Journal of Animal and Veterinary Advances 12 (13): 1147-1150, 2013
ISSN: 1680-5593

Temporal and Spatial Relationship among Pinopodes, Implantation Sites and Implantation Window

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Abstract: The aim of this study was to explore the temporal and spatial relationship among pinopodes, implantation sites and implantation window and to suggest a method to obtain a sufficient number of pinopodes for further study. The endometrium was divided into two groups: the natural pregnancy group and the pseudopregnancy group. The distribution and density of pinopodes in different phases were observed by scanning electron microscopy in order to determine the phase and location that pinopodes enriched on the surface of endometrium. In the natural pregnancy group, a small number of tiny and round pinopodes started to protrude on the endometrial surface from day 3.5 of pregnancy. On day 4.5, a large number of pinopodes protruded from the endometrial surface, concentrating in where the implantation sites were located. On day 5.5, pinopodes started to regress. Pinopodes reached their peak at the implantation sites on the phase of implant window. In comparison, in the pseudopregnancy group, pinopodes reached their largest scale and density on day 3 of pseudopregnancy and earlier than they were in the natural pregnancy group. Pinopodes concentrated in the crypts of anti mesometrial side. In the natural pregnancy group, the peak of pinopodes appeared temporal and spatial coincident with implantation sites and implantation window. In the pseudopregnancy group which was earlier than they were in the natural pregnancy group. Whether in the natural pregnancy group or in the pseudopregnancy group, researchers can obtain endometrium containing pinopodes without interference of other tissues containing none or scarce pinopodes. This makes it possible to better understand the biochemical characteristic of pinopodes by studying biological molecules in pinopodes. This also helps to further explain the mechanism of recognition in other words, the relationship between the maternal endometrium and embryo in the process of apposition and attachment during the early implantation.

Key words: Pinopodes, implantation sites, implantation window, pseudopregnancy, apposition

INTRODUCTION

As the first step of pregnancy, implantation has been divided into four steps including apposition, attachment, adhesion and invasion. The communication between uterus endometrium and embryo is needed (Lee and DeMayo, 2004). At the stage of the implantation window, luminal epithelial cells are replaced by pinopodes which are bulbous protrusions which were first discovered by scanning electronic microscope in rodents and have been shown to be responsible for the uptake of uterine secretion (Cavagna and Mantese, 2003; Nikas, 1999; Stavreus-Evers, 2005). Pinopodes developed at implantation window and associated with uterus receptivity. In mice, they were discovered on days 4-5 of pregnancy and then became to regress (Bentin-Ley *et al*., 1999; Murpy, 2000). At the time of implantation of mice, it would have a unique uterine blue reaction. The attachment reaction coincides with a localized increase in stromal vascular permeability at the site of the blastocyst as can be demonstrated by i.v. injection of a macromolecular blue dye (Dey *et al*., 2004). The first sign of the attachment reaction (apposition stage) in the process of implantation occurs in the mice and rats on the evenings of days 4 and 5, respectively and in coincidence with implantation window (Murpy, 2000). However, no research has indicated an explicit temporal and spatial relationship among pinopodes, implantation sites and implantation window.

Pinopodes are too small that they are difficult to be observed by using conventional staining methods except scanning electron microscope and to make it worse the same piece of tissue can not be used for another experiment. Therefore, it is necessary to find conventional methods to get a lot of pinopodes for subsequent experimental study.

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MATERIALS AND METHODS

Animals: Young virgin NIH mice (8-12 weeks old) were obtained from the Animal Center of the National Research Institute for Family Planning (NRIFP). Mice were housed in polypropylene cages with free access to regular food and water. They were maintained under controlled conditions of a 12 h light/dark cycle (6:00 am to 6:00 pm). Experimental and surgical procedures were approved by the Animal Ethics Committee of NRIFP.

Treatments: The mice were divided into two groups: the natural pregnancy group and the pseudopregnancy group. In the natural pregnancy, the female mice were mated with fertile males and checked for a vaginal plug in the next morning (midnight of mating night as day 0). Implantation sites were detected by intravenous injection of 0.5% Evans blue dye as previously described (Deb *et al*., 2006). Mouse uterine tissues were collected on day 2; 11:00, 22:00 h, day 3; 11:00, 22:00 h, day 4; 01:00, 06:00, 11:00, 16:00, 21:00 and day 5; 01:00, 06:00, 11:00 and 16:00.

The pseudopregnancy group was induced by intramuscular injection of 10 IU Pregnant Mare Serum Gonadotropin (PMSG, Tianjin Experimental Animal Center, Tianjin, China). And then 10 IU hCG (Livzon Pharmaceutical Group Inc.) was used to trigger ovulation after 48 h. The mice were daily intramuscular injected progesterone (1 mg, Progesterone injection publishers, Beijing Zizhu Pharmaceutical Co., Ltd. Beijing, China). On day 1 the mice were treated with progesterone. The uterine tissues were collected every 4 h from day 2. At least three natural pregnant or pseudopregnant mice were included in each time point in each study.

