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Expression of Toll-like Receptors in Human Endometrial Epithelial Cells and Cell Lines

Steven L. Young^{1,2,3}, Terri D. Lyddon¹, Rebecca L. Jorgenson², and Michael L. Misfeldt²

¹Department of Obstetrics and Gynecology, School of Medicine, University of Missouri

²Department of Molecular Microbiology and Immunology, School of Medicine, University of Missouri

³Department of Animal Sciences, University of Missouri, Columbia, MO, USA

Abstract

PROBLEM—Are toll-like receptors (TLRs) expressed by human endometrium and endometrial cell lines?

METHODS OF STUDY—Expression of each TLR mRNA species was determined by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of proliferative-phase human endometrium, separated endometrial epithelial cells, and the Ishikawa and RL95-2 endometrial epithelial cell lines. U-937 and SKW 6.4 cell lines were used as positive controls. Functional relevance of PCR findings was tested by enzyme-linked immunosorbent assay (ELISA) analysis of IL-8 production after stimulating cell lines with ligands for TLR2-5.

RESULTS—TLR1-6 and 9 mRNA species were detected in both whole endometrium and separated endometrial epithelial cells. Ishikawa cells expressed TLR2 and TLR5, while RL95-2 cells expressed TLR3, 5, and 9. Response of RL95-2, Ishikawa, and U-937 cells to TLR2-5 ligands was consistent with RT-PCR findings except response to flagellin by Ishikawa cells.

CONCLUSION—These studies provide the first evidence of TLR expression in the endometrium of any species and suggest the usefulness of endometrial cell lines to study TLR function.

Keywords

Cytokine; endometrium; interleukin 8; Toll-like receptor 3; Toll-like receptor 4; Toll-like receptor

INTRODUCTION

Cytokines and chemokines (chemotactic cytokines) were originally described as paracrine and autocrine mediators of immune cell function. More recently, investigators have established endometrial epithelium and stroma as rich sources of cytokine expression and important targets of cytokine action.¹ Evidence suggests that endometrial cytokines can regulate reproductive and immune functions, including embryo implantation, immune tolerance of the embryo, host defense, and clearance of menstrual material. Furthermore, abnormal endometrial cytokine expression may be pathogenetic in disorders including recurrent pregnancy loss, pre-term labor, and endometriosis.²

Toll-like receptors (TLRs) are a newly discovered family of integral membrane receptors, which can stimulate cytokine and chemokine production after ligand recognition.³ The 10

Correspondence to: Steven L. Young.

Address reprint requests to Steven L. Young, Department of Obstetrics and Gynecology, University of Missouri School of Medicine, N625 HSC, One Hospital Drive, Columbia, MO, USA. E-mail: youngst@health.missouri.edu.

known TLRs (TLR1-10) comprise a family of structurally related receptors that recognize specific products of bacteria, fungi, parasites, and viruses as well as endogenous ligands associated with cell damage.⁴ Despite the central importance of cytokines and chemokines in endometrial function, no published reports have examined the expression or function of TLRs in the endometrium of any species.

Of the 10 TLR species, natural ligands have been identified for TLR1-6 and 9. TLR1 and 6 are only known to act in conjunction with TLR2. The goal of the described experiments was to examine the expression of each of the 10 known TLR mRNA species, and the related molecule CD14, in whole human endometrium, separated endometrial epithelial cells, and endometrial epithelial cell lines. The functional relevance of the reverse transcriptase-polymerase chain reaction (RT-PCR) results was tested by assessing endometrial cell line production of interleukin-8 (IL-8) in response to treatment with ligands for TLR2-5.

MATERIALS AND METHODS

Cells and Culture Conditions

Whole endometrium and separated epithelial and stromal cells were generous gifts from Dr Kathy Timms (Columbia, MO, USA) and Dr Bruce Lessey (Greenville Hospital, Greenville, SC, USA). In both cases tissue was obtained with informed consent of subjects using IRB approved protocols and the cells were separated as described.⁵ Subjects were previously fertile women of reproductive age undergoing tubal sterilization less than 14 days from their previous onset of menses. All subjects were free of any known disease of reproductive tissues and no evidence of disease within the peritoneal cavity was seen during laparoscopy. No subjects were taking any hormonal medication. Cytokeratin and vimentin immunostaining has been used by both laboratories to assess purity of cell preparations and has typically demonstrated greater than 98% purity. Sample purity was further assessed in our laboratory by RT-PCR for CD45 (a pan-leukocyte marker) using conditions listed below.

