FULL PAPER *Anatomy*

Differential Expressions of Toll-Like Receptor Genes in the Vagina of Pregnant Mice

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ABSTRACT. The mammalian immune system is classified into two categories, innate and adaptive immunity, and innate immunity is an immunological first line of defense for the mucosal immune system. Toll-like receptors (TLRs) play critical roles in innate immunity, as they recognize specific molecular patterns found in microbial pathogens, and 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. Despite the importance of TLRs in mucosal immunity, little is known about their expression in the female genital mucosa during gestation. In the present study, gene expressions of TLRs 1 to 9 were investigated together with NF-κB and FoxP3 gene expressions in the vaginae of pregnant mice to understand the immune response of the female genital mucosa during pregnancy. We found that mRNA expressions of TLR4, TLR5, TLR6, TLR7 and TLR9 were significantly decreased during the late gestation period, whereas temporary increases were seen in the middle gestation period. Gene transcriptions of TLR1, TLR2, TLR3 and TLR8 were not changed specifically during the gestation period. The mRNA expression of NF-κB was not changed at any time during the gestation period, while the FoxP3 mRNA expression was increased in the middle gestation period. These results suggest that expressions of particular TLRs would be down-regulated during gestation so as to maintain the pregnant state.

KEY WORDS: innate immunity, mouse, pregnancy, toll-like receptor, vagina.

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The mucosa of the female genitalia is always exposed to microorganisms from the external environment, because the genital tract is continuously opened to the outside adjacent to the anus, with the aim of mating or delivering. To prevent microbial infection, the mucosal immune system is well developed in the genital mucosa, while tolerating the presence of embryos or fetuses during pregnancy. The mucosal immune system is classified into two categories in mammals, innate and adaptive immunity, and innate immunity functions as an immunologic first line of defense line [8, 21]. Toll-like receptors (TLRs) are generally known to be essential in innate immunity [15]. TLRs recognize specific molecular patterns of microbial pathogens as ligands, and the activation of TLRs is important not only for the initiation of the innate immune response but also for the induction of subsequent antigen-specific adaptive immune responses [9, 22].

Mammalian TLRs comprise a large family of at least 15 members, and to date, 10 and 13 TLRs have been identified in humans (TLRs 1 to 10) and mice (TLRs 1 to 13), respectively [2, 11]. TLR10 is non-functional in mice, and no functional proteins of TLR11 to TLR13 have been recognized in humans [2, 11]. TLRs are also expressed in the female genital mucosa [13, 20]; however, the activation of TLRs has been reported to induce the pregnancy failure [3, 16]. These facts indicate that TLRs are fundamental for the formation of suitable microenvironment in the genital mucosa. Consequently, it is necessary to clarify the variations of TLR expressions during pregnancy if we are to understand the immune response in the genital tract, but to date, only a few reports have been published on this subject [7, 10], and there is no report about the TLR expressions through the gestation period in the genital tract. The present study was performed to elucidate the gene transcriptions of TLRs 1 to 9 during the gestation period in the mouse vagina. We also investigated gene transcriptions of NF-κB, a key transcription factor of the TLR signaling pathway, and FoxP3, a master transcription factor of regulatory T cells.

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 ac-

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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
NF-kB	FW-AGGCTTCTGGGCCTTATGTG RV-TGCTTCTCTCGCCAGGAATAC	NM_008689.2
FoxP3	FW-TTTACTCGCATGTTCGCCTACTT RV-TCAAATTCATCTACGGTCCACACT	NM_001199347.1
GAPDH	FW-CATGGCCTTCCGTGTTCCT RV-GCGGCACGTCAGATCCA	M32599.1

FW: forward; RV: reverse.

cording to the Guide for the Care and Use of Laboratory Animals at Tottori University.

Tissue preparation: Pregnant mice on pregnant days (P) 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 vagina (middle portion between the vaginal orifice and the cervix of the uterus) was dissected and immediately immersed in RNA iso Plus reagent (TaKaRa, Otsu, Japan).

