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Expression of toll-like receptors 2, 3, 4, and 9 genes in the human endometrium during the menstrual cycle

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Abstract

Innate immunity in the endometrium has fundamental significance for reproduction. Although toll-like receptors (TLRs) play central roles in innate immune responses, their expression in the human endometrium remains to be fully elucidated. We have examined the gene expression of TLR2, TLR3, TLR4, and TLR9 in endometrial tissues by real-time quantitative PCR and *in situ* hybridization. The expression levels of the four genes in endometrial tissues varied in a similar pattern during the menstrual cycle; the levels were high in the perimenstrual period and low in the periovulatory period. Expression of the four genes was detected in both epithelial cells and stromal cells throughout the menstrual cycle. Expression levels were higher in epithelial cells for TLR3 and in stromal cells for TLR4, while they were comparable in epithelial cells and stromal cells for TLR2 and TLR9. These findings imply that differential spatio-temporal expression patterns of TLRs subserve proper innate immunity of the endometrium.

Keywords: Toll-like receptor; Endometrium; Expression; *In situ* hybridization

1. Introduction

The endometrium is a pivotal component of the reproductive organs, which nourishes implanting embryos. However, it is vulnerable to the spread of microorganisms from the vagina and cervix, resulting in endometrial infection that may impair normal fecundity. Thus, protective mechanisms against unwanted infection should be inherent in the endometrium to achieve successful reproduction.

Toll-like receptors (TLRs) are central regulators of innate immune responses (Takeda et al., 2003). Currently, 10 different human TLRs have been described, and each TLR recognizes distinct pathogen-associated molecular patterns. TLRs are thought to be important for host defense against external pathogens in mucosal systems, such as the intestinal and respiratory tracts. We have previously demonstrated the presence and possible implication of TLR4, a receptor for LPS, in the human endometrium (Hirata et al., 2005).

Other TLRs have also been shown to be present in the human endometrium. Young et al. (2004) detected TLR1–6 and TLR9 mRNA in both human endometrial tissues and separated endometrial epithelial cells, while Schaefer et al. (2005) demonstrated the expression of TLR1–9 mRNA in cultured human endometrial epithelial cells. An immunohistochemical study showed the presence of TLR1–6 in various locations in the human

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female reproductive tract, including the endometrium (Fazeli et al., 2005).

The endometrium changes its gene profile during the menstrual cycle under the influence of ovarian hormones. Accordingly, it is feasible that immunity in the endometrium alters during the menstrual cycle. However, changes of TLR expression in the endometrium during the menstrual cycle remain to be elucidated. Bacteria and viruses are common pathogens that could have contact with the endometrium. We hypothesized that proper innate immune system against these pathogens in human endometrium work with the help of TLR2, TLR3, TLR4, and TLR9 that recognize peptidoglycan from Gram-positive bacteria (Yoshimura et al., 1999), viral double-stranded RNA (Alexopoulou et al., 2001), LPS from Gram-negative bacteria (Poltorak et al., 1998) and unmethylated CpG DNA in bacterial genomes (Hemmi et al., 2000), respectively. In the present study, therefore, we have examined the gene expression profile of TLR2, TLR3, TLR4, and TLR9 through the menstrual cycle. We addressed their variation during the menstrual cycle using real-time quantitative RT-PCR and *in situ* hybridization.

2. Materials and methods

2.1. Patients and samples

Endometrial tissues were obtained from patients undergoing operations for benign gynecological conditions. All patients had regular menstrual cycles, and none had received hormonal treatment at least 6 months before surgery. Specimens were dated according to the criteria of Noyes et al. (1950) and classified as early proliferative, mid-proliferative, late proliferative, early secretory, mid-secretory, and late secretory phases. Tissues for mRNA extractions were snap-frozen in liquid nitrogen and stored at −80 °C. Tissues (5 mm × 5 mm) for *in situ* hybridization were fixed in 10% neutral buffered formalin for 16 h, stored in 70% ethanol and wax embedded. The experimental procedures were approved by the institutional review board of University of Tokyo, and signed informed consent for use of the endometrial tissue was obtained from each woman.

2.2. Isolation and culture of human endometrial stromal and epithelial cells

Isolation and culture of human endometrial stromal cells (ESC) and epithelial cells (EEC) was processed as described previously (Hirata et al., 2005; Koga et al., 2001). Fresh endometrial biopsy specimens collected in sterile medium were rinsed to remove blood cells. Tissues were minced into small pieces and incubated in DMEM/F-12 containing type1 collagenase (0.25%) and deoxynuclease1 (15 U/ml) for 120 min at 37 °C. The resultant dispersed endometrial cells were separated by filtration through a 40 μm nylon cell strainer (Becton Dickinson and Co., Franklin Lakes, NJ). Endometrial epithelial glands that remained intact were retained by the strainer, whereas dispersed ESC passed through the strainer into the filtrate.

ESC in the filtrate were collected by centrifugation, and resuspended in phenol red-free DMEM/F-12 containing 10% charcoal-stripped fetal bovine serum (FBS), 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B. ESC were seeded in a 100 mm culture plate and kept at 37 °C in a humidified 5% CO_2/95% air atmosphere. At the first passage, cells were plated into 12-well culture plates (Becton Dickinson and Co.) at a density of 2×10^5 cells/ml. Cells, reaching confluence in 2 or 3 days were used for the experiments.

EEC were collected by backwashing the strainer with DMEM/F-12 containing 10% charcoal-stripped FBS, seeded in a 100 mm plate and incubated at 37 °C for 60 min to allow contaminated ESC to attach to the plate wall. The non-attached EEC were recovered and cultured in the culture medium at a density of 2×10^5 cells/ml into 12-well culture plates. Cells which reached confluence in 3 or 4 days were used for experiments. The purity of both the stromal and epithelial cell preparations was more than 95% as judged by positive cellular staining for vimentin or cytokeratin, respectively.

2.3. Treatment of cells

To examine effects of hormone on TLR2, TLR3, TLR4, or TLR9 mRNA expression, EEC and ESC were incubated with 2.5% charcoal-stripped FBS in the presence of 10 ng/ml (36.7 nM) estradiol (E), 100 ng/ml (318 nM) progesterone (P), E plus P (EP), or 0.1% ethanol vehicle (control). EEC were incubated with hormone treatment for 24 and 48 h. ESC were incubated for 3, 8, 24, 48, and 72 h.

2.4. RNA extraction, RT and real-time quantitative PCR of TLR2, TLR3, TLR4, and TLR9

Using an RNeasy Mini kit (QIAGEN, Hilden, Germany), total RNA was extracted from biopsies or cultured cells. One microgram of total RNA was reverse-transcribed in a 20 μl volume using an RT-PCR kit (TOYOBO, Osaka, Japan). Standard PCR was