Ishikawa cells were initially supplied by Dr Bruce Lessey (Greenville Hospital, Greenville, SC, USA) and were cultured in MEM supplemented with 10% fetal bovine serum (FBS), 12 mM L-glutamine, and 100 mcg/mL gentamycin. RL95-2 cells were purchased from ATCC (Manassas, VA, USA) and were cultured in DMEM/F12 without phenol red, supplemented with 10% FBS, 12 mM L-glutamine, and 100 mcg/mL gentamycin. U-937 and SKW 6.4 cells were obtained from ATCC and cultured in RPMI 1640 supplemented with 10% FBS, 12 mM L-glutamine, 1 mM Na pyruvate, 0.5 mM beta-mercaptoethanol, and 100 mcg/mL gentamycin. Cell pellets were stored in RNeasy lysis buffer (Qiagen, Crawfordsville, IN, USA) for subsequent RNA isolation.

The TLR3 active ligand [Poly(I:C)] and inactive control [poly(dI:dC)] were obtained from Amersham (Piscataway, NJ, USA). Purified lipopolysaccharide (LPS), phorbol myristate acetate (PMA), and ionomycin (I) were obtained from Calbiochem (San Diego, CA, USA). Highly purified *S. aureus* peptidoglycan was obtained from FLUKA (now Sigma, St Louis, MO, USA) and highly purified *S. typhimurium* flagellin (F) was obtained from Apotech (Epalinges, Switzerland).

RNA Isolation and Reverse Transcription

The conditions of RNA isolation, cDNA generation, and PCR amplification were highly standardized to allow qualitative comparison between experiments. Total RNA was isolated using RNeasy lysis buffer (Qiagen, Crawfordsville, IN, USA) per manufacturers instructions except that the DNase I treatment was performed twice and second DNase treatment was performed with twice the concentration of DNase to further decrease contaminating genomic

DNA. The second DNase step was needed with some samples to remove trace amounts of contaminating genomic DNA detected by bands in the 'no reverse transcriptase' (no-RT) control lanes for the primer sets which do not span an intron (TLR1, 6, 8, and 10). RNA was quantitated using RiboGreen and the supplied ribosomal RNA standard (RiboGreen[®] RNA Quantitation Kit; Molecular Probes, Eugene, OR, USA) read by epifluorescence in a 96-well format (Fusion Plate Reader; Perkin-Elmer, Wellesley, MA, USA). First-strand cDNA was produced from 100 ng of total RNA using First Strand cDNA Synthesis Kit and the provided random hexamer primers in a total reaction volume of 20 μ L according to the manufacturer's instructions (Roche, Indianapolis, IN, USA).

PCR and Electrophoresis Conditions

Hot-start PCR was performed in a 25 μ L volume using 200 nM forward and reverse primers, 1 μ L first strand cDNA, 3 mM MgCl₂, 1.25 U AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA) with the provided buffer. dUTP was used in place of dTTP and 0.05 U of uracil N-glycosylase (Epicentre, Madison, WI, USA) was used to prevent false positives from carry over. Primers for TLR1,⁶ TLR3,⁶ TLR5,⁶ TLR8,⁷ CD14,⁸ β -glucuronidase (GUS) (D. Doueck, Personal Communication), protein kinase R (PKR),⁹ and CD45¹⁰ were obtained from the literature. All other primer sequences were designed using Primer 3 software (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). All PCR reactions were conducted on a Mastercycler Gradient thermocycler (Brinkmann, Westbury, NY, USA) and utilized 30 cycles of amplification and empirically-derived annealing temperatures shown in Table I. The primers for TLR5 and TLR9 were also run at 32–35 cycles because of light bands at 30 cycles and in all cases the findings at increased cycle numbers confirmed the findings at 30 cycles.

