Secretory role for human uterodomes (pinopods): secretion of LIF

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The differentiation of human endometrial epithelium is a dynamic event, which occurs throughout the menstrual cycle in preparation for pregnancy. The appearance of uterodomes (pinopods) in this regard was first introduced in rodents with an established pinocytotic function, whereas little evidence was available in humans in this context. This study was undertaken to identify the potential physiological roles of uterodomes in the implantation process. To address this, endometrial biopsies from early, mid- and late luteal phases of the menstrual cycle of 23 fertile female patients with regular menses were used. Scanning and transmission electron microscopies (SEM and TEM) as well as immunofluorescence and immunogold TEM were performed to study the morphological changes and the expression pattern of leukaemia inhibitory factor (LIF) at uterodomes. Our results illustrated a high level of LIF expression in the human uterodomes, which was colocalized with the well-known biochemical markers of exocytosis, including syntaxin-1, 25-kDa synaptosomal protein (SNAP-25) and vesicle-associated membrane protein-2 (VAMP-2). Our immunophenological and immunocytochemical findings illustrated a secretory function for human uterodomes for the first time. In conclusion, this novel function for uterodomes provides an important clue in detection of their physiological function(s) during the process of the plasma membrane transformation.

Key words: endometrial receptivity/implantation/LIF/pinopod(e)/secretion/uterodome

Introduction

Though implantation could occur in any human tissue, the endometrium is the only tissue, which is not receptive to embryo implantation except during a restricted frame of time called the ‘implantation window’ (Minas et al., 2005). During the implantation window, endometrium undergoes a number of essential changes in the plasma membrane of its epithelial cells, characterized as ‘the plasma membrane transformation’ (Murphy and Shaw, 1994). In many mammalian species, the appearance of a large rounded projection on the apical surface of epithelial cells forms part of the plasma membrane transformation (Murphy, 2000a).

The first observations of pinopods were made in mice (Nilsson, 1958) and rats (Warren and Enders, 1964). Early speculation on the function of these variously named projections focused mostly on apocrine secretion (Nilsson, 1972). However, uptake of an electron-dense tracer by these projections was reported later (Enders and Nelson, 1973). Thereafter, the term ‘pinopod(e)’ from the Greek ‘drinking foot’ was coined to these projections to signify their pinocytotic function. Recently, in recognition of several ultrastructural differences between pinopods of rodents and those of humans as well as their functional differences, the more general term ‘uterodome’ was suggested (Guillomot et al., 1986; Murphy, 2000b; Adams et al., 2002). The expression of several biomolecules in uterodomes was examined wildly so far (Lessey et al., 2001; Stavreus-Evers et al., 2001; Nardo et al., 2002). However, reports on the appearance timing of uterodomes and its relevance to infertility are controversial (Acosta et al., 2000; Stavreus-Evers et al., 2001; Aghajanova et al., 2003; Usadi et al., 2003). Recently, a strong temporal correlation between the appearance of uterodomes and the expression of leukaemia inhibitory factor (LIF) in human endometrium was reported (Aghajanova et al., 2003).

Substantial evidence indicates that members of the interleukin (IL)-6 family of cytokines, including LIF are the key regulators of human implantation (Kondera-Anasz et al., 2004). Evidence of a role for LIF in the implantation process came from experiments using female transgenic mice homozygous for LIF gene deficiency, in which embryo implantation did not occur (Stewart et al., 1992). Thereafter, discrete mutations in the LIF gene were shown to correlate to infertility (Giess et al., 1999). Furthermore, it was reported that the endometrial biopsies of women with proven fertility demonstrated a striking increase in the expression of LIF and LIF messenger RNA during the mid- and late luteal phases of the normal menstrual cycle (Charnock-Jones et al., 1994; Kojima et al., 1994). Accordingly, in vivo and in vitro experiments illustrated that infertile women produced less LIF (Laird et al., 1997; Hambartsoumian, 1998). On the other hand, in the absence of LIF, the mouse embryos were reported to develop normally only up to the blastocyst stage (Stewart et al., 1992) and consistently, the development of human embryos was improved in vitro under LIF treatment (Sargent et al., 1998). Finally, in addition to the regulatory role of LIF for growth and differentiation of human trophoblasts (Kojima et al., 1995), high levels of LIF were reported in the ampullary region of human fallopian tube (Keltz et al., 1996), which suggested an important role for it in the early embryonic development in vivo.
In clinical medicine, implantation failure is considered as a major impediment to a successful in vitro fertilization pregnancy rate. Despite the recent advances in reproductive medicine, the exact role played by uterine receptivity in respect of infertility remains unclear. Thus, it is of prime importance to investigate and control the characteristic features of a receptive endometrium. As uterodomes appear to be the preferred site of embryo–endometrial interactions in vitro (Bentin-Ley et al., 1999) and were considered as the biological marker of uterine receptivity in vivo (Johannisson and Nilsson, 1972; Nikas, 1999; Adams et al., 2001), we further studied the morphology and function of uterodemes in humans. In spite of several reports on both LIF and uterodomes in human reproduction, no information exists in respect of their spatial colocalization and the functional significance of human uterodomes. Thus, this study was carried out to investigate the subcellular localization of LIF in human uterine epithelial cells as well as to examine a potential physiological function for uterodomes in humans.

