FULL PAPER *Anatomy*

Gene Transcriptions of Toll-Like Receptors in the Mouse Uterus during Gestation

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ABSTRACT. Toll-like receptors (TLRs) play critical roles in innate immunity by recognizing a broad range of microbial components as ligands. The activation of TLRs is an important step not only for the innate immune response, but also for the development of the subsequent antigen-specific adaptive immune response. However, little is known about TLR expression in the female genital mucosa during gestation. In the present study, gene transcriptions of TLRs 1 to 9 were investigated in both the mesometrial side and the anti-mesometrial side of the uterus during gestation in the mouse reproductive organ during the gestation period. In the mesometrial side, gene transcriptions of TLR 1, 3, 4 and 9 were decreased in the late gestation period, whereas an increase of gene transcriptions of TLR 4 and 9 was seen in the early gestation period. In the anti-mesometrial side, gene transcriptions of TLR 1 and 9 were also decreased in the late gestation period, and TLR 9 gene transcription was increased in the early gestation period. On the other hand, gene transcriptions of TLR 3 and 4 were not changed in the late gestation period, but they were increased in the early gestation period. Gene transcriptions of TLR 2, 5, 6, 7 and 8 were not changed statistically in either side during the gestation period. These results suggest that the expressions of particular TLRs may be regulated in the uterus during the gestation period to maintain the pregnant state.
KEY WORDS: innate immunity, mouse, pregnancy, toll-like receptor, uterus.

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The female reproductive organ assumes a special role during pregnancy. The endometrium has to tolerate fetuses during the gestation period to avoid harm from the maternal immune system, and the mucosal immune system of the endometrium has to simultaneously prevent the invasion of external microorganisms. The mammalian immune system is classified into 2 categories, innate and adaptive immunity, and innate immunity is an immunological first line of defense for mucosal immunity [9, 24]. Toll-like receptors (TLRs) play critical roles in innate immunity by recognizing a broad range of microbial components as ligands, and activation of TLRs is an important step in the development of the subsequent antigen-specific adaptive immune response [10, 18, 25]. TLRs comprise a large family of at least 15 members in mammals, and 10 and 13 TLRs have been identified in humans (TLRs 1 to 10) and mice (TLRs 1 to 13), respectively [2, 13]. TLR 10 is non-functional in mice, and no functional proteins of TLRs 11 to 13 have been recognized in humans [2, 13]. TLR expression is also seen in the female genital mucosa [16, 23], and the activation of TLRs has been reported to lead to pregnancy failure [3, 7, 20, 22]. These facts indicate that TLRs are fundamental to the formation of a suitable microenvironment in the genital mucosa. The variations of TLR expression should be clarified in the genital mucosa during gestation, but to date there have been only a few reports on this matter [8, 11]. In the present study, we investigated the gene transcriptions of TLRs 1 to 9 in the uterus of pregnant mice to understand the genital immune response.

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MATERIALS AND METHODS

Animals: Sexually mature male and female BALB/c mice (CLEA Japan, Tokyo, Japan) were reared under conventional laboratory housing conditions that allowed us to obtain pregnant mice. The day when the plug was found was defined as the first day of pregnancy. Non-pregnant mice were also prepared as a control. This experiment was approved by the Institutional Animal Care and Use Committee (Permission number: 10-T-47), and all procedures were conducted according to the Guide for the Care and Use of Laboratory Animals at Tottori University.

Tissue preparation: Pregnant mice on gestational days (GD) 2, 7, 10, 13 and 18 and non-pregnant mice on diestrus were sacrificed (N=3, respectively) by *i.p.* administration of 200 mg/kg pentobarbital sodium (Dainippon Sumitomo Pharmaceuticals, Osaka, Japan). The uterus (middle portion between the cervix of the uterus and the ostium of the uterine tube) was dissected and immediately immersed in RNA iso Plus reagent (TaKaRa, Otsu, Japan). Thereafter, the uterus was divided into the mesometrial side and the anti-