The PCR product(s) were separated by electrophoresis in a 2% agarose gel (3:1 agarose; Amresco, Solon, OH, USA) at 120–140 volts followed by staining with 1:10,000 dilution of SYBR[®] Green (BioWhittaker, Rockland, ME, USA). A 100 bp ladder (Invitrogen, Carlsbad, CA, USA) was included with all experiments to confirm the expected band size. The bands were visualized by ultraviolet transillumination at 302 nm and digital images were obtained using a GelLogic 100 (Kodak, Rochester, NY, USA). In order to score relative expression, samples to be compared were run on the same gel and digital images were scored by eye as intense (++), weak to moderate (+), or absent (–). In some cases, three samples were similar with one outlier and the score was based on the three similar ones.

IL-8 ELISA

IL-8 quantitation was performed using a commercial IL-8 sandwich enzyme-linked immunosorbent assay (ELISA) DuoSet (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. In our hands, the ELISA assay had a sensitivity less than or equal to 16 pg/mL (the lowest standard used in the standard curve). Variability between separate samples run in parallel was minimal as shown in the error bars in Fig. 2. The data were obtained by measuring A₄₅₀ using a 190+ plate reader with SoftMAX Pro software (Molecular Devices, Sunnyvale, CA, USA).

RESULTS

RNA was extracted from whole human endometrium, separated epithelial and stromal cells, the human endometrial epithelial cell lines, Ishikawa and RL95-2, a human monocyte cell line, U-937, and a human B lymphocyte line SKW 6.4. The RNA samples were reverse transcribed and the first strand cDNA was subjected to PCR for TLR1-10 and the related molecule CD14 using the primers and conditions described in Table I.

Expression of each TLR species was examined for at least four independent proliferative-phase endometrial specimens and each experiment was repeated at least twice. Each experiment also included a no-RT control for each sample and a water control for each primer pair. The first strand cDNA was also subjected to RT-PCR detection of mRNA coding for two control genes, CD45 and GUS. The CD45 primers used allow detection of all known isoforms of CD45 and were used to assess for RNA from leukocytes which could potentially contaminate the specimen. Primers for the constitutively expressed 'housekeeping' gene, GUS, were used to control for RT efficiency and pipetting error. GUS was chosen as a control because limited variability was seen across specimens examined in these studies and in other preliminary studies using other cycle days (data not shown). The variability was assessed using both endpoint and real-time RT-PCR.

All stromal samples had detectable levels of CD45, precluding the use of RT-PCR to distinguish between TLR expression by stromal cells and resident endometrial leukocytes (data not shown). Therefore, the data from stromal cells are not reported. The epithelial samples were variable for CD45 signal, but included samples with complete absence of CD45 signal and the results reported are consistent with samples lacking CD45 signal.

An example of PCR results for TLR3, TLR4, and the constitutive positive control gene, GUS, is shown in Fig. 1. The results demonstrated expression of TLR3 in proliferative-phase, whole endometrial specimens and separated endometrial epithelial cells. Among the epithelial cell lines, marked TLR3 expression was detected in RL95-2 cells, but no expression was detected in Ishikawa cells. As expected U-937 cells did not express detectable levels of TLR3.¹¹⁻¹³ The pattern of TLR4 expression was distinct from the pattern seen with TLR3. TLR4 was expressed by whole endometrium, separated epithelium, and U-937 cells, but no expression by RL95-2 or Ishikawa cells was detected.

A summary of findings using primer sets for all of the TLRs is shown in Table II. The summaries of primary endometrial tissue and cells represent evaluation of at least four independent proliferative-phase endometrial samples. Definitive expression of TLR1, 2, 3, 4, 5, 6, and 9 was detected in whole endometrium. Endometrial epithelium expressed the same TLR species, but qualitatively appeared to have reduced expression of TLR5 and TLR6.

