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Original Article

Endometrial L-selectin ligand is downregulated in the mid-secretory phase during the menstrual cycle in women with adenomyosis

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ABSTRACT

Objective: Defects in L-selectin ligand (LSL) expression have been reported to cause implantation failure, but little is known about LSL expression in adenomyosis. This study evaluates LSL expression throughout the menstrual cycle in women with adenomyosis.

Materials and methods: Endometrial samples were obtained from reproductive-aged women with adenomyosis who underwent hysterectomy. A total of 42 endometrial biopsies were included. There were 12 women in proliferative phase, 10 in early-secretory phase, 9 in mid-secretory phase, and 11 in late-secretory phase. Immunohistochemistry, western blotting, and RT-PCR were performed to evaluate LSL expression. A non-parametric Kruskal–Wallis one-way analysis of variance with multiple comparisons was performed to examine differences among menstrual phases.

Results: Immunohistochemistry analysis with MECA-79 shows that LSL is expressed with weak intensity in the endometrium in all phases. In the luminal epithelium, MECA-79 reactivity increased from the proliferative to the late-secretory phase but decreased in the mid-secretory phase. There were significant differences in the mean histological scores (HSCOREs) among the proliferative, early-secretory, and late-secretory phases ($p < 0.05$). Five LSL genes were detected in the adenomyotic endometria: *PODXL*, *EMCN*, *CD300LG*, *GLYCAM1*, and *CD34*. The mRNA expression of LSL genes occurred differentially among phases. Moreover, *PODXL* differed significantly among phases ($p < 0.05$).

Conclusions: LSL expressions were downregulated in the luminal epithelium of adenomyotic endometria in the mid-secretory phase. The mRNA expressions of LSL genes also had differential expression patterns throughout the menstrual cycle, especially for *PODXL*. Our study showed that adenomyosis may cause abnormalities of LSL production in the mid-secretory phase, which may contribute to impaired endometrial receptivity and implantation failure.

Introduction

Adenomyosis is a common but benign uterine disease characterized by the presence of ectopic endometrial glands and stroma within the myometrium. In addition, the surrounding myometrium usually reveals hypertrophy [1]. The manifestation of the disease ranges from grossly visible nodules called adenomyoma to smaller forms that are only detectable by microscopy. Adenomyosis affects nearly 20% of the population of females of reproductive age. The most common symptoms are dysmenorrhea, abnormal uterine bleeding, hypermenorrhea, and infertility. Approximately 35% of women with adenomyosis are asymptomatic [2].

The relationship between adenomyosis and infertility is still unclear, but severe adenomyosis has a negative impact on fertility and could impair pregnancy rates when using artificial reproductive techniques [3,4]. Some reports show that women with

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adenomyosis have poorer reproductive outcomes [5,6]. The pathophysiology of infertility associated with adenomyosis is related to gene dysregulation, altered uterine peristaltic activity, altered endometrial function, impaired implantation, altered decidualization, and abnormal concentrations of intrauterine free radicals [7]. Thus far, there has been little molecular data to explain the mechanism behind subfertility and infertility in adenomyosis. Furthermore, little is known about the effects of adenomyosis on known biomarkers of endometrial receptivity, such as L-selectin ligand (LSL).

LSLs are glycoproteins that bind to L-selectin (CD62P) on leukocytes in high endothelial venules (HEVs) of the lymph nodes [8]. Interestingly, evidence indicates that the initial attachment of an embryo to the endometrium depends on the binding of L-selectin expressed by the trophoblast and oligosaccharide-based ligands expressed by the endometrium [9,10]. More and more evidence is revealing that the expression of LSL might reflect endometrial receptivity [11]. In our previous study, LSL was expressed differentially between different phases of the natural cycle in the human endometrium. It was upregulated in the secretory phase and downregulated in the proliferative phase. The expression of LSL reached its highest level in the luminal epithelium in the mid-secretory phase, which coincides with the window of implantation [12]. Another study of our team revealed that oocyte donors had lower LSL expression than controls on cycle day 19 in the luminal epithelium and from cycle days 19–24 in the glandular epithelium. This time frame corresponds with the implantation window [13]. These results suggest that controlled ovarian hyperstimulation is associated with reduced LSL expression in the secretory phase, particularly during the implantation window.

Defects in LSL expression lead to implantation failure and early pregnancy loss [9]. Recently, a study showed that the absence of LSL during the implantation window in the secretory endometrium could lead to recurrent implantation failure (RIF) in patients who suffer from unexplained infertility [14]. A screening test for RIF's patients who lack LSL had a predictive value of 100% with 50% sensitivity and 100% specificity. The positive predictive value was 100%, and the negative predictive value was 87%. The study concluded that LSL plays a vital role in early human implantation. Screening for the absence of LSL might help many patients with RIF to avoid repeatedly undergoing failed treatment cycles [14].

