Endocrine Journal 2008, **55** (5), 879–887

Expression and Localization of Galectin-9 in the Human Uterodome

YUJI SHIMIZU, MARYAM KABIR-SALMANI*,**, MEHRI AZADBAKHT***, KAZUHIRO SUGIHARA#, KEIJI SAKAI AND MITSUTOSHI IWASHITA

Department of Obstetrics and Gynecology, Kyorin University School of Medicine, Shinkawa 6-20-2, Mitaka, Tokyo 181-8611, Japan
**Cellular and Molecular Biology Research Center, Shahid Beheshti Medical University, Tehran, Iran*
***Department of Genetic Medicine, National Institute for Genetic Engineering, Pajoohesh Blvd, Tehran 14155-6343, Iran*
****Department of Biology, Razi University, Kermanshah, Iran*
#Department of Obstetrics and Gynecology, Hamamatsu University School of Medicine, 1-20-1, Handayama Higashi-ku, Hamamatsu 431-3192 Japan

Abstract. Galectin-9 has been recently considered as a novel marker for the mid- and late-secretory phases of human endometrium and decidua. The aim of this study was to investigate the subcellular distribution of galectin-9 in the endometrial epithelium, especially during the frame of the implantation window. Endometrial biopsies in the proliferative, early, and mid-secretory phases from women with regular menstrual cycle were studied using several approaches, including scanning electron microscopy, immunostaining for light and transmission electron microscopies (TEM), immunoblotting, and statistical analysis of the area-related numerical densities of galectin-9-bound nanogold. Images of immunostaining for light microscopy demonstrated a strong expression of galectin-9 at the luminal and glandular endometrial epithelium in the mid-secretory phase compared to the proliferative and early secretory phases. Data of immunoblotting revealed a molecular weight of 36 kDa band with high intensity in the mid-secretory samples. Photomicrographs of immunogold staining for TEM illustrated the localization of galectin-9 in the uterodomes. Statistical and morphometric analysis showed a significantly higher area-related numerical density of galectin-9-bound nano-golds in the uterodomes compared to that of the uterodome-free areas of the luminal epithelium ($p<0.001$). This is the first demonstration of the molecular localization of galectin-9 in the bulbous ultrastructure of the human endometrial epithelium, called uterodomes. High expression of galectin-9 at uterodomes during the frame of implantation window suggests that galectin-9 can be considered as a marker of endometrial receptivity and should play an important role during the initial events of human embryo implantation.

Key words: Endometrium, Galectin-9, Uterodome, Implantation Window

(*Endocrine Journal* **55**: 879–887, 2008)

THE human endometrium, a unique tissue because of its morphology and function, allows for implantation of the blastocyst only at a limited time in mid-secretory phase, the so-called window of implantation [1]. In many mammalian species, the appearance of uterodomes (pinopods) is one of the most significant morphological features of transformation occurring on the plasma membrane during endometrial development in preparation for pregnancy [2–3]. Initiation of pregnancy requires attachment and invasion of the blastocyst to the epithelial barrier, which necessitates finely tuned adhesion molecules and immune responses [4]. Accordingly, at the molecular level, the regulated spatial and temporal expression pattern of distinct galectins, which demonstrate several parallels in their function with receptive endometrium, was suggested to play a role in the regulation of endometrial receptivity.

Galectins are S-type mammalian lectins, which play

Received: April 14, 2008
Accepted: May 9, 2008
Correspondence to: Mitsutoshi IWASHITA, M.D., Ph.D., Department of Obstetrics and Gynecology, Kyorin University School of Medicine, Shinkawa 6-20-2, Mitaka, Tokyo 181-8611, Japan

