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RESEARCH ARTICLE

Expression Patterns of Toll-Like Receptor 4 in Pig Uterus during Pregnancy

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ABSTRACT

Toll-like receptor 4 (TLR4) plays a significant role in modulation of immunological tolerance in reproductive tract of human female. To better understand the functions of TLR4 in establishing and maintaining the pregnancy in pigs, this study was performed to investigate the cellular expression and distribution patterns of TLR4 in the uterus of pregnant Yorkshire pigs. For this purpose, the uterine tissues from pregnant gilts were obtained on days 15, 26, 50 and 95 of gestation. The distribution of TLR4 in the uterus was analyzed by immunohistochemical technique, using an antibody against TLR4. TLR4 was found in uterine luminal epithelium, uterine glands, trophoblasts and placental blood vessels; TLR4 expression in the trophoblast cells and uterine luminal epithelium, on days 15 and 26 of gestation was higher than that on days 50 and 95 of gestation, and there was nearly same expression between days 50 and 95 of gestation. In uterine glands, the expression of TLR4 protein was nearly equal at all stages of gestation. These results indicated that TLR4 is expressed in different histological layers of the uterus during the pregnancy and it changes with the advancement in gestational stages. These results provided novel information about cellular distribution and the functions of TLR4 in modulating immune tolerance in pig uterus during pregnancy.

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INTRODUCTION

Toll like receptors (TLRs) play an important role at the maternal-fetal interface and the normal term placenta has shown mRNA expression levels for TLR-1 to TLR-10, besides different co-receptors along with some accessory proteins such as cluster of differentiation 14 (CD14), myeloid differentiation primary response gene 88 (MyD88), toll-interleukin 1 receptor (TIR) domain-containing adaptor protein (TIRAP) and TIR domain-containing adapter-inducing interferon-β (TRIF) (Girling and Hedger, 2007). The placenta forms an interface between developing fetus and maternal tissue and acts as a unique immunologically important site along with the potential to accept a partially allogeneic fetus (Romero *et al*., 2006). During normal pregnancy, the developing fetus and placenta are not rejected by the maternal immune system. This modulation in immunological tolerance between fetus-derived trophoblast cells and maternally-derived cells contributes to embryo development and survival in the uterus until parturition (Du *et al*., 2014). In the absence of this tolerance, the maternal immunological system would destroy the developing fetus in the same way as it would do with an invading pathogen (Vanders *et al*., 2015).

Toll-like receptor 4 (TLR4) is known to be a pathogen recognition receptor (PRR), that is associated with the detection of a pathogen-associated molecular pattern (PAMP), and builds up an immune reaction against invading pathogens that results in inflammatory responses. It has been noted that regulated immune reactions can subside bacterial threats; conversely, unregulated reactions could build up the sepsis, a life-threatening state that is not easy to treat (Anwar *et al*., 2015).

TLRs also play an important role in implantation as well as in early pregnancy in human (Eriksson *et al*., 2006; Horne *et al*., 2008). These are found in the pregnant uterus and their expression has been detected not only in the first trimester but also in late pregnancy (Horne *et al*.,

2008). It is described that trophoblast cells have shown TLR2 and TLR4 expressions (Holmlund *et al*., 2002; Ma *et al*., 2007). Moreover, TLR2 and TLR4 expressions are also reported in the amniotic epithelium in the pregnant woman. Furthermore, spontaneous labor at term or preterm is related with high expressions of TLR2 and TLR4 proteins and mRNA in the chorioamniotic membranes. These findings suggest that the innate immune system has a significant role in parturition (Kim *et al*., 2004; Romero *et al*., 2006). The existence of TLR4 in female reproductive system has been reported at mRNA level in human (Pioli *et al*., 2004), bovine (Herath *et al*., 2006) and pig (Linton *et al*., 2008). Accordingly, it suggests that the pregnancy of mammals is closely related to uterine TLR4 levels.

It is also known that the placenta of pig has different structure than that of the human and mouse, consequently, the uterine endometrium in porcine may have some special physiological measures during pregnancy (Su *et al*., 2014).

