Cell-surface morphological events relevant to human implantation

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Morphological evidence on early stages of human implantation is limited to very few sporadic observations. The nature of implantation which requires the presence of both maternal and embryonic tissues, combined with the currently existing ethical constraints on human studies, appear to preclude generation of new data. However, research on relevant animal and in-vitro models as well as studies on human endometrium and in-vitro embryos, allow some indirect insights to this phenomenon. This review summarizes information on cell-surface morphological events relevant to implantation initiation, derived from scanning electron microscopy studies on the above systems. A central part of this article deals with the formation of epithelial cell projections known as pinopodes, for there is increasing evidence suggesting that these structures are closely associated with the development of endometrial receptivity for blastocyst implantation in humans.

Key words: endometrial cell culture/endometrial pinopodes/human blastocyst/implantation/uterine receptivity

Introduction

The first week of human life in vivo remains largely a mystery. Our knowledge is limited, based on some rare observations on surgical specimens (e.g. Hertig et al., 1956; Croxatto et al., 1972) and some more recent ones (Buster et al., 1985; Formigli et al., 1987), following uterine lavage for embryo donation. According to assumptions based on this and other clinical data, ovulation and fertilization occur in the oviduct on day 14 of an ideal cycle. The zygote, after a series of mitotic divisions arrives to the uterus at the morula stage, on day 18. On day 19 a blastocyst is formed, which sheds of the zona pellucida and on day 20 starts to implant to the endometrium. Meanwhile, the endometrium under the control of steroid hormones has undergone changes which have led to the development of receptivity. The onset of implantation can be seen as a successful meeting of two separate processes, both of which arise from the ovary, i.e. embryo development and endometrial maturation. Synchrony between these functions is important, thus defining a bifactorial, transient period when implantation can be initiated, called the implantation or nidation window (Psychoyos, 1976, 1986). According to the classical concept (Enders, 1994), implantation initiates with apposition of the blastocyst, when it stops moving and comes into close proximity with the uterine epithelium. Adhesive interactions actively develop between the apposing membranes of trophoblastic and surface endometrial cells, marking the adhesion stage. Adhesion is followed by epithelial penetration and placentation, which then varies between different species.

There is no information on the initial stages of human implantation. Since this process requires the presence of both embryonic and maternal tissues, it is only possible to study it in vivo. Despite the fact that more information about these phenomena is currently needed, it has become more difficult to obtain, since the development of in-vitro fertilization (IVF) has imposed severe...
moral constraints on this research. Nevertheless, data deriving from animal studies have provided a solid body of knowledge on implantation. In addition, changes of the human endometrium that initiate implantation seem to appear spontaneously in each menstrual cycle and, therefore, they can be studied independently of the presence of an implanting blastocyst. Furthermore, in-vitro culture of human zygotes to the blastocyst stage allows for studies on the embryonic partner. Finally, the development of in-vitro models of human implantation using endometrial cell cultures and co-cultures with embryos has also yielded useful information.

The molecular interactions which occur during implantation between embryonic and maternal compartments have been extensively discussed (Kimber, 1994; Makrigiannakis et al., 1995; Psychoyos et al., 1995; Tabibzadeh and Babaknia 1995; Giudice, 1997; Lessey, 1998; Simón et al., 1998; Smith et al., 1998), along with certain subcellular changes accompanying the transformation of epithelial plasma membrane and cytoskeleton (Murphy, 1995). This review summarizes some well-documented morphological findings on cell surface phenomena related to the beginning of implantation, as viewed by scanning electron microscopy (SEM) on the above-mentioned systems. In addition, some preliminary observations deriving from a number of unpublished studies are given.

Animal studies on implantation initiation

The basics of our understanding on the hormonal control of implantation has been established by studies on the rat model, pioneered by Psychoyos (1966) during the 1960s. A minimum of 3 days priming with progesterone in spayed animals drives the endometrium into a prereceptive or neutral state. This state allows ovum survival but it does not permit implantation. Addition of a very small amount of oestrogen (called nidatory oestrogen) at any time thereafter induces within 24 h a phase of receptivity which lasts for only 12 h. Blastocyst implantation can start only during this brief phase of receptivity, called the ‘nidation window’. A refractory state follows, during which the uterus is hostile to the ovum. Exit from this state and re-establishment of the cycle requires progesterone withdrawal for at least 48 h.