a role in the regulation of cell growth, adhesion, apoptosis, inflammation, and immunomodulation [5–8], all of which are intimately involved in endometrial function during the complex process of embryo implantation. According to their architecture, this family of glycan-binding proteins is classified in those containing one-carbohydrate-recognition domain (CRD) (proto-type), those containing two-CRD joined by a linker non-lectin domain (tandem-repeat) and those that have one-CRD attached to an N-terminal peptide (chimera-type) [9]. Galectins modulate cell to cell adhesion and cell to extracellular matrix interactions, and are known as ligands for integrins and galectins that recognize specific oligosaccharide structures on glycoproteins and glycolipids [10]. Galectins bind to cell adhesion molecules, such as laminin and fibronectin [11], and are possible candidates for supporting the binding between endometrial epithelial cells and the blastocyst. Gray *et al.* suggested that galectins act as cellular and molecular markers for the endometrial receptivity in sheep [12]. Recently, cycle-dependent expression of galectin-1 in stromal cells and galectin-3 in the glandular epithelial cells has been reported in the human endometrium [13]. Later, galectin-9 was identified as a new endometrial epithelial marker for the mid- and late-secretory and decidual phases in human endometrium, in which galectin mRNA was shown to be exclusively expressed in the endometrial epithelial cells but not in stromal or immune cells [14].

Uterodomes are considered as the biological marker of uterine receptivity *in vivo* [15–16] and the preferred site of embryo-endometrial interactions *in vitro* [17]. Furthermore, uterodomes are the initial sites that contact with the floating blastocyst and play an important role in accumulation and secretion of biomolecules to the uterine cavity during the initial phase of pregnancy [18]. To elucidate the significance of galectin 9 in the events of implantation, we determined the expression and subcellular distribution pattern of galectin-9 in human endometrium especially in uterodomes, which show a significant temporal increase during the mid-secretory phase of the menstrual cycle [14].

Materials and Methods

Patients and samples

Endometrial biopsies were collected from the anterior wall of the uterine cavity of 36 female patients (mean age 33.6 years, range 25–41 years) in the proliferative (days 10–12 of regular menstrual cycle, n = 8), early secretory phase (days 16–18 of regular menstrual cycle, n = 10), and the mid-secretory phase (days 20–24 of regular menstrual cycle, n = 16). Samples were dated according to the last menstrual period, histology, and serum levels of estradiol, progesterone, and LH. The patients were selected from those referred to our center at Kyorin University Hospital for some kind of medical indication. All cases were selected from fertile women having a regular cycle, who had delivered at least one live child. None of the patients were being hormonally treated or using steroidal contraceptives or intrauterine devices for at least 3 months prior to the sampling, nor were they suspected to have malignancies in uterus. Ethical approval for this study was granted by the Ethics Committee of Kyorin University, School of Medicine, Tokyo, Japan. All patients gave their informed consent for collection and investigational use of tissues before sampling.

Biopsies from each patient (two specimens from each patient) were divided into several small portions to be used for light microscopy, immunohistochemistry, immunogold staining for transmission electron microscopy (TEM), and immunoblotting as described in the respective sections. For endometrial dating according to the histopathological criteria of Noyes *et al.* [19], the paraffin-embedded biopsies were stained with hematoxylin and eosin and evaluated by an experienced observer who was blind to the objectives of this study.

Immunostaining for light microscopy

For DAB (3,3'-diaminobenzidine tetrahydrochloride) staining using optimal cutting temperature (OCT) compound-embedded samples, cryostatic sections (4 μm thick) were mounted on slides, fixed with ice-cold 100% acetone (Sigma-Aldrich, St. Louis, MO) for 10 min, and air-dried. After blocking endogenous peroxidase activity by immersing the slides in 0.3% hydrogen peroxide (H_2O_2) in methanol for 10 min at room temperature, nonspecific background bindings were blocked with 5% BSA for 30 min at room temperature. Samples were then incubated overnight at 4°C with goat polyclonal anti-galectin-9 IgG (Santa Cruz Biotechnology, Santa Cruz, CA) at a concentration of 1.5 μg/ml in 10 mM phosphate buffered saline

(PBS). After washing with PBS three times for 5 min each, the sections were incubated with horseradish peroxidase (HRP)-conjugated donkey anti-goat IgG (1.7 μg/ml) for 1 hr at room temperature. Following washing in PBS (three times, 5 min each), peroxidase activity was detected by incubating the specimens for 10 min at room temperature with DAB-H_2O_2 reaction solution (0.5 mg of DAB/ml PBS, containing 0.005% H_2O_2). After washing the slides with distilled water, they were counterstained and mounted. Control experiments included staining without the primary antibody and substitution of galectin-9 antibody by goat IgG (Jackson ImmunoResearch, West Grove, PA) at the same concentration of the primary antibody. These experiments were repeated al least four times using different preparations of four different samples.

