αvβ3 Integrin Express on Mid-luteal Human Endometrium: An Immunogold and Immunofluorescent Staining Study

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Introduction: The implantation is a complex procedure that involves many molecules. One of these molecules is integrin specially αvβ3, which serves as receptor for components of extracellular matrix to act as bridging molecules between the blastocyst and the endometrial surface during the implantation process. By blocking αvβ3 interactions, the implantation can be impaired.

Methods: The endometrial biopsies obtained from the anterior wall of the uterine cavity of 12 women. Each biopsy divided into three pieces; one fixed in 10% neutrally buffered formaldehyde for light microscopy and immunofluorescent study. The second fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for immunogold electron microscopy and the third fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for scanning electron microscopy. Afterwards, the biopsies evaluated by immunofluorescent, immunogold and scanning electron microscopy techniques.

Results: Immunofluorescent staining demonstrated that αvβ3 integrin express only on luminal surface epithelium and glandular epithelium of mid-luteal phase. Immunogold staining images in mid-luteal phase samples showed that αvβ3 integrin express on ciliated, non-ciliated (pinopdes) cells and junctional complexes. While, no reactivity observed on the endometrial surface, using the negative control antibody or in specimens incubated without primary antibody in any of the specimens.

Conclusion: The results showed that αvβ3 integrin express only on luminal surface epithelium and glandular epithelium in the mid-luteal phase of human endometrium and it may play a key role during the process of the embryo implantation. Targeting integrins may provide a new avenue for the development of contraceptive technologies, and the loss of this integrin in certain infertility states may signify the presence of implantation defects that reduce cycle fecundity in women.
1. Introduction

The endometrium remodels throughout the menstrual cycle, and exhibits only a short period of receptivity, known as the “window of implantation” (1). The endometrium becomes receptive to blastocyst 6-8 days after ovulation and remains receptive for 4 days (cycle days 20-24) (2).

The implantation is a complex procedure that can be divided into three distinct steps: opposition, attachment, and invasion (3). Shortly after the opposition step, an integrin-dependent adhesion occurs. This allows the blastocyst to attach firmly to the uterine wall and trophoblasts transmigrate across the luminal epithelium, burying the embryo beneath the uterine wall (4).

To achieve implantation, many molecules (hormones, cytokines, integrins, enzymes, etc) involve in the dialogue between the human blastocyst and the maternal endometrium (5). Integrins are cell-surface adhesion receptors that play key role in mediating numerous physiological processes, including inflammation, migration, adhesion, and proliferation (6). Integrins composed of an alpha and a beta subunit. Each subunit comprises an extracellular domain, a transmembrane region and an intracellular domain (7). Integrins serve as receptors for components of extracellular matrix such as osteopontin, fibronectin and collagens. These components have the capacity to act as bridging molecules between the blastocyst and the endometrial surface during the adhesion phase of the implantation process (8-11). The role of integrins in implantation has been widely reviewed (12-20). The extensive work of Lessey et al showed that three integrins (α1β1, α4β1, and αvβ3) express in uterine epithelium during implantation window (12-15). In other studies, it was reported that the best characterized cell adhesion molecules on the luminal surface of the endometrium are αvβ3 integrin and its ligand osteopontin, repeatedly found in genome-wide studies of human receptive endometrium (21-24). Blocking αvβ3 interactions in mouse or rabbit models impairs implantation (25, 26).

The purpose of this study was to establish the expression of αvβ3 integrin as a marker of endometrial receptivity in human by immunogold and immunofluorescent staining study and prove its role in implantation.

2. Materials & Methods

2.1. Endometrial Specimens

Endometrial biopsies obtained from the anterior wall of the uterine cavity of 12 women. The samples divided into three equal groups; proliferative, early luteal and mid-luteal phases. The design of the study approved by the ethics committee of Kyorin University and informed consent obtained from all participating women. All women were fertile with regular menstrual periods (25-35 days). The mean age was 37 years (range 25 – 45) and none of them used steroidal contraceptive or an intrauterine device for at least 3 months before sampling. Each biopsy divided into three pieces; one fixed in 10% neutrally buffered formaldehyde for light microscopy and immunohistochemistry. The second portion fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for immunogold electron microscopy and the third in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for scanning electron microscopy.

For endometrial dating, according to the histopathological criteria of Noyes, et al., (27), the paraaffin-embedded biopsies stained with hematoxylin and eosin and evaluated by an experienced observer who was blind to the study.

2.2. Scanning Electron Microscopy

For scanning electron microscopy preparation, endometrial tissues fixed for at least 24 hr in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and post fixed for 1hr in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4). The samples dehydrated in a graded series of ethanol (50%, 70%, 90%, 99.5% and 100%), critical-point-dried with carbon dioxide by using a freeze drying device (JFD–300, JEOL, Tokyo, Japan), mounted and coated with gold in a sputter coater (JFC-1300 Auto Fine Coater, JEOL, Tokyo, Japan). Finally, using a scanning electron microscope (JSM- 5600 LV SEM, JEOL, Tokyo, Japan), the samples were examined. The specimens in phase showed pinopodes but no pathological features divided into two experimental groups: early luteal (days 15-19) and mid-luteal (days 20-24).