Pre-labeled the endometrium: All the uterine tissues for scanning electron microscopy were longitudinally cut from the mesometrial side. Blue dye reaction was determined prior to uterine collection from day 3, 22:00 in the natural pregnancy group. When the uterus was dyed in blue, the uterine tissues were fixed with the thin needles on the sites where were blue dyed.

Scanning electron microscopy: All the uterine tissues for scanning electron microscopy were gently rinsed in saline, fixed in 2.5% (wt/vol) glutaraldehyde then postfixed in 1% (wt/vol) osmium tetroxid (Simmons and Kennedy, 2000). The samples were dehydrated in increasing concentrations of ethanol and dried in a critical-point drier using carbon dioxide, mounted on the specimen holder and coated with platinum. Specimens were examined under a S2500 SEM (Hitachi, Tokyo, Japan) for detection of pinopodes.

RESULTS AND DISCUSSION

In the natural pregnancy group, pinopodes can not be detected on the endometrial surface of day 2.5 (Fig. 1a). On day 3.5, sporadic pinopodes began to protrude from the endometrial surface. The diameter was about 1.6 μm (Fig. 1b). On day 4.5, a large number of pinopodes protruded on the endometrial surface (Fig. 1c). Pinopodes concentrated at the implantation sites coincident with the pre-labeled sites (Fig. 1d and e). The diameter grew to about 3.4 μm. On day 5.5, the number of pinopodes decreased and the shape of pinopodes shrank (Fig. 1f). The number, density and maturity of pinopodes varied depending on the different implantation sites.

In the pseudopregnancy group, pinopodes were induced by progesterone. On day 2, a small number of pinopodes started protrude on the endometrial surface (Fig. 2a). On day 3 of pseudopregnancy, the number and density of pinopodes reached their peak. Pinopodes were scattered from the oviduct end to the cervical end, the maximum density appeared in the upper 1/3 part near the oviduct end. The density of pinopodes was high in the crypts at the antimesometrial side (Fig. 2b). On day 4, the number of pinopodes decreased and the shape shrank in a way similar to what happened in the natural pregnancy group on day 5.5 (Fig. 2c). The maturity of pinopodes was not synchronized in different regions of endometrium as the maturity declined gradually from the oviduct end to the cervical end. Specifically, only one pinopode protruded in each endometrial epithelial cell (Fig. 2d).

Compared with the natural pregnancy group, the pinopodes in the pseudopregnancy group appeared earlier than they in the natural pregnancy group. In the natural pregnancy group, the pinopodes reached their peak at the implantation sites in the implantation window. There was a synchronized relationship among pinopodes, implantation sites and implantation window. Whereas in the pseudopregnancy group, the pinopodes reached their peaks at the upper 1/3 part of the antimesometrial side on day 3.

Pinopodes are generally considered as an important morphology marker of constructing endometrial receptivity and opening of implantation window and they seem to be directly involved in the adhesion of the blastocyst to the endometrial surface (Bentin-Ley *et al*., 1999; Cavagna and Mantese, 2003; Murpy, 2000; Nikas, 1999; Stavreus-Evers, 2005).

LIF and LIFR play a significant role in the process of implantation. LIF expression in gland epithelial cells and LIFR expression in lumen epithelial cells increase when pinopodes form but LIF expression is mainly in gland epithelial cells while pinopodes contribute more to the