Immunoprecipitation and immunoblotting

Immunoblotting was performed to compare the expression of galectin-9 in the proliferative, early and mid- secretory phases of human endometrium at the biochemical level. Endometrial spieces were washed with ice-cold PBS three times and were solubilized with lysis buffer (1% Nonidet P-40, 150 mM NaCl, 1 mM EGTA, 0.25% sodium deoxycholate, and 50 mM Hepes, pH 7.5) containing protease inhibitors (1 μg/ml aprotinin and leupeptin, 1 M AEBSF, 0.5 mg/ml pepstatin) using sonication. Insoluble materials were then removed by centrifugation at 15,000 × g for 15 min and supernatant were immunoprecipitated with 6 μg anti-galectin-9 antibody (R&S Systems, Minneapolis, MN) overnight at 4°C. The immune-complexes were incubated with protein-G agarose at 4°C for 3 hr. Immobilized protein-G agarose was sedimented by centrifugation at 10,000 × g for 1 min and washed four times with same lysis buffer. The protein concentrations were measured using protein detection kit (Bio-Rad, Munich, Germany) according to the manufacturer's instruction and the samples were adjusted to the same amount of final protein concentrations per ml of supernatant. The supernatant from each tube (20 μl) was then assayed for galectin-9 by immunoblotting. In brief, samples were subjected to 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under the reducing conditions and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes with 0.45 μm pore size (BioRad Laboratories, Hercules, CA). After blocking with Tris buffer saline (10 mM Tris, 140 mM NaCl, pH 7.4) containing 3% BSA for 2 hr at room temperature, blots were exposed to anti-galectin-9 antibody (R&D Systems) overnight at 4°C. After several washes with washing buffer (Tris buffer containing 0.5% (v/v) Tween 20), immunoreactive proteins were identified by 30 min incubation with horseradish peroxidase-conjugated donkey anti-goat monoclonal IgG diluted with blocking buffer (1 : 10000) at room temperature. Following several washes, the immunoreactivity was detected with the ECL system (Amersham, Tokyo, Japan) and exposure to Kodak X-AR film (Eastman Kodak Co., Rochester, NY) for 1–15 min at room temperature. This experiment was repeated in triplicate using three different samples.

Scanning electron microscopy (SEM)

SEM was performed to evaluate the presence of uterodomes in the endometrial specimens obtained at the early and mid-secretory phases of the menstrual cycle. For SEM preparation, endometrial biopsies were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 hr at 4°C and postfixed using 1% OsO_4 in 0.1 M phosphate buffer (pH 7.4) for 1 hr. The specimens were then dehydrated in a graded series of ethanol (50%, 70%, 90%, 99.5% and 100%), critical-point-dried with carbon dioxide 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, the specimens were observed under a scanning electron microscope (JSM-5600 LV SEM, JEOL, Tokyo, Japan).

Immunostaining for transmission electron microscopy (TEM)

Immunogold staining for TEM was performed according to previous reports [20–21]. Briefly, specimens were divided into 2 mm^3 blocks and fixed in 4% PFA in 0.1 M phosphate buffer (pH 7.4) for 24 hr at 4°C. After dehydration in a graded series of ethanol (50%, 70%, 90%, 99.5%, and 100%), they were embedded in Lowicryl white resin (London Resin Co., London, UK). They were then cut into ultrathin sections. After pretreatment with 5% BSA for 10 min at room temperature, they were incubated overnight at 4°C with goat polyclonal anti-galectin-9 IgG (1.5 μg/ml) or with normal goat IgG (1.5 μg/ml), as a negative

control. Following washing in PBS (5 times for 5 min each), the ultrathin sections were incubated overnight at 4°C with 18 or 12 nm colloidal gold-conjugated donkey anti-goat antibody diluted with PBS at 1 : 20 (Jackson ImmunoResearch, West Grove, PA). The ultrathin sections were then washed in PBS followed by washing in distilled water and were stained with uranyle acetate and observed under a transmission electron microscope (JEM-1010; JEOL, Tokyo, Japan). Immunostainings were performed at least in triplicate to confirm the reproducibility.