Each epithelial cell line expressed a distinct subset of the TLRs expressed by endometrial epithelium. RL95-2 and Ishikawa cells were concordant for expression of TLR5 and lack of expression of TLR4, 6, 7, 8, 9, and 10. Endometrial epithelial cell line expression of TLR2 was detected only in Ishikawa cells, while expression of TLR3 was detected only in RL95-2 cells. Expression of CD14, a molecule structurally-related to the external domain of TLRs, was also examined because it functions as a co-receptor with TLR4 for LPS. CD14 expression was detected in whole endometrium as well as purified endometrial epithelium. Expression was present at low levels in RL95-2, SKW 6.4, and U-937 cells and absent in Ishikawa cells.

In order to test the functional significance of PCR detection of mRNA, the response to specific TLR ligands was tested in the RL95-2, Ishikawa and U-937 cell lines. Figure 2 demonstrates the production of IL-8 from each cell line in response to treatment with a TLR3 agonist, double-stranded RNA [poly(I:C)], a TLR4 agonist, LPS, and a TLR5 agonist, *S. typhimurium* flagellin. Treatment with poly(dI:dC), a dsDNA, was used as a negative control for non-specific nucleic acid effects and treatment with PMA (10 ng/mL) and I (500 ng/mL) was used as a positive control stimulus for IL-8 production.

IL-8 production by RL95-2 cells was induced by treatment with poly(I:C) and flagellin, but not LPS (Fig. 2A), consistent with the expression of TLR3 and TLR5 and lack of expression of TLR4. Production of IL-8 by U-937 cells (Fig. 2B) was induced by treatment with LPS, but not poly(I:C), consistent with the expression of TLR4 and lack of expression of TLR3. Ishikawa

cell production of IL-8 could not be induced by poly(I:C), LPS, or flagellin treatment, consistent with the lack of expression of both TLR3 and TLR4, but inconsistent with demonstrated expression of TLR5. Ishikawa cell production of IL-8, however, could be detected after treatment with PMA and I.

Since TLR2 acts as a heterodimer with either TLR1 or TLR6, expression of TLR2 without TLR1 or 6 by Ishikawa cells and absent expression of TLR2 by RL95-2 cells would predict lack of response to the TLR2 ligand, peptidoglycan by both cell lines. No response of Ishikawa or RL95-2 cells to peptidoglycan could be demonstrated (data not shown).

Since it has been suggested that PKR can also act as a receptor for dsRNA,¹⁴⁻¹⁶ we sought to determine whether the difference in the observed response to dsRNA by the cell lines could be explained by differential expression of PKR. RT-PCR detection of PKR mRNA expression in RL95-2, Ishikawa, and U-937 cells was demonstrated (Fig. 3). Since each cell line clearly expressed PKR mRNA, and only RL95-2 cells expressed TLR3 mRNA, the IL-8 response of the cell lines to dsRNA was likely due to TLR3 and not PKR.

DISCUSSION

Endometrial epithelium is the first uterine cell layer likely to encounter microbes ascending the female reproductive tract. Thus, we hypothesized that TLRs, known to function as innate pathogen detectors, should be expressed by endometrial epithelium. The data that purified endometrial epithelial cells express TLR1-6 and 9 supports the hypothesis. The validity of the PCR data is enhanced by close correlation between PCR and functional outcome of treatment with ligands for TLR 2, 3, and 4 in the cell lines.

Both whole endometrial samples and purified epithelium lack detectable mRNA expression for TLR 7, 8, and 10. Since B-cells are the predominant cell type that expresses TLR10, the absence of endometrial TLR10 expression is consistent with data suggesting that B-cells are rare in endometrium.^{17,18} To our knowledge, no studies have previously examined expression of TLR7 or TLR8 in epithelial cells, nor have any published data described non-pharmacologic ligands for these receptors. Thus, implications of the lack of TLR7 and TLR8 expression in human endometrium are unclear.¹⁹

TLR4 expression in primary endometrial epithelial cells contrasts with lack of expression in cervicovaginal epithelium.²⁰ A potential explanation of this difference is that gram negative bacteria in the upper genital tract are likely to be associated with infection, whereas bacteria are commonly found as commensal organisms in the lower genital tract. Thus, TLR4 expression by vaginal epithelium might cause unnecessary inflammation due to the presence of commensal organisms. An analogous situation is found in gut epithelium, where expression of TLR4 is limited to areas which do not encounter commensal bacteria.²¹ Alternatively, differences between the two cell types may represent effects of endometrial separation and/or long-term culture of cervicovaginal cells.