During the phase of receptivity, the ultrastructure of the luminal epithelium undergoes striking modifications. In the rat, the apical membranes of the secretory cells lose their microvilli and develop large ectoplasmic protrusions resembling sponges (Potts and Psychoyos, 1967). Intrauterine administration of tracers showed that these structures are involved in an apico-basal transport of fluid and macromolecules towards the stroma (Enders and Nelson, 1973). Due to the pinocytotic function, these organelles were termed pinopodes (from the Hellenic term πινοε = drink and ποδες = feet). Pinocytosis results in the closure of the lumen and closer contact between the endometrial and trophoblastic cells. It may also facilitate capture of embryonic signals by the epithelial cells. The appearance of pinopodes is associated with the thinning of the glycocalyx, resulting in the reduction of the negative surface charge which occurs during receptivity (Anderson, 1989). There is abundant experimental evidence that pinopodes provide a specific marker for uterine receptivity in rats (Psychoyos and Mandon, 1971a,b; Sarantis et al., 1988; Martel et al., 1991). Structures resembling pinopodes, present at the time of implantation, have been described in a number of animals, including mice (Parr and Parr, 1974), baboons (A.Psychoyos, personal communication), cows (Guillomot et al., 1986) and other mammals.

Surface ultrastructure of the human endometrium

The endometrial epithelium consists of two types of cells, which are easily distinguishable in SEM: the secretory and the ciliated cells. The morphology of ciliated cells does not change much during the cycle. In contrast, the secretory cells bear microvilli and undergo hormone-dependent changes including the formation of pinopode-like structures (Nilsson, 1962). The first attempt to describe these changes throughout the menstrual cycle was by Martel et al. (1981). On the basis of this work, the surface endometrial ultrastructure is examined more systematically, using sequential (up to four) endometrial biopsies, taken from the same individual every 2 or 3 days during the same menstrual cycle requires progesterone withdrawal for at least 48 h.
Morphology of human implantation cycle (G. Nikas, O.H. Oevelioglu, J.P. Toner and H.W. Jones Jnr, unpublished). This study aims to describe consistent morphological features occurring during the cycle. Some of these features appear to be as follows: during the proliferative phase the cells vary greatly in size and their shapes are either elongated or polygonal. Bulging is minimal, the intercellular clefts are barely marked, and the microvilli are short and slender. As the proliferative phase progresses, the microvilli develop. After ovulation, by day 16, cell bulging increases. On day 17, the microvilli reach their maximum development, being long and thick and upright. On day 18, the tips of the microvilli appear swollen. On day 19, a pronounced and generalized cell bulging appears. The microvilli decrease in number and length and fuse or disappear. Smooth and slender membrane projections form, arising from the entire cell apex (developing pinopodes). On day 20, the microvilli are virtually absent and the membranes protrude and fold maximally (fully developed pinopodes). Fully developed pinopodes assume many shapes resembling mushrooms or flowers (Figure 1). On day 21, bulging decreases and small tips of microvilli reappear on the membranes, which are now wrinkled, and the cell size starts to increase (regressing pinopodes). By day 22, the pinopodes have largely disappeared and the microvilli develop further. Day 23 is characterized by a further increase in the size of cells which begin to appear dome-shaped and covered with short, stubby microvilli. It should be mentioned that the above changes refer to an ideal 28 day cycle. In reality, although the sequence of changes is unvarying, the actual cycle days when these changes occur including pinopode formation, may be different in each woman. This point appears to be of clinical interest and it is more extensively discussed elsewhere (Nikas, 1999).

Another point which is worth mentioning is the consistent changes of the lateral cell to cell contacts between the epithelial cells (see Figure 2). In cross-sections of the epithelium before pinopode formation, the cells appear columnar and regular, with tight contacts between them. In the presence of pinopodes, these contacts are looser and the cell height decreases. This finding is consistent with the reported decrease of cell polarity (Denker, 1993) and number of tight junctions (Murphy et al., 1992) in humans, or the suppression of connexin expression in rats, during receptivity (Grummer et al., 1996). The rounded shape remains during the post-pinopode period, but the cell contacts appear to re-establish and the uterine cavity is sealed again. Looking deeper into the glandular epithelium, structures morphologically identical to pinopodes are present at the same time when they flourish on the surface. It is interesting that surface pinopodes prefer to form around gland openings.

