a co-ordinated series of events that would allow establishment of a timely dialogue between a receptive endometrium and an intrusive blastocyst. The members of the molecular repertoire that make endometrium receptive to implantation are gradually being recognized. Among these are the cytokines, integrins, heat shock proteins, tastin and trophinin. In addition, the expression of a second set of genes including *tumour necrosis factor* α (*TNF*-α) and *ebaf*, may be the appropriate signal for the closure of the 'implantation window', for making the endometrium refractory to implantation and for preparing it for the menstrual shedding.

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Key words: α crystallin β/ebaf/endometrial receptivity/ implantation window/ TNF-α

Causes of infertility

A substantial number of women do not become pregnant. As shown in Guttmacher's classic table (Table I), about 7% of couples can be considered infertile after they have tried for 2 years to attain pregnancy (Guttmacher, 1956). In the USA, in 1982, nearly one in five married women of reproductive age reported that, during their lifetime, they had sought professional help for infertility (Mosher and Pratt, 1991) and, in 1988, 8.4% (a total of 4.9 million) of women aged 15–44 years reported impaired fecundity (Mosher and Pratt, 1993). In the USA, after all the standard clinical investigations are done and known causes of infertility attributable to tubal and pelvic pathologies, male factor, ovulatory dysfunction and unusual problems are ruled out, a substantial number (10%) of infertility cases remain of unknown aetiology. These cases are designated as 'unexplained infertility' (Crosignani *et al*., 1993; Lobo, 1993; Speroff, 1994; Blacker *et al*., 1997). The underlying basis for the infertility in patients with unexplained infertility is not known. In some of these women, an 'endometrial factor' may be involved in the infertility and there may be a primary derangement in the expression of the endometrial genes crucial to implantation. In addition, human endometrium is the end organ for the signals derived from the hypothalamic–pituitary–ovarian axis. Therefore, the aetiological factor(s) causing infertility may secondarily cause an alteration in the molecular repertoire of the endometrial receptivity. In either case, such aberrations may be specific and thus may allow diagnosis of the infertility. At the present time, only limited information exists regarding the genes that account for endometrial receptivity in humans and whose aberrant expression is associated with infertility. Therefore, before we can gain an insight into the molecular lesions in infertility, we should attempt to identify the molecular signals that confer receptivity to endometrium or lead to its resistance to implantation.

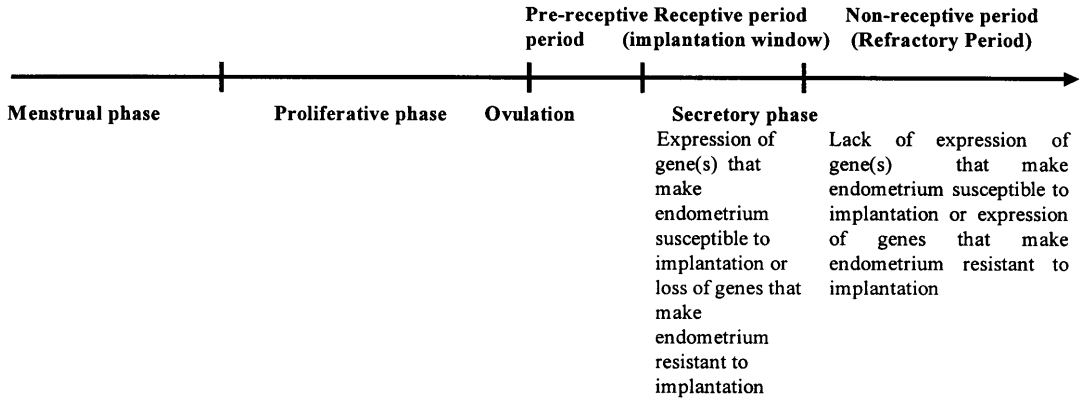

Figure 1. An hypothesis for explaining the presence of an 'implantation window' in human endometrium.