Statistical analysis was performed by taking the mean of 50 fields per test substance for six replicates. For semiquantitative morphometric analysis, 300 fields (each field equaled to 8.04 μm^2) randomly chosen from each experimental group that were obtained at the early and mid-secretory phases were taken by photography and counted by an observer who was blind to the identity of the grids. The number of immunogold particles was counted in all selected areas at the same magnification (×15,000). After calculating the area-related numerical densities of immunogold particles, statistical analysis was carried out by taking the mean number of immunogold particles and the area-related numerical density of immunogold particles was expressed as mean ± SEM. Statistical significance was evaluated using ANOVA with Scheffe's test and differences were considered statistically significant if $p<0.05$.

Results

Immunohistochemistry for Light Microscopy

DAB staining was performed to compare the expression pattern of galectin-9 in the endometrium at proliferative, early and mid-secretory phases of menstrual cycle. Images of DAB immunostainings demonstrated a strong immunoreactivity for galectin-9 in mid- secretory phase endometrium (Fig. 1, panel Aa, b) compared to that of the samples obtained from the proliferative phase (Fig. 1, panel Af) and early secretory phase (Fig. 1, panel Ae) of the menstrual cycle. No immunoreactivity was observed in the negative controls, in which the primary antibody was excluded to assess the level of antigen-independent binding (Fig. 1, panel Ac, Ad). In all biopsies taken from the mid-secretory phase endometrium, immunoreactivity was more intense in both glandular and luminal epithelium, while a very faint reaction was observed in the stromal cells and in other components (data not shown).

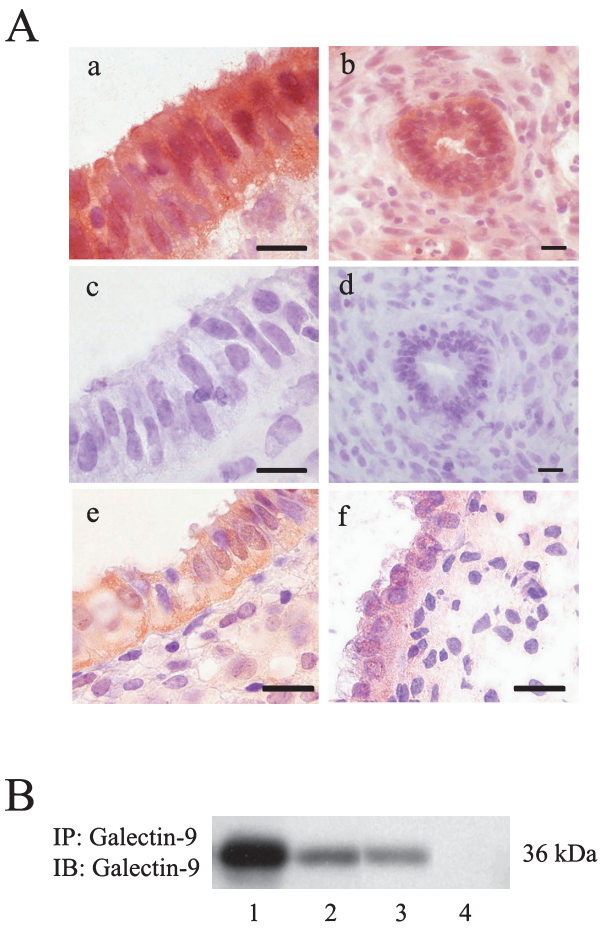

Fig. 1. Panel A) DAB immunostaining for galectin-9 in the biopsies of the human endometrium in the proliferative, early, and mid-secretory phases of a normal menstrual cycle. A more intense immunoreactivity for galectin-9 was observed in the luminal and glandular epithelial cells in the biopsies of the mid-secretory phase (a, b) compared to the proliferative (f) and early-secretory (e) phases. Negative controls, in which the primary antibody was excluded (c, d) no immunostaining was observed. Scale bar = 10 µm. Panel B) SDS-PAGE and immunoblotting for galectin-9 demonstrates a 36 kDa protein mass band corresponding to galectin-9. The results showed a significantly higher amount of galectin-9 expression in the specimens obtained in mid-secretory phase (lane 1) compared to proliferative (lane 2) and early-secretory (lane 3) phases of the regular menstrual cycle. Blotting with the negative control antibody resulted in no band (lane 4). Endometrial biopsies obtained in the mentioned phases were lysed, separated by SDS-PAGE, transferred electrophoretically to PVDF membrane, and analyzed by immunoblotting.