Our finding of TLR5 expression by endometrial epithelium is consistent with TLR5 expression by multiple epithelial cell types, including corneal, gastric and intestinal.²²⁻²⁴ Although mRNA for TLR5 was expressed by both RL95-2 and Ishikawa cells, IL-8 production in response to flagellin was detected only in RL95-2 cells. The reason for Ishikawa cell unresponsiveness to flagellin remains unclear. The lack of responsiveness is not due to the inability of the cells to produce IL-8 as demonstrated by PMA/I treatment. The lack of Ishikawa response is also not due to a generalized absence of TLR signaling molecules since transfection of Ishikawa cells with a TLR3 expression plasmid confers TLR3 response (R. Jorgenson, personal communication). Interestingly, a recent study demonstrated that some responses to *Salmonella* flagellin require both TLR4 and TLR5.²⁵ Thus, the lack of Ishikawa response may

involve alteration in a signaling mechanism other than that used by TLR3, lack of interaction of TLR5 with another TLR species, or an alteration in TLR5 sequence or expression not detected by these studies.

U-937 cells have been shown to express TLR2 and TLR4 mRNA under basal conditions.^{26, 27} Our data confirm the findings of TLR2 and TLR4 expression and extend the observations to the other known TLR species.

The findings regarding TLR3 expression and function are unique in many respects. No previous study, to our knowledge, has demonstrated an endometrial epithelial response to dsRNA. Furthermore, TLR3 expression has only been described in a handful of cell types, including immature dendritic cells, intestinal epithelial cell lines, glial cells, and cultured cervical and vaginal cells.^{8,11,12,20,28,29} The only cell line which has been reported to contain natively-expressed, functional TLR3 is the MRC5 lung fibroblast line.³⁰ Thus RL95-2 cells, provide a second model system to study TLR3 expression and function. RL95-2 cells also lack TLR4 expression and endotoxin (LPS) response. Therefore, studies of TLR3 effects in RL95-2 cells would be relatively free of effects of endotoxin, whose presence as a possible contaminant has generated concerns in the study of other TLR ligands.⁴

The ligand for TLR3, dsRNA, is produced by virtually all viruses at some point in their life cycle and our data demonstrating TLR3 expression and function suggest a previously unappreciated role for endometrial epithelium in viral defense. The chemokine produced in response to TLR3 stimulation, IL-8, is thought to play an important role both as a chemoattractant for monocytes and neutrophils and as an important factor in endometriosis pathogenesis,³¹ suggesting a potential role for TLR3 in endometriosis pathogenesis and endometrial inflammation.

This paper, to our knowledge, provides the first description of expression of any TLR in the endometrium of any species. Expression of multiple TLR mRNA species by endometrial epithelium suggests a role for TLRs in regulating endometrial cytokine and chemokine expression and therefore a potential role in the regulation of endometrial immune and reproductive functions.

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Fig. 1.

RT-PCR Detection of TLR3, TLR4, and GUS. Whole endometrium, separated epithelial cells, and relevant cell lines were analyzed for TLR3, TLR4, and GUS mRNA by RT-PCR. In lanes designated with a plus sign, reverse transcriptase (RT) was used to generate first-strand cDNA; while lanes designated with a minus sign represent identical reactions omitting the reverse transcriptase enzyme. Whole endometrium and separated endometrial cells are represented by two independent samples. H designates a control where water replaces the first strand cDNA. The expected sizes of the PCR products are TLR3, 305 bp; TLR4, 201 bp; and GUS, 83 bp. The molecular weight markers (M) are a 100 bp ladder with a stronger staining 600 bp fragment. RL95, RL95-2 cells; Ishi, Ishikawa Cells.

Fig. 2.