2.3. Immunofluorescent Staining

For immunofluorescent study, the paraffin sections (4 µm) dewaxed in xylene and rehydrated in decreasing concentrations of ethanol and, finally distilled water. Endogenous peroxidase blocked by 0.3% hydrogen peroxide in methanol for 10 min and nonspecific antibody binding
blocked by incubation in 5% BSA in PBS for 30 min. After this treatment, the sections washed three times (5 min each) with PBS and incubated overnight at 4 °C with the appropriate primary antibodies diluted in PBS (anti-αvβ3 integrin IgG 1:100). For control, the sections incubated overnight at 4°C with the same concentration of mentioned antibody, normal mouse serum (substituted for mouse anti-αvβ3 integrin) primary antibody. The sections rinsed in PBS extensively and counter-stained with proper fluorescent-labeled secondary antibody (Alexa 568-labeled goat anti-mouse IgG 1:250) appropriately and incubated for 1 hr at room temperature. Finally, these experiments were repeated four times in different endometrial samples taken from proliferative and luteal phases.

2.4. Immunostaining for Electron Microscopy

Immunogold labeling performed to ultrastructural distribution of αvβ3 integrin molecules according to the previous reports (28). Briefly, the specimens divided into blocks (sized 2 mm3) and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for at least 24 hr at 4°C. After dehydration in a graded series of ethanol (50%, 70%, 90%, 99.5%, 100%), they were embedded in Lowicryl White Resin (London Resin company Ltd, London, UK) and ultrathin sections were cut. Then ultrathin sections washed with PBS and pretreated with 5% BSA for 10 min at room temperature. After a PBS rinse, they incubated with mouse anti-αvβ3 integrin IgG (1:100) or with normal mouse serum as control for overnight at 4°C. Following washing with PBS 5 times (5 min each), the sections incubated with the colloidal gold (12 nm in diameter)-conjugated goat anti-mouse colloidal gold-conjugated IgG (Jackson Immuno Research Laboratories Inc., West Grove, PA, USA) for 1 hr at room temperature (1:20 dilution), the sections washed with PBS for 5 times and then distilled water 3 times (5 min each). The ultrathin sections stained with uranyl acetate and then examined with a transmission electron microscope (JEM-1010; JEOL, Tokyo, Japan). Using above technique, four different samples from each experimental group were examined.

3. Results

Regarding the images from scanning electron microscopy (SEM), endometrial epithelium in secretory phase showed two different types of cells: ciliated and nonciliated cells, that the latter cover the majority of luminal surface (Figure 1). Membrane projections on the apical pole of non-ciliated cells appear as fine microvilli and dome-like projections defined as progressing, developed and regressing pinopodes (4). Under scanning electron microscopy, in the early luteal phase (dating 15-19) progressing pinopodes and in mid luteal phase (dating 20-24) fully developed pinopodes were seen (Figure 1B, C) while in proliferative phase no pinopodes were detected (Figure 1A)

Immunofluorescent staining demonstrated that αvβ3 integrin express only on luminal surface epithelium and glandular epithelium of mid-luteal phase. During the three phases, αvβ3 integrin was present in the stroma (Figure 2).

Figure 1. Scanning electron microscopy (SEM) photomicrographs of luminal surface of human endometrial biopsies taken from proliferative (A), early (B) and mid-luteal (C) phases of normal menstrual cycle to identify developmental stage of pinopodes. Notice that there are not any pinopodes in proliferative phase, few isolated pinopodes are detectable in specimens from early luteal phase, numerous developed pinopodes in the mid-luteal phase. Scale bars = 10 µm.
Immunogold staining images in the mid-luteal phase samples showed that αvβ3 integrin express on ciliated, non-ciliated (pinopdes) cells and junctional complexes. However, no reactivity was observed on the endometrial surface using the negative control antibody or in specimens incubated without primary antibody in any of the specimens (Figure 3).

Figure 2. Immunofluorescent staining for αvβ3 integrin in proliferative (p), early luteal (E.L), and mid-luteal (M.L) phases of human endometrium. Lane 1 show specific staining for αvβ3 integrin; lane 2, DAPI staining and lane 3, Merged (magnification ×400). Notice that αvβ3 integrin express on stromal tissue in the three phases but, it expressed only on luminal surface epithelium and glandular epithelium of mid-luteal phase.