Table I. Time required for conception in couples who will attain pregnancy

Months of exposure	% Pregnancy
3	57
6	72
12	85
24	93

Receptivity of human endometrium to implantation

Human endometrium undergoes characteristic phases of proliferation and secretion. Exquisite mechanisms control the processes that drive endometrium through these phases and prepare this tissue for implantation (for a review, see Tabibzadeh and Babaknia, 1995). Implantation is a complex process which initially requires the interaction of the blastocyst and, subsequently, the developing embryo and placenta with the endometrium. In implantation, both endometrial and embryonic factors are involved. Some studies show that a 100% pregnancy rate cannot be achieved by increasing the number of embryos (Medical Research International Society for Assisted Reproductive Technology, 1992). This suggests that other factor(s), presumably of endometrial origin, may be involved in the implantation. These as yet unidentified factors are likely to account for the endometrial receptivity to the implantation process. Presence of endometrial receptivity was first established in the rat and later validated in other species (Psychoyos, 1973a,b, 1976, 1980, 1986, 1993; Psychoyos and Casmiri, 1980). These studies show that endometrium can be maintained in various states which include neutral, receptive and non-receptive or refractory phases. It is postulated that in humans, an 'implantation window' also exists (Navot *et al*., 1984, 1986, 1991; Paulson *et al*., 1990a,b; Psychoyos, 1993; for a review, see Tabibzadeh and Babaknia, 1995). This phase is followed by a 'non-receptive' phase when the endometrium becomes refractory to the implantation process (Strauss and Gurpide,1991; Psychoyos, 1993) (Figure 1).

The presence of receptive and non-receptive phases during the menstrual cycle is consistent with the hypothesis that receptivity of endometrium to implantation by the blastocyst is a regulated process. By removal of the uterine epithelium, Cowell (1969) showed that endometrium controls the implantation of the blastocyst. After removal of the endometrial epithelium, blastocysts could implant in endometrium completely independent of any hormonal control (Cowell, 1969). An additional feature of this control is regulation of the depth of the invasion of the endometrium by the trophoblasts. For example, the trophoblasts of the implanting blastocysts can invade, quite deeply, tissues other than endometrium (for review, see Denker, 1993). Despite the invasive behaviour *in vitro*, in some animals such as pig, the trophoblasts attach but do not invade the uterine epithelium (Dantzer, 1985; Keys and King, 1990). When transplanted ectopically in the uterine wall, however, the same cells can invade tissues. Therefore, it is speculated that two sets of molecules may exist in endometrium. One set of molecules makes endometrium receptive whereas a second set makes endometrium resistant to implantation. Therefore, presence or absence of the 'implantation window' may depend on the regulated and timed expression of these molecules in endometrium (Figure 1).

Period of endometrial receptivity

There is no general agreement as to the dates and duration of the 'endometrial receptivity period' or 'implantation window' during normal menstrual cycles. For example, it was suggested that the implantation window is confined to the postovulatory days (POD) 5–7 of the normal menstrual cycle (Psychoyos, 1993). Rogers and Murphy (1989),

however, concluded that the human implantation window must be at least 3.5 days, whereas Formigli *et al*. (1987) suggested that the period of endometrial receptivity may be as long as 7 days. Based on the available data, however, it can be concluded that the endometrial implantation window in humans opens several days after ovulation and closes several days prior to menstruation.

Molecular repertoire of endometrial receptivity

Endometrial receptivity may depend on timed and regulated expression of specific set of genes and presence of the implantation window may require establishment of a molecular dialogue between the blastocyst and the endometrium. For example, in the mouse, endometrial epithelial cells express H-type carbohydrate (Wilson *et al.*, 1990, Raboudi *et al.*, 1992), and the abembryonic ectoderm acquires the ability to specifically bind H-type-I structures around the time of implantation (White and Kimber, 1994). So far, little information exists in humans about the genes whose expression in endometrium is essential to its interaction with the blastocyst. Most of our current knowledge regarding expression of such genes has been derived from animal studies. Studies in experimental animals have revealed potential molecular cues that are implicated in endowing endometrium with this receptivity. An increased expression of leukaemia inhibitory factor (LIF) was found in the endometrial glands in the mouse prior to the implantation of the blastocyst. This increased expression was found to be under maternal control (Bhatt *et al.*, 1991). Later, the role of LIF in this process was shown by eliminating the *LIF* gene by homologous recombination (Stewart *et al.*, 1992). LIF-deficient mice were infertile. These animals ovulated, and their ova could be fertilized. The blastocysts, however, failed to implant in the endometrium of these animals but successfully implanted in the endometria of normal surrogate female mice (Stewart *et al.*, 1992). These data suggest that specific molecules are involved in the receptivity of the endometrium to implantation by the blastocyst in the mouse. We may not infer from animal studies, however, that in humans the same set of endometrial genes is implicated in endometrial receptivity and implantation. For example, despite the data that amphiregulin may be important in implantation in the mouse (Das *et al.*, 1995), throughout the entire pregnancy in humans, no immunoreactivity for amphiregulin was detectable in the gestational endometria (Lysiak *et al.*, 1995).