**Materials and methods**

**Endometrial specimens**

Endometrial biopsies were obtained from the anterior wall of the uterine cavity of 23 female patients at early luteal (five samples), mid-luteal (10 samples) and late luteal (eight samples) phases of a regular menstrual cycle. All cases were selected from fertile women with regular cyclic menses who had delivered at least one live child. The mean age was 36 years old (range, 36–42), and none of them had used steroid contraceptives or intrauterine devices for at least 3 months before sampling. For endometrial dating, according to the histopathological criteria of Noyes (Noyes et al., 1950), the cryosections were stained with hematoxylin and eosin and evaluated by an experienced observer who was blind to the study. To have access to scanning electron microscopy (SEM), transmission electron microscopy (TEM) and light microscopy of each sample, two biopsy pieces were taken from the same patient. One piece was fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer solution (pH 7.3) for 2 h at 4°C, treated with sucrose, embedded in optimal cutting temperature (OCT) compound (Miles, Elkhart, IN, USA) and kept at −80°C until use. The second piece was divided into three small sections to be used for SEM, Epon Resin block for standard TEM and Lowicryl white resin block for immunogold TEM. All patients gave informed consents for collection and the investigational uses of tissues. This study was approved by the Ethics Committee of Kyorin University, School of Medicine, Tokyo, Japan.

**Scanning and TEMs**

SEM was performed for morphological studies and to confirm the presence of uterodemes in the endometrial specimens from the different stages of luteal phase biopsies. For SEM preparation, endometrial tissues were fixed in 2.5% glutaraldehyde and post-fixed for at least 1 h in 1% osmium tetroxide. Then, samples were dehydrated in a graded series of ethanol (50, 70, 90, 99.5 and 100%), critical-point-dried, mounted and coated with gold in a sputter coater (JFC-1300 Auto Fine Coater, JEOL, Tokyo, Japan). Finally, the samples were observed under a scanning electron microscope (JSM-5600 LV SEM, JEOL, Tokyo, Japan). All 23 specimens were processed for SEM to study the morphology of uterodemes in different samples. For TEM, biopsies of endometrium were fixed immediately in phosphate-buffered 2.5% glutaraldehyde (pH 7.4), post-osmicated, and then dehydrated in graded concentrations of alcohol. For standard double staining, the specimens were embedded in Epon resin (Embed 812, Electron Microscopy Sciences, Fort Washington, PA, USA). Ultrathin sections were cut perpendicular to the epithelium and were double-stained using uranyl acetate and lead citrate. Then, samples were observed under a transmission electron microscope (TEM-1010C; JEOL, Tokyo, Japan). All samples were observed under transmission electron (TE) microscope.