Immunoblotting

Results obtained from immunoblotting for galectin-9 experiments demonstrated the existence of a protein band of 36 kDa, corresponding to galectin-9 molecule in the proliferative, early and mid-secretory phases biopsies of the endometrium (Fig. 1, panel B). These results showed a significantly higher concentration of galectin-9 in the specimens obtained in mid-secretory phase (lane 1 in Fig. 1, panel B) compared to proliferative and early-secretory phases of regular menstrual cycle (Fig. 1, panel B lane 2, 3). Staining with negative control antibody resulted in no band (Fig. 1, panel B lane 4), which confirmed the specificity of the antibody used.

Scanning electron microscopy

Morphological studies of endometrial biopsies by SEM are usually performed to monitor the expression of uterodomes (pinopods) and evaluation of their developmental stage during secretory phase. Generally, on the basis of the stage of their development, uterodomes are scored as developing, developed, or regressing [15]. SEM images demonstrated that the luminal endometrium as well as glandular epithelial cells were composed of two different types of cell including ciliated and nonciliated cells (Fig. 2, panel Aa, Ab). The majority of luminal epithelial cells were of the latter type. The membranous projections on the apical pole of nonciliated cells appeared as fine microvilli and dome-like projections. In the samples obtained from early-secretory phase of the menstrual cycle, we observed many progressing and few isolated developed projections (Fig. 2, panel Aa). In the samples collected at mid-secretory phase, developed uterodomes, which appeared on the majority of nonciliated epithelial cells, were dominant beside a few regressing uterodomes in the mid-secretory phase specimens (Fig. 2, panel Ab).

Identification of galectin-9 in uterodomes and its area related distribution

Immunogold staining for TEM was performed to detect the subcellular localization of galectin-9 at uterodomes as well as comparing the area-related numerical densities of galectin-9-bound nanogolds at the uterodomes compared to that of the uterodome-free areas of the luminal endometrial epithelial cell. The photomicrograph of immunogold TEM exhibited a high expression of galectin-9 at uterodomes (Fig. 2,