While the pinocytic function of pinopodes has been well studied in rodent species (Enders and Nelson, 1973; Parr and Parr, 1974), no information is available regarding pinopode function in humans. If we assume that surface and glandular pinopodes are identical, the glandular location would argue...
rather for an apocrine-secretory function than a pinocytotic one. No apparent differences on the external cell morphology between surface and glandular epithelium are evident, except that ciliated cells are less numerous in the glands. However, immunohistochemical studies show different patterns of staining between these two compartments, probably reflecting discrete functional roles. An explanation for these differences has never been given. Looking at their position, surface epithelium surrounds stroma, while glandular epithelium is surrounded by stroma. Therefore, one can speculate that glandular cells may respond more readily to humoral messages arriving either via the bloodstream, or via a paracrine communication with white blood cells present in the stroma.

One issue arising from the study of morphology is that the appearance of the endometrium is not always uniform, but regional variations are frequently encountered. To examine these variations more systematically, hysterectomy specimens are used to look for cyclical changes comparatively on uterine fundus, Fallopian tube and endocervix (G.Nikas, N.Hakris, N.Koutsodimas and S.Michalas, unpublished). Preliminary results show that the morphological changes of the tubal epithelium, except the fimbriated end which is densely covered with cilia, follow the changes of the uterine fundus, including the formation of pinopodes. In contrast, the lower uterine segment and the endocervix exhibit only weak changes on the surface morphology during the menstrual cycle, as reported also in classical histology. No pinopodes form here and this may provide some explanation as to why cervical pregnancy is extremely rare.

In parallel with the overall morphology of the endometrium, its surface undergoes distinct changes which peak at the mid-luteal phase. Teleologically, the menstrual cycle can be viewed as preparation of the endometrium to receive the embryo. The classic work on dating the endometrial biopsy (Noyes et al., 1950) describes a number of morphological events occurring during the menstrual cycle. Looking at the curves representing several cellular features, such as mitoses, pseud stratification of nuclei, basal vacuolation, secretion and stromal oedema, it is remarkable that these curves fluctuate acutely at the mid-luteal phase between days 18 and 22. In this context, the most dramatic change in cell morphology appears to be the formation of pinopodes during the window of receptivity. Clinical studies have strongly suggested that pinopodes are closely associated with the development of endometrial receptivity. Some of these studies have been presented elsewhere (Nikas, 1999).

**Surface ultrastructure of human in-vitro blastocysts**

Culture of human IVF embryos has provided abundant material for research. The first change in the surface morphology in these embryos appears
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with compaction, on day 4 morulae (Nikas et al., 1996). The blastomeres flatten against each other and the distribution of microvilli polarize at the free surfaces. After one day, the blastocoelic cavity forms, causing the inner cells of the nascent blastocyst to segregate at one pole. Consequently, the blastocyst sheds the zona pellucida and develops axial trophoectodermal polarity. At these stages, a variable degree of cell death frequently occurs in vitro, which predominantly affects the inner cell mass (ICM) and this seems to reveal the important role of the ICM on the above processes (G. Nikas and A. H. Handyside, unpublished). Blastocysts with a poorly developed ICM do not exhibit any polarity and do not hatch. These blastocysts expand and there is a progressive thinning of the zona pellucida which is finally dissolved by the trophoblast, allowing its microvilli to emerge. Exposure of the blastocoel cavity reveal that the ICM is absent or degenerated (Figure 3). In contrast, blastocysts with well developed ICMs do hatch. This happens usually on day 6, but it may occur on day 5 or day 7. Hatching apparently starts from the abembryonic pole, and this may be due to both biochemical and mechanical reasons. In mice, a trypsin-like proteinase (strypsin) localized on the mural trophoblast opens a slit on the zona (Perona and Wassarman, 1986). Contractions and expansion of the blastocyst may cause the thin abembryonic pole to protrude first through the slit.

Irrespective of the mechanism by which the embryo escapes from its zona in vitro, it seems likely that in-vivo uterine factors may also contribute to the loss of the zona. In rats, an oestrogen-dependent zona lysin is present in the receptive uterus, which rapidly dissolves the zona (Psychoyos, 1966). Hatched blastocysts exhibit a strong trophoectodermal polarity (Figure 4). The embryonic pole bears large cells, apparently syncytial, while cells at the abembryonic pole are much smaller. The cell membranes are covered with microvilli which are particularly dense at the embryonic pole.