**Immunohistochemistry for light microscopy**

Using OCT compound-embedded samples, frozen sections (4 μm thick) were cut, washed with phosphate-buffered saline (PBS), and treated with 5% bovine serum albumin (BSA) diluted in PBS for 30 min at room temperature. The sections were then incubated overnight at 4°C with relevant primary antibodies, such as a polyclonal goat antibody against human LIF, a monoclonal antibody against syntaxin-1 (Santa Cruz Biotechnology, CA, USA), a polyclonal rabbit antibody against vesicle-associated membrane protein-2 (VAMP-2) (Calbiochem Oncogene Research Products, San Diego, MA, USA), and a monoclonal antibody against 25-kDa synaptosomal protein (SNAP-25) (Sigma Chemical, St. Louis, MO, USA); at a final concentration of 1.3 μg/ml. After extensive rinsing with PBS-containing 0.01% Tween-20 (PBST), the bound antibodies were visualized by their subsequent incubation for 1 h at room temperature with appropriate fluorescent-conjugated secondary antibodies (Alexa 488-conjugated donkey anti-goat IgG, 1.5 μg/ml; Alexa 568-conjugated donkey anti-mouse IgG, 1.5 μg/ml, and Cy2-conjugated donkey anti-rabbit IgG, 1.5 μg/ml; all from Molecular Probes, Eugene, OR, USA). Nuclei were stained with a nuclear acid stain (DAPI, 1 : 1000, Molecular Probes). After washing several times with PBST (0.01%) and a final rinsing in deionized water, the specimens were mounted using 90% glycerol and 0.1 M Tris–HCl buffer (pH 8.5) containing 0.5 mM p-phenylene diamine, and observed under an AX-80 fluorescence microscope (Olympus Optical, Tokyo, Japan). Negative controls were incubated with proper nonimmunooct, rabbit and mouse IgGs (Santa Cruz Biotechnology, CA, USA) corresponding to the species of their primary antibodies. The reproducibility was verified by four repeats in four different biopsies of mid-luteal phase samples.

**Immunohistochemistry for TEM**

Immunogold staining was performed to localize the subcellular distribution of LIF in human uterodemes and to identify the colocalization of LIF and syntaxin-1, SNAP-25 or VAMP-2 in these projections. Furthermore, other areas of the uterus, including epithelial cells not covered with uterodemes and glandular epithelium were observed for detection of colocalization of LIF and syntaxin-1, SNAP-25 or VAMP-2. For immunogold TEM preparation, specimens were fixed in 4% PFA in 0.1 M phosphate buffer (pH 7.4) for at least 24 h at 4°C. After dehydration, in increasing concentrations of ethanol, from 50% (v/v) to absolute ethanol, they were embedded in Lowicryl white resin (London Resin company Ltd, London, UK), and ultrathin sections were cut. Then ultrathin sections were washed with PBS and pre-treated with 5% BSA for 30 min at room temperature. After a PBS rinse, they were incubated with anti-rabbit polyclonal LIF: 1.5 μg/ml or with normal rabbit serum as control overnight at 4°C degree. Following several washing with PBS, sections were incubated overnight at 4°C with one of the following nanogold IgG (Jackson Immuno Research Laboratories Inc., West Grove, PA, USA) including 18 nm colloidal gold-conjugated donkey anti-goat IgG, 12 nm colloidal gold-conjugated donkey anti-rabbit IgG or 12 nm colloidal gold-conjugated donkey anti-mouse IgG (all diluted 1 : 20 in PBS), appropriately. Then sections were washed with PBS, rinsed with distilled water and stained with uranyl acetate. The specimens were observed under a TEM microscope (JEM-1010; JEOL, Tokyo, Japan). For negative controls, all the abovementioned procedures were performed without incubating with primary antibodies. In order to examine the reproducibility, six different samples were examined using above-mentioned technique from each mid- and late luteal phase biopsies.

**Results**

**SEM and TEM**

Morphological studies of endometrial biopsies using SEM are usually performed to monitor expression of uterodemes (pinopods) and evaluation of their developmental stage during the luteal phase. Generally, on the basis of the stage of their development, uterodemes are scored as developing, developed and regressing (Nikas et al., 1999). In the samples obtained from early luteal phase of the menstrual cycle, we observed many progressing and few isolated developed projections (Figure 1A). Developed uterodemes, which appeared on a majority of non-ciliated epithelial cells, were dominant beside few regressing uterodemes in the mid-luteal phase specimens (Figure 1B), and samples from the late luteal phase biopsies exhibited mostly regressing uterodemes (Figure 1C). Observation of morphological features of uterodemes under high magnification in these specimens revealed that...
Secretion of LIF through uterodomes

Some holes existed on the apical pole of some of these projections, especially in biopsies from mid- and late luteal phases (Figures 2A and 3A). Release of secretory vesicles could be detected on the opening of one of these holes (B). C, cilia; P (U), pinopod (uterodome), SV, secretory vesicle, CJ, cell junction. Scale bars = (A) 20 µm, (B) 1 µm, (C) 2 µm and (D) 0.5 µm.