Figure 3. Immunogold labeling showed the expression of αvβ3 integrin on ciliated (A, B) and non-ciliated (pinopod) cells (C, D) in the mid-luteal phase of human endometrium samples. Expression of αvβ3 integrin was seen on cilia (CI), pinopod (P) and junctional complex (JC).

No reactivity observed on the endometrial surface in the control samples (E, F). Scale bars = 5µm
4. Discussion

Embryo implantation is a part of mammalian reproduction (29). Initial step of embryo implantation is cell adhesion of trophectoderm of blastocyst and endometrial luminal epithelial cells of uterus, at their respective apical cell surfaces. This occurs despite generally the non-adherent nature of apical cell surfaces of epithelial cells; thus, integrin plays a key role in this adhesion (30).

The discovery of integrins in the human endometrial epithelium and stroma (12, 31, and 32) led to this expectation that these molecules are somehow involved in the procedure leading to successful pregnancy. Recent evidences demonstrate that integrins regulated in the uterus of rodents (33, 34).

Integrins are surface glycoproteins which have α and β subunits. During the adhesion phase of the implantation, these components act as bridge between the blastocyst and the endometrial surface. During implantation, Integrins appear on the blastocyst, surface of glandular and luminal endometrial epithelium (32).

In this study, the interesting finding is the localization of \( \alpha \beta_3 \) integrin in the apical membrane projections in mid-luteal phase of human endometrial epithelium, so called pinopodes.

Pinopodes on the epithelial surface are visible in light microscopy, but other structures may be mistaken for pinopodes (35); using these techniques, it would not be possible to determine their stage. Thus, in this study, scanning electron microscopy used to confirm the presence of pinopodes in the endometrial tissue. Further, using the indirect immunogold technique in this study permitted to evaluate the distribution of \( \alpha \beta_3 \) integrin specifically in pinopodes during opening of the implantation window.

The results obtained from immunogold transmission electron microscopy displayed an increase in expression of \( \alpha \beta_3 \) integrin at uterine pinopodes of mid luteal specimens compared to early luteal phase. Furthermore, the results show that \( \alpha \beta_3 \) integrin distributed with higher density at area near the cell membrane of pinopodes comparing to similar neighboring areas without pinopode. These findings enhance the significance of pinopode formation in preparation for embryo attachment. In photomicrograph from immunogold transmission electron microscopy, \( \alpha \beta_3 \) integrin observed over cytoplasm, nucleus and cell membrane of specimens from mid luteal phase endometrium. However, no reactivity was observed on the endometrial surface using the negative control antibody or in specimens incubated without primary antibody in any of the specimens (Fig.3).

The increase of expression of \( \alpha \beta_3 \) integrin in this study was confirmed by the previous reports (13, 36). Lessey et al reported that the glandular and luminal epithelium affected by independent alterations in integrin expression throughout the menstrual cycle. The expression of one integrin (the \( \alpha \beta_3 \) vitronectin receptor) on both luminal and glandular epithelium coincides with the time of embryo attachment; also they demonstrated that aberrant expression of this integrin is associated with infertility (13). Lessey et al detected one integrin in the glandular epithelium during postovulatory days 5 and 6. Subunit \( \alpha \) is present at the epithelial level during the early and late secretory phase and at the stromal level during the whole cycle; \( \beta_3 \) is present at the epithelium during the late secretory phase and in the stroma during the menstrual cycle. In the luminal epithelium, the expression of \( \alpha \beta_3 \) starts on day 20 of the cycle, continues until the end of the cycle, and persists during early pregnancy (12, 32).

Yelian et al showed that \( \alpha \beta_3 \) integrin appears to be specifically present in the endometrium during the window of implantation in both humans and mice. Ligand binding to \( \alpha \beta_3 \) is dependent on the three-amino acid sequence arg-gly-asp (RGD), which is involved in embryo attachment and outgrowth in vivo (37).

Cheresh et al reported that \( \alpha \beta_3 \) integrin is critical for angiogenesis. Neutralization of this integrin during implantation can reduce embryo survival by preventing new vessel formation at the site of implantation (38).

Mid-secretory phase increase in endometrial epithelial \( \alpha \beta_3 \) resulted from an increase in \( \beta_3 \) after day 19 (15). Aberrant \( \alpha \beta_3 \) integrin associated with unexplained infertility and other endometrial pathologies (12, 32, 39, and 40). Integrins \( \alpha \beta_3 \) and \( \alpha \beta_6 \) are also present in endometrium (41). Blocking \( \alpha \beta_3 \) interactions in mouse or rabbit models impairs implantation (25, 26).

5. Conclusions

The results showed that \( \alpha \beta_3 \) integrin express only on luminal surface epithelium and glandular epithelium in the mid-luteal phase of human endometrium and it may play key role during the process of the embryo implantation. Targeting integrins may provide a new method for development of contraceptive technologies, and the loss of this integrin in certain infertility states may signify the
presence of implantation defects reducing cycle fecundity in women.

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References