Expression of IL-8 in Response to TLR Ligands. Untreated cells (U) or those treated with 10 mcg/mL poly(I:C) (dsRNA), 10 mcg/mL poly(dI:dC) (dsDNA), 100 ng/mL LPS, 50 ng/mL F, or 10 ng/mL PMA and 500 ng/mL I (PMA/I). For each treatment, media was collected after 24 hr from three separate cultures of RL95-2 (A), Ishikawa (B), and U-937 (C) cells and was analyzed for IL-8 content by ELISA. Data were analyzed by one way analysis of variance with Tukey's *post-hoc* test. * $P < 0.05$ as compared with U. Pharmacologic treatment (PMA/I) was analyzed separately from ligand treatments as the large stimulation with PMA/I otherwise skewed the sample from normality.

Fig. 3.

Detection of Protein Kinase R (PKR) mRNA in RL95-2, U-937, and Ishikawa Cells. The expected band size is 301 base pairs. In lanes designated with a plus sign, reverse transcriptase (RT) was used to generate first-strand cDNA; while lanes designated with a minus sign represent identical reactions omitting the reverse transcriptase enzyme. The molecular weight markers (M) are a 100 bp ladder with a stronger staining 600 bp fragment. RL95, RL95-2 cells; Ishi, Ishikawa Cells.

Table I
PCR Primers

	Forward (5'-3')	Reverse (5'-3')	Product size (bp)	Anneal (°C)
TLR1	CTATACACCAAGTTGTCAGC	GTCTCCAACCTCAGTAAGGTG	220	61
TLR2	GTACCTGTGGGGCTCATTGT	CTGCCCTTGCAGATACCATT	191	62
TLR3	GATCTGTCTCATAATGGCTTG	GACAGATTCCGAATGCTTGTG	305	60
TLR4	ACAACCTCCCCTTCTCAACC	AACTCTGGATGGGGTTTCCT	201	61
TLR5	CTAGCTCCTAATCCTGATG	CCATGTGAAGTCTTTGCTGC	438	59
TLR6	AGGTGCCTCCATTATCCTCA	GAATCCATTGGGAAAGCAG	211	59
TLR7	CATGCTCTGCTCTCTTCAACC	CGATCACATGGTTCTTTGGA	201	59
TLR8	GCCAGCGAGTCTCACTGAACCT	GCCAGGGCAGCCAACATA	558	61
TLR9	TTCCCTGTAGCTGCTGTCC	ACAGCCAGTTGCAGTTCACC	207	58
TLR10	GGCCAGAACTGTGGTCAAT	CTGCATCCAGGGAGATCAGT	199	61
CD14	GGTGCCGCTGTGTAGGAAAGA	GGTCCTCGAGCGTCAGTTCCT	454	66
GUS	CTCATTTGGAATTTTGCCGATT	CCGAGTGAAGATCCCCTTTTTA	83	60
PKR	GCCTTTTCATCCAAATGGAATTC	GAAATCTGTTCTGGGCTCATG	301	60
CD45	GGCAAAGCCCAACACCTT	TGTGGTTGAAATGACAGC	95-577 ^a	60

Anneal, annealing temperature; GUS, β -glucuronidase; PKR, protein kinase R.

^aBand size varies depending on isoform.

Table II
Expression of TLR mRNAs by proliferative phase endometrial epithelium and cell lines^a

	TLR1	TLR2	TLR3	TLR4	TLR5	TLR6	TLR7	TLR8	TLR9	TLR10	CD14	GUS
Whole endometrium	+	++	+	+	++	++	-	-	+	-	++	+
Endometrial epithelium	+	++	+	+	+	+	-	-	+	-	+	+
RL95-2	-	-	++	-	++	-	-	-	+	-	+	+
Ishikawa	-	+	-	-	+	-	-	-	-	-	-	+
U-937	-	+	-	++	+	-	-	-	+	-	+	+
SKW 6.4	+	-	+	+	-	++	++	-	+	++	+	+

^a subjective staining intensity of PCR bands: -, no band detected; +, weakly to moderately stained band; ++, intensely stained PCR band.