Immunohistochemistry for light microscopy

Immunofluorescence double staining for LIF and VAMP-2 (Figure 7, panels a–c), syntaxin-1 (Figure 7, panels d–f), and SNAP-25 (Figure 7, panels g–i) in human endometrial biopsies that were obtained from mid-luteal phase of regular menstrual cycles showed that LIF was colocalized with syntaxin-1, SNAP-25 and VAMP-2 in the surface epithelial cells. The colocalization of these proteins is shown in merged images by change of original colour as a result of mixing of overlapped colours. In all panels, blue colour demonstrates nucleus staining by DAPI. Negative controls exhibited no reactivity for any of these proteins (data not shown).

Immunohistochemistry for TEM

Photomicrographs of TEM using immunogold particles displayed a high expression of LIF at uterodomes of luminal epithelial cells of the mid-luteal phase biopsies of human endometrium (Figure 6A). Immunogold particles were absent from the negative control group (Figure 6B).
Double staining for LIF and one of the syntaxin-1, SNAP-25 or VAMP-2 proteins by using different size immuno-labelled nanogolds, demonstrated that LIF colocalized with these markers of exocytosis at human endometrium. In some areas, colocalization of LIF with syntaxin-1, SNAP-25 or VAMP-2 could be detected as clusters of different size-immunoconjugated nanogolds (Figure 8, panels B, E and H). No reactivity was observed in any of endometrial specimens incubated without primary antibodies in the control groups (Figure 8, panels C, F and I). Uterodome-free area of the surface epithelium exhibited less or no colocalization of LIF and syntaxin-1, SNAP-25 or VAMP-2 (Figure 9; panels B, D and F). Photomicrographs of glandular epithelial cells, which were covered with uterodomes revealed that LIF and syntaxin-1 were colocalized in biopsies from mid-luteal phase of the normal cycle (Figure 10; panel B).

**Discussion**

The morphological and immunocytochemical studies of uterodomes of human endometrial biopsies from early, mid- and late luteal phases of regular menstrual cycles demonstrated a secretory role for them for the first time. Furthermore, to our knowledge, this is a first demonstration of subcellular localization of LIF in human uterodomes, which were shown to secrete through exocytosis pathways. Our observations revealed several differences in human uterodomes from those shown in rats and mice (Enders and Nelson, 1973; Parr and Parr, 1982), both in their distribution patterns and ultrastructures. Although pinopods of rodents were reported as organelle-free structures, images of TEM in this study are consistent with some previous reports that illustrated that several membranous organelles such as nucleus, mitochondria, Golgi complex and rough ER existed in human uterodomes (Pschoyos and Nikas, 1994; Dockery et al., 1997; Bentin-Ley et al., 1999 and Murphy, 2000b). Furthermore, in contrast to observation of large pinocytotic vacuoles in pinopods of rats and mice, our observation of human uterodomes under TEM microscope did not exhibit pinocytotic vacuoles (Enders and Nelson, 1973; Parr and Parr, 1977). A more interesting finding of our observations was detection of uterodomes around the opening and inside the lumen of uterine glands. This is the first illustration of uterodomes on the uterine glandular epithelium. Observations of these projections under TE and SE microscopes revealed that some holes existed on the apical pole of them. Interestingly, it was noticed that some of apical plasma membranes were ruptured and detached, and clusters of secretory vesicles were detectable around these ruptures. Rupture of the apical membranes of uterodomes in this study seemed very similar to those reported in the epithelial cells of the apocrine-secreting rat coagulating gland (Wiche et al., 2003). Based on these observations, we were encouraged to investigate the possibility of a secretive role for human uterodomes.

On the basis of a recent report that exhibited a strong temporal correlation between the appearance of uterodomes and secretion of LIF in humans (Aghajanova et al., 2003), and considering LIF as one of the most important components of histotrophs, which is necessary for the survival and growth of the conceptus as well as its key regulatory role in the endometrial receptivity (Carson et al., 2000); spatial interrelation of uterodomes with LIF and the possibility of secretion of LIF through uterodomes was studied. Photomicrographs of immunogold staining for TEM in this study clearly exhibited a high level of expression of LIF in human uterodomes. This is the first illustration of subcellular localization of LIF in bulbous ultrastructures of human endometrial epithelium referred to uterodomes.
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Figure 6. (A) Immunogold labelling for leukaemia inhibitory factor (LIF) (12 nm gold particles) using ultrathin sections of human endometrial biopsies from mid-luteal phase of a normal menstrual cycle. A high level of LIF expression in human uterodomes is clearly exhibited in this panel. (B) No immuno-reactive particle is detectable in the control group. Scale bars = 1 µm.