To date, little is known about the patterns of LSL distribution in the different phases during the menstrual cycle in adenomyosis. In addition, little is known about whether adenomyosis impairs LSL expression in the human endometrium. Therefore, this study evaluates the expression pattern of LSL in different phases during the menstrual cycle in adenomyosis and explores the mRNA expressions of LSL genes in these phases. We hypothesized that adenomyosis could impair endometrial LSL expression in the mid-secretory phase (the period of embryo implantation).

Materials and methods

All procedures conformed to the Declaration of Helsinki for research involving human subjects. The Institutional Review Board of Cathay General Hospital approved the use of human specimens (*CGHIRB No.:* CT9681). Formal informed consent was obtained from all patients before sample collection. The medical records of all patients were reviewed retrospectively.

Sample collection

Participants were included using the following criteria: 1) age ranging from 35 to 50 years; 2) regular menstrual cycle (28–35 days); 3) body mass index (BMI) less than 28; 4) no hormone therapy within at least 2 months before surgery; 5) no other gynecological diseases, such as pelvic inflammatory disease (PID), cancers, endometrial hyperplasia, submucosal myoma or endometrial polyps; 6) and no sexually transmitted diseases (STD). Patients were excluded from the study if they had 1) pregnancy, 2) coagulopathy, 3) psychological diseases, or 4) any other apparent reproductive tract pathology.

Endometrium tissue samples were collected from women with adenomyosis who underwent hysterectomy from August 2008 to July 2009. Samples with evidence of endometritis, endometrial polyps, endometrial hyperplasia, or other endometrial pathologies were excluded. There were 42 endometrial biopsies, which included 12 from the proliferative phase (days 7–14), 10 from the early-secretory phase (days 15–18), 9 from the mid-secretory phase (days 19–24), and 11 from the late-secretory phase (days $\geqq$ 25). In addition, 11 endometrial samples were obtained to compare the LSL mRNA expression from menopausal women with uterine prolapse who underwent vaginal hysterectomy. Tissue samples were frozen at −80 °C until immunohistochemistry analyses and RNA isolation.

Immunohistochemistry

One portion of the obtained tissues was fixed in buffered formalin and evaluated by a pathologist with experience in endometrial dating. Separate dating of the luminal and glandular epithelium was carried out according to the criteria reported by Noyes et al. [15]. A second portion of each biopsy sample was used to investigate the expression of LSL by immunohistochemistry. The expression of LSL was examined by immunolocalization with rat monoclonal antibody MECA-79 (BD Biosciences, San Jose, CA), which recognizes a high-affinity LSL carbohydrate epitope containing SO3 → 6GlcNAc [16].

The assay was performed as described previously [12,17]. Briefly, the samples were fixed in 10% buffered formaldehyde for 24 h and embedded in paraffin. Paraffin sections with thicknesses of 3–4 mm were prepared on positively charged slides prior to the immunohistochemical analysis. The sections were dewaxed with xylene, followed by descending grades of methanol solutions to distilled water. They were then pretreated at 90° for 20 min with Citra Buffer (Vector H3300, Vector Laboratories, Burlingame, CA) in a steamer (HA900; Black & Decker, Hampstead, MD).

The tissue sections were labeled with MECA-79 at a concentration of 3.3 μg/mL using a dilution of 1:30 in phosphate-buffered saline (PBS). Positive and negative (no antibody) controls were established using a section of tonsil and a section of endometrium tissue, respectively. For antigen retrieval, the slides were incubated in CC1 buffer (Ventana) for 1 h on plates heated at 100 °C with a Benchmark XT processor. The primary antibody incubation was performed for 32 min at a dilution of 1:30 and 37 °C. Positive binding of MECA-79 was detected by biotinylated rabbit anti-mouse secondary antibody (at a dilution of 1:800 with PBS), which cross-reacts with the rat primary antibody.

After the hybridization of primary and secondary antibodies, the HRP conjugated avidin-biotin peroxidase (ABC) complex was analyzed using a Ventana DAB Detection Kit (Ventana-Biotek Solutions Inc., Tucson, AZ). The slides were counterstained with hematoxylin, dehydrated, cleared, and mounted in DPX mountant. Finally, they were evaluated using an Eclipse 80i optical microscope (Nikon, Tokyo, Japan). The intensity of staining of the antibody was then analyzed in both the glandular and luminal epithelium by a semi-quantitative histological scores (HSCOREs) system [18]. HSCOREs for the tissue section were derived as the sum of the component HSCOREs weighted by the fraction of each component observed in the tissue section. The HSCOREs were calculated using