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR): Total RNA of the vagina 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 TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, Foxp3 and NF-κB 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: Differences in TLR mRNA expressions for the 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 expression in the vagina: The expressions of TLR mRNAs were significantly changed with TLR4, TLR5, TLR6, TLR7 and TLR9 during the gestation period (Fig. 1A-1I). TLR4 mRNA was 2.1-fold higher on P7 compared with the non-pregnant vagina ($P<0.01$), but it was 0.5-fold lower on P13 ($P<0.05$) (Fig. 1D). TLR5 mRNA was 0.3, 0.4, 0.4 and 0.4-fold lower on P2, 7, 13 and 18, respectively, compared with the non-pregnant vagina ($P<0.01$ on P2 and 7 and $P<0.05$ on P13 and 18) (Fig. 1E). TLR6 mRNA was 0.5, 0.4, 0.2 and 0.2-fold lower on P2, 7, 13 and 18, respectively, compared with the non-pregnant vagina ($P<0.05$ on P2 and $P<0.01$ on P7, 13 and 18) (Fig. 1F). TLR7 mRNA was 1.6-fold higher on P10 compared with the non-pregnant vagina ($P<0.01$), but it was 0.5, 0.4 and 0.4-fold lower on P2, 7 and 13, respectively ($P<0.01$) (Fig. 1G). TLR9 mRNA was 2.2-fold higher on P7 ($P<0.01$) compared with the non-pregnant vagina, but it was 0.6-fold lower on P18 ($P<0.05$) (Fig. 1I). Gene transcriptions of TLR1, TLR2, TLR3 and TLR8 were not changed statistically during the gestation period, though they showed a tendency to decrease in the