Many changes in the structural morphology (for a review, see Martel *et al.*, 1987; Psychoyos, 1993) and enzyme activity (Tseng and Gurpide, 1974; King *et al.*, 1981; Satyaswaroop *et al.*, 1983), as well as the expression of specific proteins, such as complement C3 (Hasty *et al.*, 1994), MUC1 (Aplin *et al.*, 1994, Hey *et al.*, 1994), PP12 (Rutanen *et al.*, 1986), prolactin (Maslar and Riddick, 1979), cathepsin D (Maudelonde *et al.*, 1987), α_1-PEG (Waites *et al.*, 1988), α_2-PEG (Joshi *et al* 1980; Julkunen *et al.*, 1986), various integrin molecules (Lessey *et al.*, 1992, 1994; Tabibzadeh, 1992), cytokines (Tabibzadeh *et al.*, 1994, 1995a,b) and heat shock proteins (Tabibzadeh *et al.*, 1996), have been described in human endometrium during the secretory phase. Among these, the expression of a few proteins, including integrins (Lessey *et al.*, 1992, 1994; Tabibzadeh, 1992), interleukin-6 (Tabibzadeh *et al.*, 1995b), MUC1 (Aplin *et al.*, 1994; Hey *et al.*, 1994), trophinin and tastin (Fukuda *et al.*, 1995), seems to coincide with the period of endometrial receptivity, suggesting that these molecules may be implicated in rendering endometrium receptive to implantation.

We recently attempted to identify the genes expressed during the implantation period by using representational difference analysis (RDA; Gruidl *et al.*, 1997). When the cDNAs of a proliferative endometrium were used as the driver and the cDNAs of a postovulatory day 5 endometrium were used as the tester, a number of bands were identified by RDA. DNA of the cloned RDA products revealed that the majority of the clones contained a fragment of a cDNA identical to that of α-crystallin B chain (Gruidl *et al.*, 1997). Northern blot analysis showed that the expression of the *α-crystallin B* chain mRNA was absent during the proliferative phase. The expression of the mRNA of α-crystallin B chain first appeared in the secretory phase, progressively increased during this phase and peaked in the late secretory endometria (Gruidl *et al.*, 1997). The pattern of expression of *α-crystallin B* chain mRNA in the endometrium of mature cycling baboons (*Papio anubis*) was similar to that seen in human endometrium (Gruidl *et al.*, 1997). As revealed by Western blot analysis, the expression of the α-crystallin B chain protein in human endometrium followed a pattern of expression similar to its mRNA. At the cellular level, the immunoreactive protein first appeared in the surface epithelial cells of human endometrium within the implantation window. Within the same period, there was no significant immunoreactivity in the underlying glandular cells. During the mid and late secretory phases, the intensity of staining in the epithelial cells was enhanced and an intense immunoreactivity was developed in the glandular epithelium. α-Crystallin B chain was virtually an epithelial product and no immunoreactivity for this protein was detectable in the stromal cells, endothelial cells or lymphoid cells. The expression of α-crystallin B chain could be regulated, both by medroxy progesterone acetate as well as by oestrogen withdrawal, in human endometrial carcinoma

cells (EnCa-101) transplanted to nude mice (Gruidl *et al.*, 1997). The function of the α-crystallin B in human endometrium may be to act as a molecular chaperone for the proteins whose proper functioning is required during the implantation process. Tumour necrosis factor α (TNF-α) is a cytotoxic cytokine that is secreted during the secretory phase. α-Crystallin B chain may protect the endometrium against the potential cytotoxic damage of TNF-α during the critical period of the implantation window.