Taken together, human and other mammalian uterine cell lines express several TLR genes. However, the function of TLR4 in pregnancy is poorly known in the pig. Moreover, the information about the cellular distribution of TLR4 in pregnant pig uterine tissue is scarce. So, the present study was performed to compare the expression patterns of TLR4 protein in the pig uterus and to explore its potential role in mediating innate immunity at different gestational stages during pregnancy.

MATERIALS AND METHODS

This experiment was performed according to protocols approved by Animal Care and Use Committee for Biological Studies, Hubei Province, PR China.

Animals: In total, 12 pure-bred Yorkshire gilts were purchased from Pig Breeding Farm of Huazhong Agricultural University, and reared under conventional housing conditions. They were divided into four groups depending on the stage of the gestation i.e. 15, 26, 50 and 95 days, with three animals in each group.

Tissue collection and preparation: Immediately after slaughtering of pregnant pigs, the uteri were harvested and transported in an ice-box to the laboratory, where they were dissected longitudinally. Three healthy implantation sites were sampled per gilt. The samples (tissues from pregnant uterine wall) were fixed in 4% paraformaldehyde-0.1M phosphate for 24h for immunohistochemical examination.

Immunohistochemical staining: The localization of TLR4 protein in uterine tissues by immunostaining was accomplished by following our previously described method (Huang *et al.,* 2014), using the primary rabbit anti-TLR4 antibody (BA1717; Boster, China). In brief, 4 μm tissue sections were cut and then mounted on polylysine-coated slides. After deparaffinization and rehydration, the slides were treated with 3% H_2O_2 for 10 minutes. After antigen retrieval in citrate acid buffer (pH 6.0), the sections were treated with 5%BSA (SV0002-rabbit IgG kit; Boster, China) for 10 minutes and then incubated with the diluted TLR4 antibody with 100-fold concentration overnight at 4°C. All slides were washed in PBS and then incubated with horseradish peroxides (HRP) conjugated anti-rabbit IgG (SV0002-rabbit IgG kit; Boster, China) for 30 minutes at 37°C. The slides were stained with 3,3'-diaminobenzidine (DAB) (AR1022; Boster, China) and counterstained with hematoxylin for 10 seconds. In the same way, negative control sections were incubated with PBS instead of the primary antibody.

Quantification of immunohistochemical staining: The protein expression of TLR4 as obtained with positive immuno-staining was examined by light microscope (BH-2; Olympus, Japan). Three sections of each implantation site were taken, and 10 horizons were intercepted randomly in different tissue regions (uterine epithelium and uterine gland) under 20 high-power fields. The density mean value of the TLR4 expression was calculated using a computerized image analysis program, Image-Pro Plus version 6.0 (Media Cybernetics, USA). Finally, the graphic representations were performed with Prism software 5.0 (GraphPad Software, Inc., San Diego, USA).

Statistical analysis: The data are shown as mean±SD, and the significant difference was analyzed using SPSS 17.0 with one-way ANOVA for group comparisons.

RESULTS

Distribution pattern of TLR4 expression: Under light microscope, the positive expression products of TLR4 appeared brown-yellow and the nucleus was blue. TLR4 was observed in different layers of the uterine tissue at different gestational stages (Fig. 1). TLR4 was localized in the endometrial glandular epithelial cells (UG) at all stages (15, 26, 50, 95 days). It was strongly detected in the endometrial luminal epithelium (LE) only on 15 and 26 days of gestation and weakly expressed on 50 and 95 days of gestation. The trophoblast cells (Tr) also expressed TLR4 on days 15 and 26 (Fig. 1-A, B), but the expression was absent in these cells on days 50 and 95 (Fig. 1-C, D). Moreover, the expression of TLR4 in endometrial luminal epithelium was higher than that on trophoblasts on days 15 and 26 of gestation. Furthermore, slight positive immunostaining was also observed in the walls of placental vessels, indicating the expression of the TLR4 in blood vessels of the placenta (Fig. 1-E).