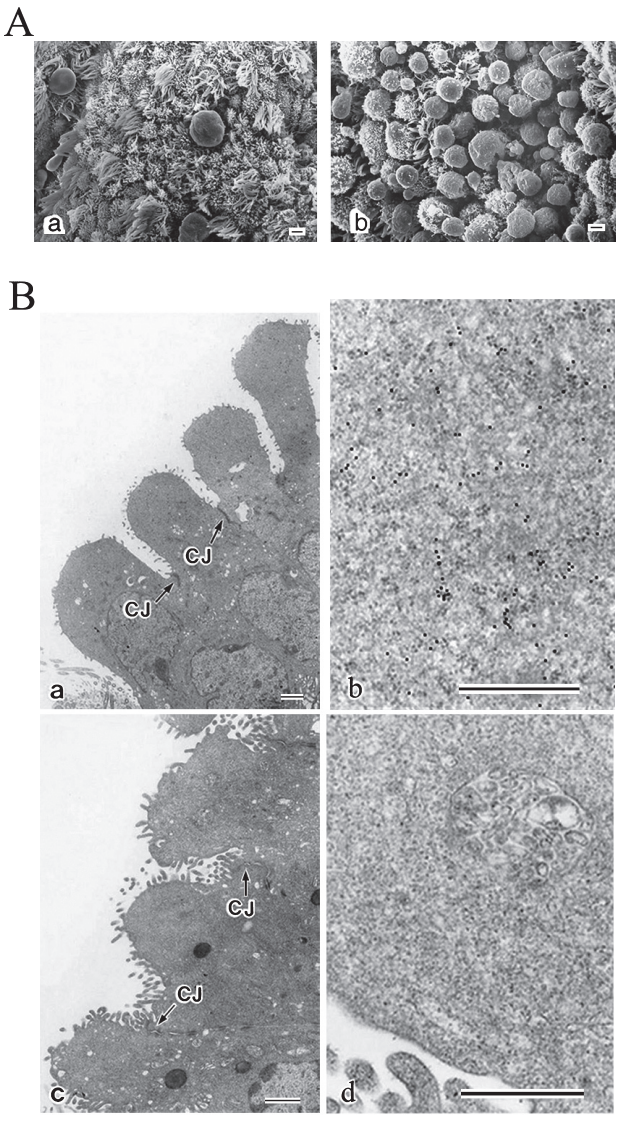

Fig. 2. Panel A) SEM photomicrographs from the human endometrium early (a) and mid- secretory (b) phases of a normal menstrual cycle. Note the numerous fully developed uterodomes among the majority of uterine epithelial cells in the mid-secretory phase specimen. Scale bar = 5 μm. Panel B) Immunogold labeling for galectin-9 (12 nm gold particles) using ultrathin sections of human endometrium in the mid-secretory phase (a, b) of a normal menstrual cycle and control (c, d) to detect the subcellular distribution of galectin-9 in uterodomes. These photomicrographs clearly exhibit the expression of galectin-9 in uterodomes of mid-secretory phase specimen (b). In negative controls, no immunogold particles were seen (d). Scale bars = 2 μm (a, c) and 0.2 μm (b, d). CJ: cell junction.

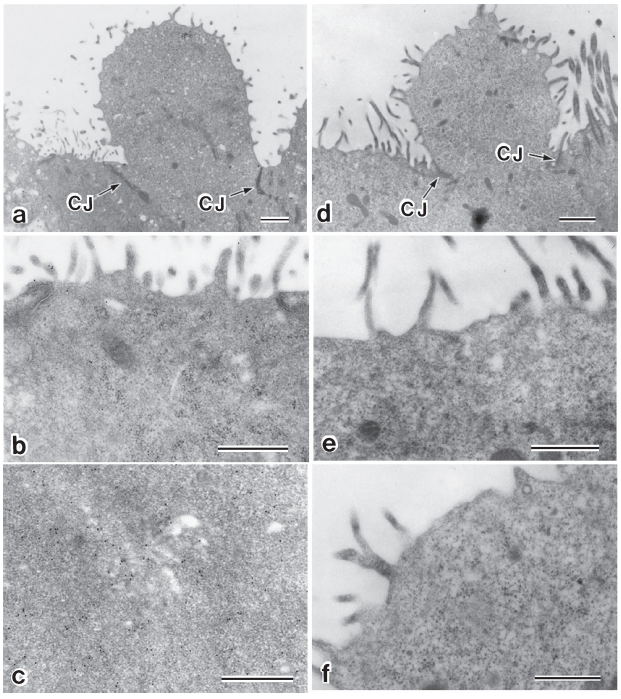

Fig. 3. TEM photomicrographs of the mid-secretory phase endometrium using immunogold staining to compare the distribution pattern of galectin-9 in the uterodomes and uterodome-free area of epithelial cells of the mid-secretory phase endometrium. Fig. 3a shows immunogold labelling for galectin-9, and Fig. 3d shows negative control in low magnification. In the uterodome-free area, less nanogolds were detected (Fig. 3b) compared to the uterodomes (Fig. 3c). No immunoreactivity was observed in the negative controls and no gold particles were observed in negative controls (Fig. 3d, e, f). Scale bars = 3 μm (a, d) and 0.3 μm (b, c, e, f). CJ: cell junction.