Molecules signalling closure of the implantation window

If implantation fails, the endometrium is shed during the menstrual period. Therefore, it is conceivable that a specific molecular repertoire prepares endometrium for the menstrual shedding. The same molecular repertoire may signal the closure of the implantation window and act to make endometrium refractory to implantation. Little information is available regarding the molecules that make endometrium resistant to implantation. However, it has been suggested that MUC1 may act as an anti-adhesion molecule during embryo attachment in the mouse (Surveyor *et al.*, 1995). We have identified TNF-α as a member of the premenstrual/menstrual molecular repertoire that may play a role in menstrual shedding. This assumption is based on the following information. TNF-α is expressed by the human endometrium (Tabibzadeh, 1991; Hunt *et al.*, 1992; Tabibzadeh *et al.*, 1995e). In-situ hybridization and immunohistochemical staining showed that most of the *TNF*-α mRNA and immunoreactivity is confined to the endometrial glands (Tabibzadeh, 1991; Hunt *et al.*, 1992). Northern blot analysis showed that the amount of *TNF*-α mRNA progressively increased towards the menstrual phase (Phillipaeux and Piguet, 1993). We also showed that the amount of TNF-α released into the endometrial cavity also progressively increased towards the late secretory phase (Tabibzadeh *et al.*, 1994). In addition, TNF-α was present in the menstrual discharge in amounts far exceeding those found in the peripheral circulation (Tabibzadeh *et al.*, 1995a). Furthermore, TNF-α is cytotoxic and by virtue of damaging the endothelial cells, causes oedema (Remick and Kunkel, 1994) and bleeding in various organs (Shalaby *et al.*, 1989). Injection of TNF-α into mouse causes endometrial bleeding and apoptosis in the endometrium (Shalaby *et al.*, 1989) which are reminiscent of human menstruation (Tabibzadeh, 1996). TNF-α also causes epithelial dissociation (Tabibzadeh *et al.*, 1995c), apoptosis (Tabibzadeh *et al.*, 1994) and converts the F actin to G actin in the endometrial epithelial cells (Tabibzadeh *et al.*, 1995d), changes that are seen in the late secretory and menstrual phases (Tabibzadeh, 1996; Tabibzadeh *et al.*, 1996). Taken together, these findings suggest that TNF-α may be implicated in glandular dissociation and menstrual bleeding during the menstrual phase.

We have recently identified a novel gene of the transforming growth factor (TGF)-β family, endometrial bleeding associated factor (*ebaf*), whose expression, in human endometrium is confined to the late secretory and menstrual phase (Kothapalli *et al.*, 1997). The size of the *ebaf* mRNA species detected in all endometria was ~2.5 kb. However, *ebaf* mRNA seems to also exist as 2.1 and 1.5 kb species (unpublished data). The expression of this gene is not seen in endometrium in the proliferative, early and mid secretory phases (Kothapalli *et al.*, 1997). In-situ hybridization confirmed the findings of Northern blot analysis. Sections of late secretory endometria exhibited hybridization signals with the anti-sense *ebaf* RNA probe in the stroma. Within stroma, the mRNA expression was confined to pre-decidualized stromal cells in the upper layers of endometrium underlying the surface epithelium. In the same endometria, the stroma in the basal part of the endometrium overlying the myometrium failed to exhibit evidence of mRNA expression. In these endometria, with the exception of a few glands located near the surface epithelium, virtually no *ebaf* mRNA expression could be identified in the epithelial cells. On the other hand, sections of late proliferative endometria failed to show any hybridization signal with the anti-sense *ebaf* RNA probe (Kothapalli *et al.*, 1997). The *ebaf* mRNA was expressed in the endometria of patients with endometrial bleeding during the proliferative, early and mid secretory phases when no expression of the *ebaf* mRNA was expected (Kothapalli *et al.*, 1997). Northern blot analysis failed to reveal the presence of 2.5 kb *ebaf* mRNA in a number of normal tissues, including lung, kidney, ovary, liver, colon, rectum, spleen, lymph node and stomach (Kothapalli *et al.*, 1997).

The predicted protein sequence of ebaf showed a strong homology to the mouse lefty/stra3 (Bouillet *et al.*, 1995; Meno *et al.*, 1996). The deduced amino acid sequence of ebaf protein is 77% identical and 83% similar to lefty protein. A motif search revealed that the predicted ebaf protein contains most of the cysteine residues which are conserved among the TGF-β related proteins (Kingsley, 1994) and which are necessary for the formation of the cysteine knot structure (Daopin *et al.*, 1992; McPherron and Lee, 1993). The ebaf sequence contains an additional cysteine residue, 12 amino acids upstream from the first conserved cysteine residue. The only other family members known to contain an additional cysteine residue are TGF-βs, inhibins and GDF-3 (McPherron and Lee, 1993; Kingsley,