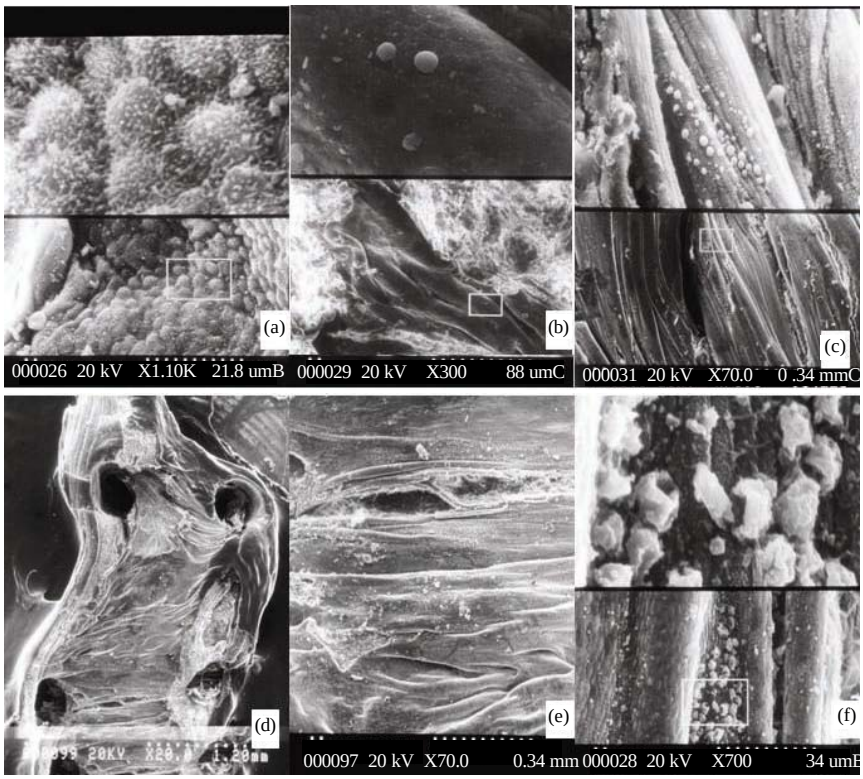

Fig. 1: Ultra-structure of the endometrium in the natural pregnant mice; a) There was no pinopodes on the endometrial surface of day 2.5; b) Sporadic pinopodes began to protrude from the endometrial surface on day 3.5; c) A large number of pinopodes protruded on the endometrial surface on day 4.5; d, e) Pinopodes concentrated at the implantation sites coincident with the pre-labeled sites and f) The number of pinopodes decreased and the shape of pinopodes shrank on day 5.5

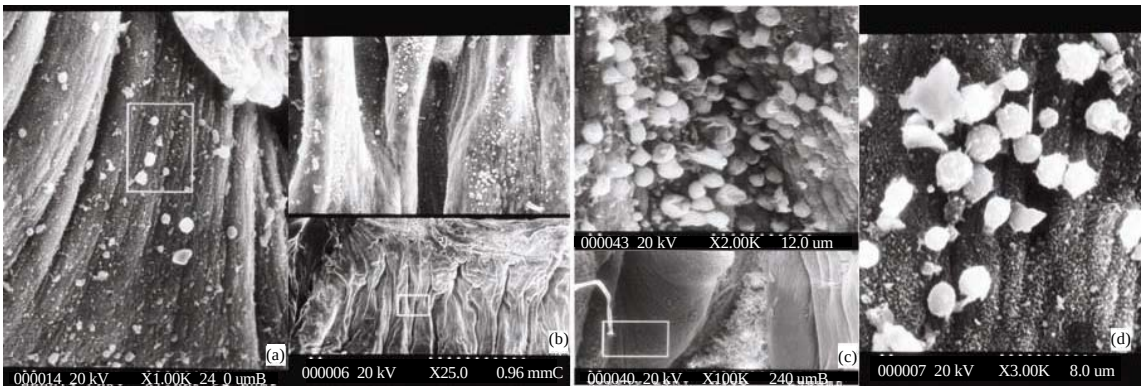

Fig. 2: Ultra-structure of the endometrium in the pseudopregnant mice; a) A small number of pinopodes started protrude on the endometrial surface on day 2; b) The number and density of pinopodes reached their peaks on day 3; c) The number of pinopodes decreased on day 4 and d) Only one pinopode protruded in each endometrial epithelial cell

recognization process (Aghajanova *et al*., 2003). Glutaredoxin expresses in pinopodes but it does not decrease when pinopodes disappeared (Stavreus-Evers *et al*., 2002a).

HB-EGF plays a major role in blastocyst implantation. The relationship between pinopodes and HB-EGF was detected by using scanning electron microscope and the immunohistological technique (Stavreus-Evers *et al*.,

2002b). It is determined that HB-EGF staining is consistent with the four stages of appearance, development, full development and regression of pinopodes. HB-EGF is located in cytoplasm of lumen epithelial cells and on the surface of pinopodes. HB-EGF may play an important role in implantation but in HB-EGF knockout mice, the implantation process is not affected by this deficiency (Iwamoto *et al*., 2003). Therefore, more evidences will be needed to prove that HB-EGF is significant for blastocyst implantation.

So far, except HB-EGF, no other special biological molecules have been found inside or on the surface of pinopodes. As mentioned, HB-EGF is not an explicit factor for implantation. Presumably, there should be some biological molecules in the pinopodes or on the surface of pinopodes and they are involved in recognizing the relationship between maternal uterus and the embryo in the early stage of embryo implantation.

In this study, the results showed that in the natural pregnancy group, pinopodes, implantation sites and endometrial implantation window were consistent in terms of time and space. This pre-labeled method helps us to obtain concentrated pinopodes by conventional technique. Secondly, after treating mice using a pre-labeled technique, researchers obtained endometrium tissues containing a large number of pinopodes without the interference by other tissues containing little or no pinopodes. This will help to get more details of implantation to identify the recognition mechanism that pinopodes mediated the relationship between the maternal endometrium and embryo in the process of apposition and attachment during the early implantation.

CONCLUSION

Whether in the natural pregnant or pseudopregnant mice, researchers can obtain concentrated and sufficient pinopodes, avoid the interference from the tissue with rare or none pinopodes. This simplifies the further study for pinopodes and helps to find the biological molecules and related to pinopodes.

ACKNOWLEDGEMENT

This study was supported by the National Key Grant of Basic Research Project (2010CB530403).

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