This is likely to be the appearance of a blastocyst which is close to attachment. No direct information is available on the morphological relationships between the human blastocyst and the endometrium at this stage. The earliest specimen of human implantation yet observed concerns a blastocyst (Carnegie no. 8020) which presumably on cycle day 21 is firmly attached and already invading the endometrium (Hertig et al., 1956). The orientation of this and older Carnegie specimens, as well as the interactions of human blastocysts and endometrial cells in vitro (Lindenberg et al., 1986), suggests that apposition and adhesion is initiated at the embryonic pole. This is also the case with other primate species studied (Enders et al., 1997). It is plausible to assume that trophoblast attachment will be established initially with the apices of the surface epithelial cells. However, the possibility

Figure 3. Scanning electron micrograph of a day 6 human blastocyst. The blastocoel cavity has been opened to expose the inner cell mass (on the left), which appears degenerated. The zona pellucida has been partially dissolved by the trophoblast, allowing its microvilli to emerge. Bar = 50 μm.

Figure 4. Scanning electron micrograph of a day 6 hatched human blastocyst. The embryonic pole (on the left) shows large cells covered with dense microvilli. The abembryonic pole (on the right), which appears partially collapsed, has smaller cells and microvilli are less numerous. Bar = 20 μm.
that trophoblast may extend projections which can make their way between the epithelial cells, cannot be excluded (Lopata, 1996). Extensive ectoplasmic flanges have been noticed on the polar trophoectoderm in expanded baboon blastocysts flushed from the uterus at a time when adhesion may have started (Enders et al., 1997).

**In-vitro models of uterine receptivity**

Endometrial epithelial cell cultures (EECs) have been used for the development of in-vitro models of uterine receptivity (reviewed by Aplin and Glasser, 1994). EECs may survive for several days and cells retain many morphological and biochemical characteristics of their counterparts *in vivo*. These cells, which are intrinsically polarized, attach to plastic substrates and form monolayers with lateral cell junctions and apical microvilli distribution. To enhance these features, more sophisticated systems have been employed, using collagen matrices or endometrial stromal cells as underlying feeder layers. If maintained under progesterone, the EECs develop pinopodes which look identical to those forming *in vivo* (Bentin-Ley et al. 1995).

The temporal expression of pinopodes in EECs appears also to follow the *in-vivo* situation (G.Nikas and J.J.Brosens, unpublished). Cells growing on plastic in the presence of oestrogen alone are covered with punctuate microvilli. Addition of progesterone for 2–3 days causes the microvilli to fuse, and after 4 days of progesterone some pinopodes appear, which become more numerous on day 5 and 6. Similar to the *in-vivo* conditions, cells bearing pinopodes appear to loosen their connections with neighbouring cells (Figure 5).

EECs seem to be suitable systems to study implantation initiation and provide evidence for the importance of pinopodes in this process. In their elegant studies, Bentin-Ley et al. (1999) have placed human blastocysts on EECs grown on stromal cells. The blastocysts adhered with their embryonic poles, exclusively in areas displaying pinopodes. The latter appeared to increase in number and size after contacting the trophoblast for 24 h. The question of whether an implanting blastocyst can induce pinopode formation or other morphological changes on the contacting endometrial epithelium remains unclear. Simón et al. (1997) used blastocyst-conditioned media to enhance both integrin expression and pinopode formation in EECs grown on plastic. While these reports support the notion that blastocysts can induce morphological changes on the uterine epithelium, further
studies are required to define these changes and to which extent they reflect the in-vivo situation.

Concluding remarks
Peri-implantation changes on the surface morphology of the mid-luteal endometrium are compatible with the existence of a short phase of receptivity in humans. The most dramatic morphological change occurring at this phase is the development of uterine pinopodes. These structures may be involved in trophoblast adhesion and/or facilitation of penetration to the stroma. In-vitro models of receptivity using endometrial cell cultures appear to mimic well some morphological events that occur in vivo, including the formation of pinopodes. Considering the difficulties involved in human studies, these models seem to be a promising tool to investigate the fascinating phenomenon of implantation initiation, which is a crucial topic for both infertility and antifertility treatment.

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