Semi-quantitative analysis of TLR4 expression: The expression of TLR4 protein decreased with the advancement of gestational stage in the maternal-fetal interface (Fig. 2). The expression of TLR4 localized at the maternal-fetal interface on days 15 and 26 was higher as compared with that on days 50 and 95 of gestation ($P<0.05$), however it did not differ between gestational days 50 and 95 ($P\geq0.05$) or in other words, TLR4 protein was nearly equally expressed at days 50 and 95 of gestation (Fig. 2). Conversely, the expression of TLR4 protein was detected nearly in equal amount in uterine glands at all gestational stages (15, 26, 50, 95 days), or in other words, the differential expression of TLR4 localized in uterine gland was not statistically significant ($P\geq0.05$) (Fig. 2).

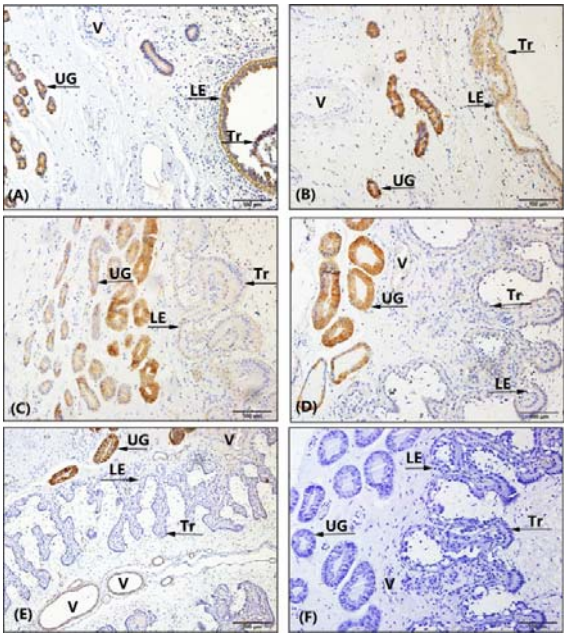

Fig. 1: Photomicrographs of the cellular distribution of Toll-like receptor 4 (TLR4) in the uterus of Yorkshire gilts at different gestational stages. TLR4 was expressed in the endometrial luminal epithelium and trophoblasts on days (A) 15, (B) 26, (C) 50, and (D) 95 of gestation. (E) TLR4 Expression in the wall of vessels in the placenta. Scale bars A-D=100μm & E=200μm. (F) Tissue section as a negative control; Scale bar=100μm (Tr, trophoblast cells; LE, endometrial luminal epithelium; UG, uterine glands; V, vessels).

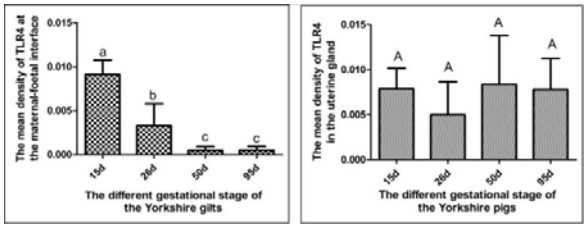

Fig. 2: The mean density for TLR4 in the uterus of Yorkshire gilts at different gestational stages. The letters "a", "b" and "c" indicate expression in the endometrium and trophoblast cells at the maternal-fetal interface within groups. The letter "A" indicate expression in uterine gland. Different letters indicate significant difference (P<0.05), and the same letters indicate non-significant difference (P≥0.05).

DISCUSSION

This study revealed a strong immunoreactivity for the TLR4 genes in the maternal-fetal interface cells, such as the endometrial epithelial cells, the trophoblast cells and the uterine gland cells in pigs. Previously, the expression of mRNA for TLR1 to TLR10 was described in the placenta (Girling and Hedger, 2007), however, the cellular expression was only reported for TLR2 and TLR4 in human placenta (Holmlund *et al*., 2002). It seems to be the first time that the expression of TLR4 at cellular level has been reported in the uterus of pregnant Yorkshire pigs in this study.