Table 1. Primer sequences for mRNA of target genes

Target gene	Sequence (5′-3′)	GenBank accession number
TLR1	FW-GTCAAAGCTTGGAAAGAATCTGAAG RV-AATGAAGGAATTCCACGTTGTTTC	NM_030682.1
TLR2	FW-GAATTGCATCACCGGTCAGAA RV-CCTCTGAGATTTGACGCTTTGTC	NM_011905.3
TLR3	FW-CGAAAGTTGGACTTGTCATCAAATC RV-ACTTGCCAATTGTCTGGAAACAC	NM_126166.4
TLR4	FW-TTCAGAACTTCAGTGGCTGGATT RV-CCATGCCTTGTCTTCAATTGTTT	BX649609.2
TLR5	FW-CAGTCCTGGAGCCTGTGTTGT RV-ACCCGGCAAGCATTGTTCT	NM_016928.2
TLR6	FW-TGAATGATGAAAACTGTCAAAGGTTAA RV-GGGTCACATTCAATAAGGTTGGA	NM_011604.3
TLR7	FW-TGCCACCTAATTTACTAGAGCTCTATCTTTAT RV-TAGGTCAAGAACTTGCAACTCATTG	NM_133211.3
TLR8	FW-GAAGCATTTCGAGCATCTCC RV-GAAGACGATTTCGCCAAGAG	NM_133212.2
TLR9	FW-CTCCATCTCCCAACATGGTTCT RV-GCCAGCACTGCAGCCTGTA	NM_031178.2
GAPDH	FW-CATGGCCTTCCGTGTTCCT RV-GCGGCACGTCAGATCCA	M32599.1

FW: Forward; RV: Reverse.

mesometrial side, where the placenta would be developed and implantation would occur, respectively.

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR): Total RNA was isolated using RNeasy Mini Kit (Life Technologies, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions, and cDNA was reverse-transcribed from 1 μg of total RNA using 200 units of SuperScript™ III RT (Life Technologies). For quantification of TLR 1, 2, 3, 4, 5, 6, 7, 8 and 9 transcripts, we used primer sequences designed by Primer Express® Software (Applied Biosystems, Foster City, CA, U.S.A.), and the GAPDH gene was chosen as an internal standard. The primer sequences are shown in Table 1. Real-time PCR was performed in duplicate with Step One Real-Time PCR System (Applied Biosystems), using the universal temperature cycles: 10 min of pre-incubation at 95°C, followed by 45 two-temperature cycles (15 sec at 95°C and 1 min at 60°C). All PCR reactions were carried out with Power SYBR® Green PCR Master Mix (Applied Biosystems), and relative mRNA levels were calculated after normalizing by GAPDH.

Statistical analysis: TLR mRNA expressions for each pregnant day were analyzed by one-way ANOVA followed by Tukey's test using Ekuseru-Toukei 2008 software (Social Survey Research Information, Tokyo, Japan). The level of significance was set at $P<0.05$. All values are expressed as the mean ± SEM.

RESULTS

Gene transcription in the mesometrial side of the uterus: Gene transcriptions of TLR 1, 3, 4 and 9 were significantly changed in the mesometrial side of the uterus during the gestation period. TLR 1 gene transcription was 0.2-fold lower on GD 13 or 18 compared with the non-pregnant uterus ($P<0.01$, respectively) (Fig. 1A). TLR 3 gene transcription was also 0.3-fold lower on GD 13 compared with the non-pregnant uterus ($P<0.05$) (Fig. 1C). On the other hand, TLR 4 gene transcription was 4.3-fold higher on GD 7 compared with the non-pregnant uterus ($P<0.05$), whereas it was 0.3-fold lower on GD 13 ($P<0.05$) (Fig. 1D). TLR 9 gene transcription was 1.8-fold higher on GD 2 compared with the non-pregnant uterus ($P<0.05$), and it was 0.3-fold lower on GD 13 ($P<0.05$) (Fig. 1I). Gene transcriptions of TLR 2, 5, 6, 7 and 8 were not changed statistically during the gestation period, whereas they showed a tendency to decrease in the late gestation period (Fig. 1B, 1E, 1F, 1G and 1H, respectively).