1994). Similar to lefty, ebaf GDF-3/Vgr2 and GDF-9, lacks the cysteine residue that is known to form the intermolecular disulphide bond (Jones *et al.*, 1992; McPherron and Lee, 1993). Therefore, ebaf appears to be an additional member of the TGF-β superfamily with an unpaired cysteine residue which may not exist as a dimer. Nevertheless, it has been suggested that, in GDF-3 and GDF-9 which also lack such a cysteine residue, hydrophobic contacts between the two monomer subunits may promote dimer formation (Kingsley, 1994). Whereas the carboxy terminus of the TGF-β family is usually CX1CX1, ebaf has a longer C-terminal sequence, CX1CX19. In addition, the members of the TGF-β superfamily are synthesized as prepro-proteins which are cleaved at RXXR site to release the mature form of the protein. The predicted protein of ebaf exhibits two such RXXR sites which are respectively located at amino acid residues 73–76 and 131–134. If one of these sequences is the cleavage site, a mature protein of 294 and 236 amino acids should be produced. The deduced amino acid sequence of lefty also contained two potential cleavage sites at amino acid residues 74–77 and 132–135, with mature proteins of 291 and 233 amino acids (Meno *et al.*, 1996). In the case of lefty, the expression of the protein in 293T cells led to formation of a non-secretory, 42 kDa, protein which is the size of the prepro-protein (Meno *et al.*, 1996).

Besides the sequence similarity, *ebaf* and *lefty* share several other features. In-situ hybridization revealed that the transient expression of *ebaf* was primarily confined to the mesenchymal cells of the endometrial stroma rather than the epithelium or endothelium. The *lefty* mRNA was also transiently expressed in the mesoderm in the left half of the gastrulating mouse embryo just before the first sign of lateral symmetry appeared (Meno *et al.*, 1996). By fluorescent in-situ hybridization, the *ebaf* gene was localized to the human chromosome 1 at band q42.1 (Kothapalli *et al.*, 1997). Similarly, the location of the *lefty* has been provisionally assigned to chromosome 1 (Meno *et al.*, 1996). Taken together, the available data show that *ebaf* is a new member of the TGF-β superfamily. In view of chromosomal localization, great homology in the cDNA, and the predicted protein sequence and other structural features, *ebaf* seems to represent the human homologue of mouse *lefty.* In addition, *ebaf* may be a member of the premenstrual and menstrual molecular repertoire that participates in menstrual shedding.

Molecular lesions in infertility

It is conceivable that various types of infertility may primarily or secondarily affect the regulatory mechanism(s) involved in implantation and lead to lesions in the molecular repertoire required in this process.

From the members of the molecular repertoire of the 'endometrial receptivity' period, with the exception of integrin $\alpha_v\beta_3$ (Lessey *et al.*, 1992), no other molecule has been described thus far whose aberrant expression is associated with or results in infertility. It has been suggested that the expression of the immunoreactivity for integrins $\alpha_4\beta_1$ and $\alpha_v\beta_3$ coincides with the putative 'implantation window' (Lessey *et al.*, 1994). Immunostaining for α_v increased throughout the menstrual cycle, while the β_3 subunit appeared abruptly on cycle day 20 on luminal and glandular epithelial cells (Lessey *et al.*, 1992). Discordant luteal phase biopsies (≥3 days out of phase) from infertile patients exhibited delayed epithelial β_3 immunostaining (Lessey *et al.*, 1992).

To gain an insight as to whether the expression of *ebaf* is disturbed in cases of infertility, we have performed Northern blot analysis on RNA extracted from endometria of women who had unexplained infertility. During the implantation window, the 2.1 kb *ebaf* mRNA was aberrantly expressed in these endometria. In addition, using a primer set framing the coding region of the *ebaf* cDNA, the polymerase chain reaction of menstrual endometrial cDNAs followed by Southern blotting revealed amplified products of *ebaf* mRNA of various sizes. Sequencing of some of the products showed that the size difference was due to deletions in the coding region of the *ebaf* cDNA (unpublished results). In the endometria of patients with infertility, products that were aberrant in size were noted during the implantation window (unpublished results). These findings show that female infertility is associated with aberrant expression of *ebaf* mRNA.

We recently identified a novel gene, *H1*, whose aberrant expression is seen in some patients with unexplained infertility (unpublished data). The expression of *H1* was seen in the normal endometria irrespective of the phase of the menstrual cycle. On the other hand, the expression of this gene was missing from the endometria of a subset of women with unexplained infertility.

Therefore, the available evidence suggests that infertility is associated with lesions in the molecular repertoire that is expressed during the periods of receptivity and refractoriness of the endometrium to implantation.

Acknowledgement

This work was supported by Public Health Research Grant CA46866 (to S. Tabibzadeh).

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