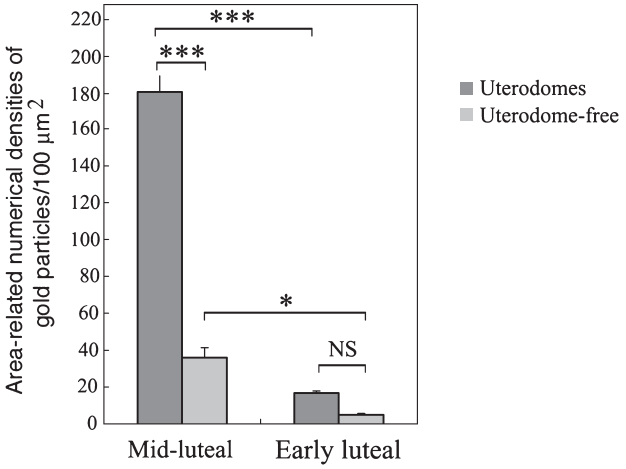

Fig. 4. Statistical analysis of the area-related numerical densities of immunogold-conjugated galectin-9 in human early and mid-secretory phase endometrial biopsies. The statistical analyses of the number of immunogold particles revealed that the distribution of galectin-9 in these specimens was significantly ($p<0.001$) higher in the uterodomes (dark gray bars) of the mid-secretory phase specimen compared to the uterodome-free areas (light gray bars) and uterodomes of the early-secretory phase. *: $p<0.05$, ***: $p<0.001$, NS: Not significant.

panel Bb) shown by nanogold-conjugated particles, while in the negative control no gold particles were observed (Fig. 2, panel Bd).

The expression pattern of galectin-9 in the epithelial cells of the mid- secretory phase endometrium displayed different area-related numerical densities (Fig. 3a–f). In the uterodome-free area fewer nanogolds were detected (Fig. 3b) compared to those in the uterodomes (Fig. 3c). No immunoreactivity and no gold particles were observed in negative controls (Fig. 3e, f).

The statistical analyses of the number of immunogold particles revealed that the distribution of galectin-9 in these specimens was significantly ($p<0.001$) higher in the uterodomes of mid- secretory phase specimen compared to the uterodome-free areas and uterodomes of early-secretory phase (Fig. 4). The results of semiquantitative morphometric assays revealed that the area-related numerical density of gold particles in the uterodomes of mid- secretory phase was more than five times that of the neighboring uterodome-free areas of the luminal epithelium and sevenfold that of the uterodomes of early-secretory phase (Fig. 4).

Discussion

An interesting and, to our knowledge, new finding of this study is the demonstration of the ultrastructural localization of galectin-9 in the apical membranous projections of the human endometrial epithelium, called uterodomes, as a dominant morphological feature of the plasma membrane transformation of endometrial epithelium in receptive endometrium. Furthermore, using an indirect immunogold staining for TEM, we compared the galectin-9 distribution pattern between the uterodomes and the uterodome-free area of endometrial epithelial cells.

Considering several parallels in the function of galectins and the regulation of human endometrium prompted some investigators to hypothesize that galectins might also be expressed in the endometrium. In a recent study by von Wolff *et al.* [13] the fingerprinting

of galectin-1 and galectin-3 was reported for the first time in the human endometrial stromal cells and glandular epithelial cell, respectively. In the same year, the same group demonstrated the expression of galectin-9 mRNA in the human endometrium, especially in the endometrial epithelial cells [14]. They introduced galectin-9 as a novel endometrial epithelial marker for the window of implantation, as well as in the early pregnancy decidua. At the protein level, the results of immunohistochemistry and immunoblot for galectin-9 in the current study revealed a strong expression of galectin-9 in the endometrial epithelial cells in the mid- secretory phase of regular menstrual cycle compared to that of the proliferative and early-secretory phases. Our findings enhance the novel findings of the recent report using galectin-9 mRNA analysis that showed a high concentration of galectin-9 specifically in human endometrial glandular and luminal epithelial cells, but not in the stromal or immune cells [14]. Consistently, they showed that galectin-9 mRNA was expressed at very low concentrations during the proliferative and early secretory phases but showed a sharp and significant increase in the mid-secretory phase [14]. Thus, galectin-9 can be considered as one of the very few epithelial markers, such as glycodelin (PP14) [22], that are strictly regulated during the menstrual cycle. A sharp and strong expression of galectin-9 during the window of implantation suggests a potential role of this lectin during embryo implantation. On the basis of a report that the initial step of embryo implantation in humans is similar to that of an inflammatory response [23], in which galectin-9 acts as a mediator of leukocyte adhesion to endothelial cells for their extravasation [11], the expression of galectin-9 in human uterine epithelial cells during the opening of the implantation window could be considered to indicate that it plays a similar role in the adhesion of the floating blastocyst-stage embryo to the uterine wall and its transepithelial migration.