To date, three modes of secretion have been characterized, which include exocytosis, apocrine and holocrine. Exocytosis is the most commonly occurring type of secretion, in which the biochemical markers such as syntaxin-1, SNAP-25 and VAMP-2 have been extensively studied. In case of apocrine mode of secretion, merely morphological data were described (Wiche et al., 2003), and a little information is available concerning the plasma membrane dynamics and its biochemical markers (Gesase and Satoh, 2003). In this study, immunofluorescence double staining for LIF and the most critical biochemical markers involved in the exocytotic mode of secretion, including syntaxin-1, synaptosomal protein of 25 kDa molecular weight (SNAP-25) and VAMP-2. These well-known biochemical markers of exocytosis form a heterotrimeric complex, referred to as a core complex, which exists between the membrane of secretory vesicles and the plasma membrane (Calakos et al., 1994; Lonart and Sudhof, 2000). The results of immunofluorescence and TEM immunogold double staining for LIF and these exocytotic markers revealed colocalization of LIF with syntaxin-1, SNAP-25 and VAMP-2 in human uterodomes both in surface and glandular epithelial cells, whereas their colocalization in uterodome-free area of the uterus was not significant. Furthermore, according to the previous morphological data concerning apocrine mode of secretion and comparing them with the photomicrographs of SEM in this study, we propose the possibility of apocrine secretion in some of human uterodomes. Taken together, these findings suggest that besides previously reported pinocytotic role of pinopodes and their role in expressing adhesion molecules on their surface and establishing the first contact between the uterine wall and the implanting blastocyst (Enders and Nelson, 1973; Kabir-Salmani et al., in press), secretion in the form of both exocytosis and apocrine occur in human uterodomes. Introducing the more suitable in vitro models as well as biochemical markers of apocrine secretion will address different biomolecules, which may be released from uterodomes in the future studies.

Clinically, it is anticipated that regulation of LIF secretion plays an important role in the physiological and pathological processes of reproduction in humans. Thus, mediating LIF secretion as well as understanding the precise mechanisms of uterodome secretion may contribute to the investigation of new clues to control the complex process of implantation. It has been reported that interleukin (IL)-1, tumor necrosis factor, platelet-derived growth factor (PDGF), transforming growth factor (TGF), and epidermal growth factor (EGF) were inducers of LIF expression in human endometrium (Arici et al., 1995). We suggest that blocking the production or action of LIF and its mediators in adult models (as with transgenic mice) will address different biomolecules, which may be released from uterodomes in the future studies.

Figure 7. Immunofluorescent images of double staining for leukaemia inhibitory factor (LIF) and vesicle-associated membrane protein-2 (VAMP-2) (a–c), syntaxin-1 (d–f) and 25-kDa synaptosomal protein (SNAP-25) (g–i) in human endometrial biopsies taken from mid-luteal phase of a normal menstrual cycle exhibit their colocalizations. Panels (a), (d) and (g) are stained for LIF, (b) vesicle-associated membrane protein-2 (VAMP-2) and (e) merge of (a) and (b); (e) syntaxin-1 and (f) merge of (d) and (e); (h) SNAP-25 and (i) merge of (g) and (h). In all panels, blue colour demonstrates nucleus staining by DAPI. In the merged images, colocalization of proteins could be detected by change of original colours, shown in yellow and point by arrowheads. Scale bar = 10 µm.

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Figure 8. Double immunogold labelling for leukaemia inhibitory factor (LIF) (shown by 18 nm gold particles) and one of syntaxin-1, 25-kDa synaptosomal protein (SNAP-25) or vesicle-associated membrane protein-2 (VAMP-2) proteins (shown by 12 nm gold particles) using ultrathin sections of human endometrium from mid-luteal phase of a normal cycle to detect the pattern of the subcellular distribution of these proteins at human uterodomes. Panels (A) and (B) are stained for LIF and syntaxin-1; (D) and (E) for LIF and SNAP-25; and (G) and (H) for LIF and vesicle-associated membrane protein-2 (VAMP-2). These photomicrographs clearly exhibited the colocalization of LIF with syntaxin-1, SNAP-25 and VAMP-2 at human uterodomes. Their colocalizations could be detected as clusters of different size immuno-labelled nanoparticles. Scale bars = (A, D and G) 2 µm, (B, E and H) 0.5 µm, (C, F and I) 1 µm.

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