Gene transcription in the anti-mesometrial side of the uterus: Gene transcriptions of TLR 1, 3, 4 and 9 were significantly changed in the anti-mesometrial side of the uterus during the gestation period. TLR 1 gene transcription was 0.1-fold lower on GD 18 compared with the non-pregnant uterus ($P<0.01$) (Fig. 2A). On the other hand, TLR 3 gene transcription was 2.9-fold higher on GD 2 compared with the non-pregnant uterus ($P<0.01$), whereas it was decreased in the late gestation period (Fig. 2C). TLR 4 gene transcription was also 2.3-fod higher on GD 2 compared with the non-pregnant uterus ($P<0.01$), but it was decreased in the late gestation period (Fig. 2D). TLR 9 gene transcription was 1.6-fold higher on GD 7 compared with the non-pregnant uterus ($P<0.05$), and it was 0.3-fold lower on GD 13 ($P<0.05$) (Fig. 2I). Gene transcriptions of TLR 2, 5, 6, 7 and 8 were not changed statistically during the gestation period (Fig. 2B, 2E, 2F, 2G and 2H, respectively).

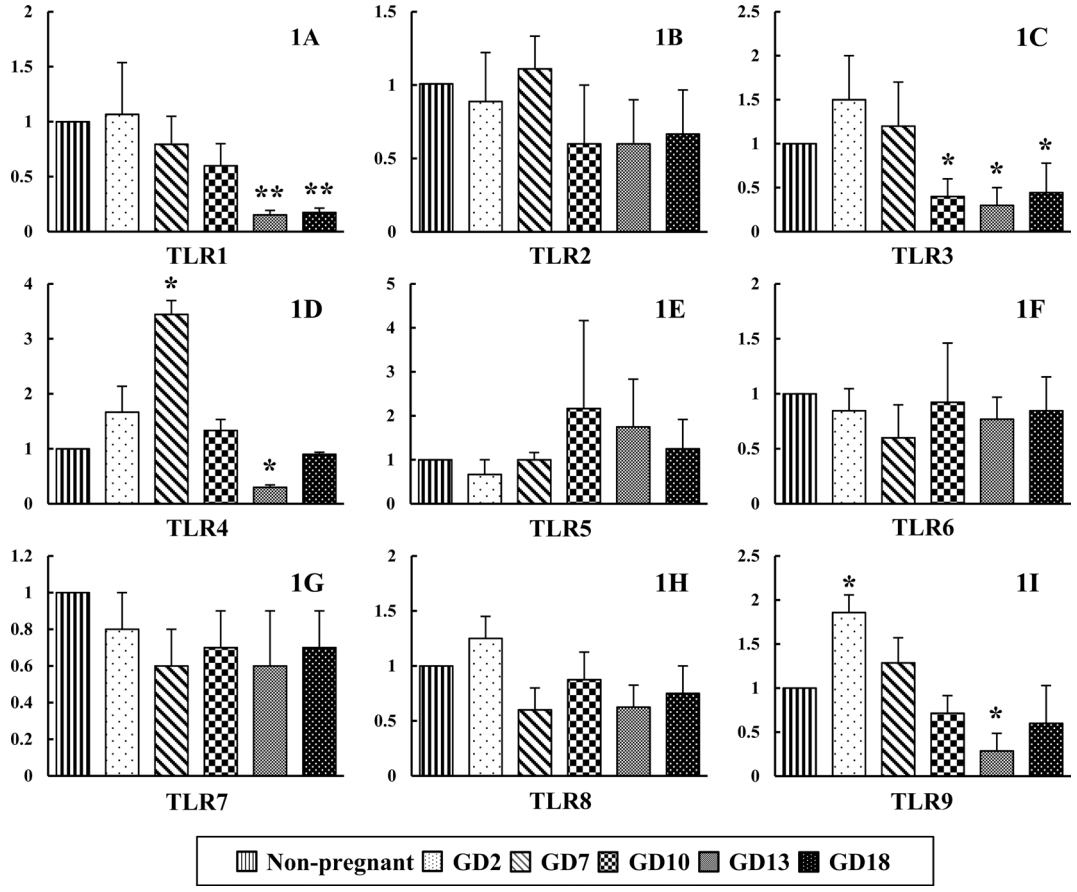

Fig. 1. Relative mRNA levels of TLRs 1 to 9 (1A to 1I, respectively) in the mesometrial side of the uterus on non-pregnant mice and mice on gestational days (GD) 2, 7, 10, 13 and 18. Each bar represents mean ± SEM. Asterisks and double asterisks indicate significant differences compared to non-pregnant mice ($P<0.05$ and $P<0.01$, respectively).

DISCUSSION

TLRs are crucial for innate immunity in the mucosa, as they recognize pathogen-associated molecular patterns of bacteria, fungi and viruses [10, 18, 25]. Specifically, TLR 1 forms a heterodimer with TLR 2 and recognizes triacyl lipopeptide, which is a component of bacteria and mycoplasma [10, 18]. TLR 3 recognizes the double-stranded RNA produced by many viruses during replication [10, 18]. TLR 4 recognizes lipopolysaccharide (LPS), which is an endotoxic component of the Gram-negative bacteria, and TLR 9 recognizes