In the present investigation, it has been shown that the expression patterns of TLR4 were dependent on gestational stages in the pig. Previous studies indicate that different TLRs expressed in the trophoblasts of placenta vary by gestational stages in human (Holmlund *et al*., 2002; Herath *et al*., 2006), mice (Sun *et al*., 2013), dogs (Chotimanukul and Sirivaidyapong, 2011) and goats (Tirumurugaan *et al*., 2010). During the early stage of pregnancy in pigs, the blastocysts are fixed to the endometrium for initial placentation around days 13-15 (Dantzer, 1985) and the placenta is established around days 26-30 of gestation (Hong *et al*., 2013; Su *et al*., 2014). The early trimester of pregnancy involving fertilization, implantation and placentation could induce series of inflammatory reactions (Kimber, 2005; Mor and Cardenas, 2010). It is proposed that the immune system of both maternal and fetal sides would be active for the recognition, communication, trafficking and repair during the early stage when the allograft invades the uterus (Mor and Cardenas, 2010).

In the present study, it was found that in the early pregnancy, TLR4 was mainly localized in the endometrial epithelium, endometrial glandular epithelial cells and trophoblasts. Previous researches have shown that the TLRs are very critical proteins, which not merely cause the activation of innate immune response but also initiate the enhancement of antigen-specific acquired immune response (Akira *et al*., 2001; Akira and Takeda, 2004). It is reported that stimulation of TLR4 protein leads to the activation of two signaling pathways; MyD88-dependent pathway and MyD88-independent pathway. The former pathway involves the activation of early phase of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) that results in inflammatory cytokines production and the latter signaling pathway causes the activation of interferon regulatory factor3 (IRF3) and involves in the further activation of the late phase NF-κB, finally both of these pathways lead to interferon-β (IFN-β) production and IFN-inducible genes expression (Akira and Takeda, 2004). The high expression of TLR4 during the early stage of pregnancy suggests that the TLR4 within the endometrium is likely necessary for recognizing each other, between the mother and the fetus, which results in successful implantation and the pro-inflammatory responses.

In the current investigation, TLR4 expression was strongly present in the uterine luminal epithelium (endometrium) as well as in the trophoblast cells at early stages of the pregnancy (15 and 26 days). Previously, it has been reported that the endometrium at the start of pregnancy becomes an immunologically privileged site and the grafts can continue to exist without being rejected for extended period of time (Yoshinaga, 2012). Furthermore, the uterine epithelium and trophoblasts communicate through molecular signals to protect the fetus from infection (Koga *et al*., 2009). This indicates that the endometrium of the pregnant uterus not only acts as an implantation site for the developing and growing fetus but also protects it from infection by the activation of signaling pathways through the expression of TLR4 gene.

In this study, we found that the TLR4 expression at the cellular level decreased in trophoblasts and uterine epithelium with advancing gestation in pigs. This low expression of TLR4 seems to result from the regulation of trophoblasts, and it could decrease the immune responses. The above results indicate that TLR4 protein may play a significant role in the growth of the fetus by modulation

of immunological tolerance in pig uterus which needs further investigation.

Furthermore, it is worth noting that the TLR4 positive immunostaining was observed in the blood vessels in the placenta. A number of bacteria and viruses can cross the placenta and infect fetal tissues. The expression of TLR4 in the placental vasculature could potentially affect the transportation at the maternal-fetal interface. Probably, the TLR4 would prevent the invading pathogenic microorganism through the placenta and may play role in the placental vascular barrier. However, the mechanism involved needs further study.

Conclusion: The current findings provide morphological evidence that TLRs recognition and response occurs not only in immune cells but also in non-immune cells such as the trophoblasts and the epithelium cells as well as in placental blood vessels. It suggests that cellular expression of TLRs may provide insight about functions of different histological layers of the tissues, whereas detection of TLRs at mRNA level can only provide information about its existence in tissues. In the present study, differential expression patterns of TLR4 at the maternal-fetal interface revealed that TLR4 may play role in implantation and fetus growth by modulation of immunological tolerance in pig uterus during the pregnancy.

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Author's contribution: HZL, KMP and MY designed the project, supervised the work and contributed in the preparation of manuscript. ARA, XHG, HBH and JXW performed the sampling, laboratory experiments, microscopy, statistical analysis and write up.

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