Considering the physiological significance of uterodomes in the initial phases of the embryo implantation and the notion that the apical poles of the luminal epithelium, that is, uterodomes, are the first areas that interact with an embryo, thus initiating the implantation cascade, we next studied the expression pattern of galectin-9 in the human mid-secretory phase endometrium. Although uterodomes are visible under light microscope, other structures could be mistaken for them [24]. Thus, SEM was performed in this study to confirm the presence of uterodomes and TEM immunostaining was carried out to study their molecular properties. Our results revealed a high expression of galectin-9 in the mid-secretory phase uterodomes. Furthermore, the statistical analysis of morphometric results for immunogold TEM in this study revealed a significantly ($p<0.001$) higher area-related numerical density of the galectin-9-bound nanogolds between the mid-secretory phase uterodomes and the uterodome-free areas. Since all the known galectins lack a classical signal sequence and a transmembrane hydrophobic segment, have a cytoplasmic localization, and are secreted as soluble proteins by a nonclassical secretory pathway [25], and based on the fact that uterodomes have a secretory function in human [18], our finding suggests that galectin-9 is released from endometrial epithelial cells at the embryo maternal interface. In this regards, several functions can be attributed to galectin-9 at the embryo-maternal interface. 1) Galectin-9 has been reported to be an important mediator in immunomodulation [26–27]. Endometrial tissue is characterized by a sensitive network of inflammatory mediators, allowing the accumulation of a spectrum of immune cells in the secretory phase [28] and migration of immune cells to the implantation site [29]. Thus, galectin-9 might contribute to the modulation of the endometrial immune system. 2) Galectins of the tandem-repeat type, such as galectin-9, play an important role in the process of cell migration and chemotaxis [5, 26, 30, 31]. Since adequate endometrial function is dependent on leukocyte migration and because implantation is characterized by the accumulation of immune cells around the implantation site, galectin-9 can be expected to play a role in the regulation of endometrial leukocytes. 3) Galectin-9 has β-galactoside-binding domains, which are involved in the adhesive interactions of various cells [32]. Consistently, Hughes [33] showed that galectins affect cell adhesion both as agonists as well as antagonists. It is known that integrin binding to its extracellular ligands is relatively weak and a stronger binding can be achieved by conformational changes of the integrin. Galectins could lead to these conformational changes by binding to extracellular parts of the integrins. This interaction is mostly mediated by galectins of the tandem-repeat type, such as galectin-9 [34–35]. Thus, it would be interesting to find an integrin ligand for galectin-9 in the future studies.

In light of the fact that the complex phenomenon of

implantation is currently considered as the major limiting factor for successful assisted reproduction techniques [36], the identification of the different biomolecules which are important in triggering the implantation cascade and the determination of their expression pattern have a prominent clinical significance. Understanding the regulation and the functions of galectin-9 in the human endometrium will broaden our knowledge of the regulation of endometrial receptivity. We suggest studying the expression pattern of galectin-9 in the endometrium of infertile women as well as establishing a galectin-9 knock-out mouse model, since no such mouse model has been described so far.

Taken together, our findings describe the ultrastructural localization of galectin-9 in the uterodomes of the human endometrium for the first time. Furthermore, the spatial expression pattern of these molecules was exhibited to be different at the uterodomes and the uterodome-free area of the luminal epithelial cells of the receptive endometrium. In conclusion, since galectin-9 expression shows a sharp and significant increase in the mid-secretory phase and the expression of galectin-9 in human uterodomes, this indicates the potential role of uterodomes in the initial phases of embryo implantation in humans.

Acknowledgments

The authors would like to thank Mr. M. Fukuda (Department of Anatomy, Kyorin University School of Medicine) for technical assistance with electron microscopy.

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