the unmethylated CpG motif, a specific DNA sequence of the bacteria, double-stranded DNA viruses and parasites [10, 18]. In the present study, gene transcriptions of TLR 1, 3, 4 and 9 or TLR 1 and 9 were significantly decreased in the mesometrial or anti-mesometrial side of the uterus in the late gestation period, respectively. These reports and our present study results indicate that the expressions of bacteria or virus-specific TLRs are decreased in the uterus during the late gestation period.

Gene transcriptions of TLRs should be activated with a view to inhibiting the invasion of microbial pathogens, but activations of TLRs would induce pregnancy failure during the gestation period. Mouse models of intrauterine inflammation demonstrate that administration of LPS or Gram-negative bacteria activates TLR 4 and induces preterm birth [3, 5, 7], and administration of CpG DNA activates TLR 9 and induces an inflammatory response in the genital mucosa, which results in fetal resorption, abortion and preterm birth [17, 19, 25]. Hence, a decrease of TLR gene transcriptions during the late gestation period seems to be necessary to avoid pregnancy failure. We also learned from our examination, however, that gene transcriptions of TLR 4 and 9 or TLR 3, 4 and 9 were significantly increased in the mesometrial or anti-mesometrial side of the uterus during the early gestation period, respectively. On the other hand, we confirmed in another experiment that the gene transcription of NF-κB, a key transcription factor of the TLR signaling pathway and antigen-specific adaptive immune response [14, 21, 22, 26], was not changed in the mouse uterus and vagina during the gestation period (unpublished data). We also observed an increase in the FoxP3 gene transcription in the mouse vagina during the middle gestation period, and FoxP3 is a master transcription factor of the regulatory T