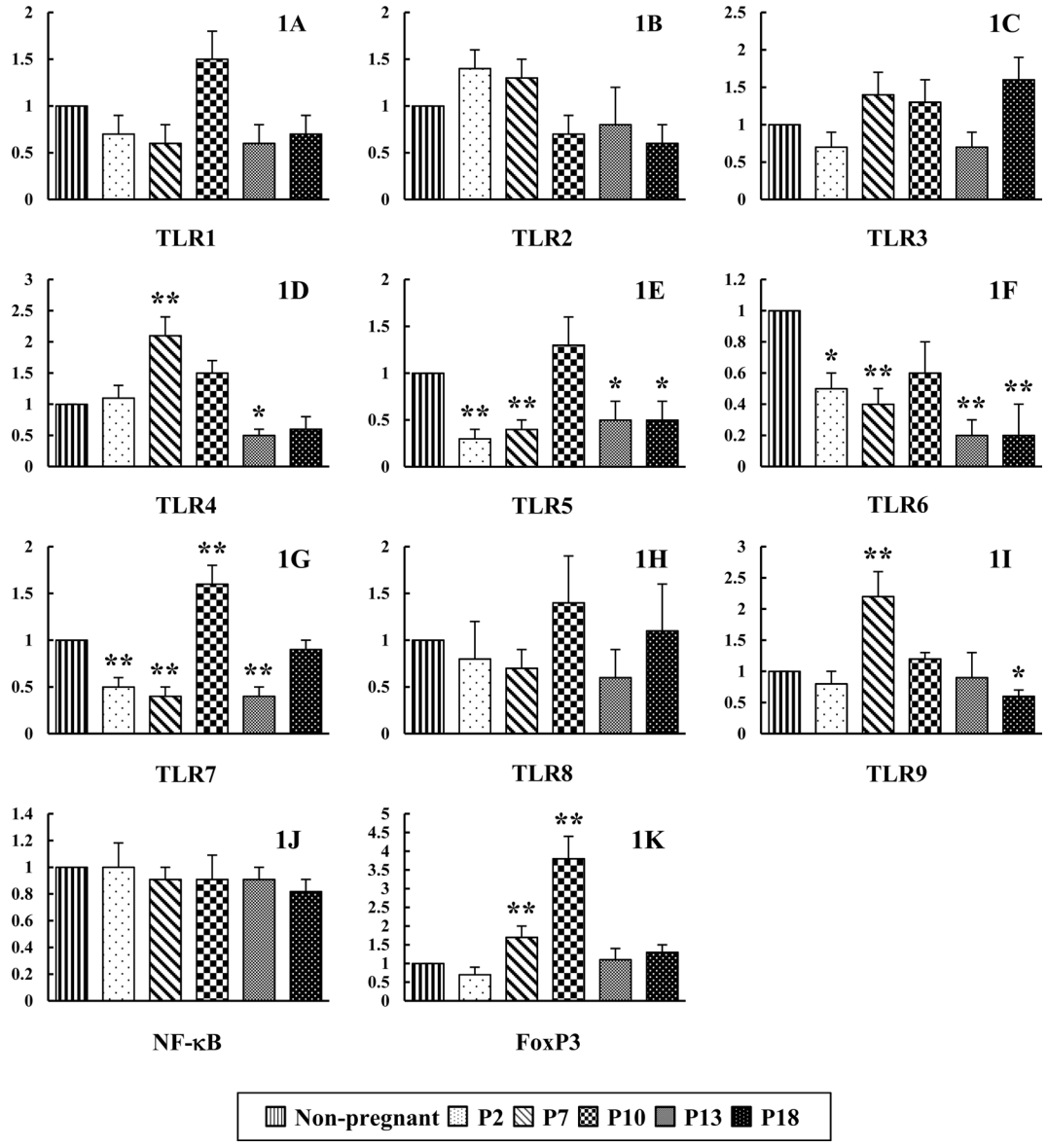

Fig. 1. Relative mRNA levels of TLRs 1 to 9, Foxp3 and NF-κB (1A to 1I, 1J and 1K, respectively) in the vagina on non-pregnant mice and on pregnant days (P) 2, 7, 10, 13 and 18. The mRNA expressions were normalized by that of GAPDH, and the vagina on non-pregnant mice was estimated as 1. Each bar represents mean ± SEM. Asterisks and double asterisks indicate significant differences compared to non-pregnant mice (P<0.05 and P<0.01, respectively).

late gestation period (Fig. 1A, 1B, 1C and 1H, respectively). On the other hand, the mRNA expression of NF-κB was not changed throughout the gestation period (Fig. 1J). However, FoxP3 mRNA was 1.7 and 3.8-fold higher on P 7 and 10, respectively, compared with the non-pregnant vagina (P<0.01) (Fig. 1K).

DISCUSSION

Results from our present study demonstrated that gene transcriptions of TLRs are changed during the gestation period in the mouse vagina. Namely, gene transcriptions of TLR4, TLR5, TLR6, TLR7 and TLR9 were decreased during the late gestation period, whereas they were increased temporarily in the middle gestation period. Individual TLRs play instructive roles in innate immune response in the genital mucosa by recognizing broad range of specific molecules of microorganisms (bacteria, fungi and viruses) [9, 15, 22]. Specifically, TLR4 recognizes lipopolysaccharide (LPS), which is an endotoxic component of the Gram-negative bacteria [9, 15]. TLR5 recognizes flagellin, which is a component of bacterial flagellum, and TLR6 forms a heterodimer with TLR2 and recognizes diacylated lipopeptides, which are components of the bacterium or mycoplasma [9, 15].

Moreover, TLR9 recognizes the unmethylated CpG motif, which is a specific DNA sequence of the bacteria, double-stranded DNA virus and parasite [9, 15]. These facts and the results from our present study indicate that the expressions of bacteria-specific TLRs are mainly changed during the gestation period in the vagina.

Gene expressions of TLRs should be activated with a view to inhibiting the invasion of pathogens, but the activation of TLRs during the gestation period would evoke pregnancy failure in the genital mucosa. Actually, reproductive mouse models demonstrated that an up-regulation of TLR4 induces intrauterine inflammation and causes preterm birth or fetal demise [3, 5]. Moreover, the administration of CpG DNA activates TLR9 and induces an inflammatory response in the genital mucosa, resulting in fetal resorption, abortion or preterm birth [12, 17, 23]. On the other hand, the expression of NF-κB mRNA was not changed throughout the gestation period in the present study. NF-κB is a representative key transcription factor of the TLR signaling pathway [18, 24], and antigen-specific adaptive immune responses are induced subsequent to an activation of NF-κB with the production of various inflammatory cytokines [9, 15, 19, 21]. In addition, the gene transcription of FoxP3 was increased during the middle gestation period in the present study. Foxp3 is specifically expressed in the $CD4^+CD25^+$regulatory T cell (Treg), which plays an important role in the negative regulation of immune response, and is required for the development of the Treg as a master transcription factor [1, 6]. Taken together, the findings of the current study and referenced work suggest that gene expressions of particular TLRs would be inhibited in the vagina during gestation. Incidentally, the gene transcription of TLR7, which interacts with the single-stranded RNA virus [9, 15], was also changed during the gestation period. These results also suggest that the vaginal mucosa would respond to viral infection during gestation.

Meanwhile, we chose GAPDH as an internal standard in the present study, which is the most common housekeeping gene to normalize gene expression levels in the RT-PCR. However, it has been proposed that commonly used housekeeping genes vary considerably under different experimental conditions, and therefore their use for normalization is limited [8]. The gene expression of GAPDH was not varied during gestation in the mice vagina, but the use of a combination of housekeeping genes might minimize the risk implicit in the use of a single housekeeping gene.

In summary, the present study results suggest that expressions of particular TLRs would be down-regulated during the gestation period so that the female reproductive organ can tolerate the fetus and maintain the pregnant state. Further studies are needed to examine the distribution of TLRs in the genital mucosa to elucidate the roles of TLRs in pregnancy.

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