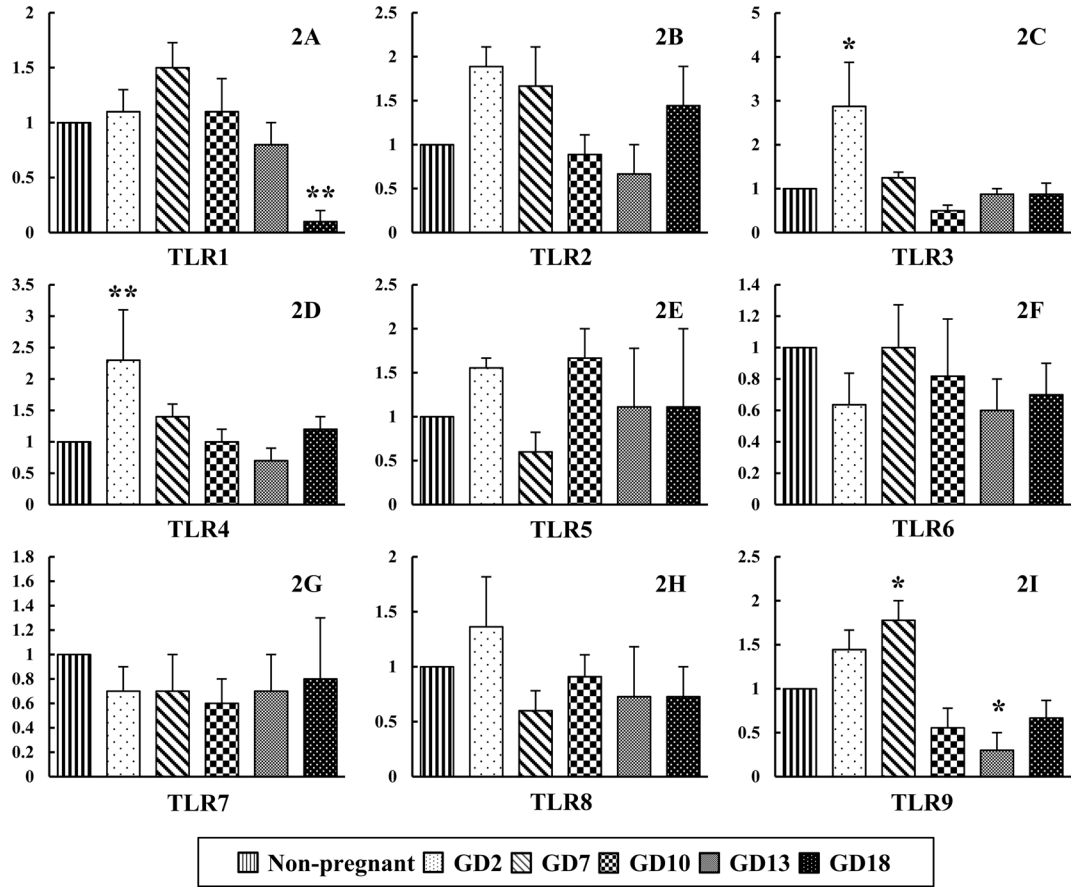

Fig. 2. Relative mRNA levels of TLRs 1 to 9 (2A to 2I, respectively) in the anti-mesometrial side of the uterus on non-pregnant mice and mice on gestational days (GD) 2, 7, 10, 13 and 18. Each bar represents mean ± SEM. Asterisks and double asterisks indicate significant differences compared to non-pregnant mice ($P<0.05$ and $P<0.01$, respectively).

cells [1, 6]. These findings suggest that activation of the adaptive immune response, which develops subsequent to the innate immune response, would be suppressed in the uterus during the gestation period. Moreover, it is also probable that particular TLRs would play other roles in the uterus during the gestation period.

Recently, it was reported that the production of human chorionic gonadotropin (hCG), which is one of the most important molecules during gestation secreted by syncytiotrophoblasts of pre-implantation embryos, is stimulated by TLR 3 and 9 in the human choriocarcinoma cell line [12]. This fact suggests the possibility that TLR 3 and 9 may be involved in the regulation of placental endocrine functions, by stimulating pre-implantation blastocysts to produce hCG and facilitate implantation. The placental development or implantation would start on GD 4 in the mucosa of the mesometrial or anti-mesometrial side of the mouse uterus, respectively [4, 7, 15, 22], which would coincide with the increase of gene transcriptions of TLR 4 and 9 or TLR 3, 4 and 9 in the mesometrial or anti-mesometrial side of the uterus, respectively. We could not elucidate the accurate reason for these occurrences in the present study, but an increase of the gene transcriptions of TLRs in both sides of the uterus during the early gestation period might be a reflection of these events.

In conclusion, our present study indicates that the expressions of particular TLRs may be regulated in the uterus during the gestation period so as to maintain the pregnant state and allow the female reproductive organ to tolerate fetuses. Further studies to examine the distribution of TLRs are needed to elucidate the mucosal immunity in the endometrium during pregnancy.

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