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Paisan Tienthai

Yumi Hoshino

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Paisan Tienthai[1*] Yumi Hoshino[2]

Abstract

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The expression of Fas/Fas Ligand (FasL) is thought to be involved in the immune privileged status of the testis, cornea and the placenta. The objective of this study was to see whether FasL mRNA was expressed in the epithelium of the porcine oviduct, where spermatozoa, oocytes and developing embryos avoid elimination by the female immune system during the estrous cycle. The distribution of Fas/FasL might provide information about immune privileged sites in the pig oviduct. The gene expression of FasL was detected in the uterotubal junction (UTJ), isthmus and ampulla of the pig oviductal epithelium, during both pre- and post-ovulatory periods, using semiquantitative RT-PCR. FasL mRNA was found in the epithelial cells from the sperm reservoir (UTJ, adjacent isthmus) and the ampulla during both pre- and post-ovulation. The level of expression between UTJ and other oviduct segments differed significantly during pre-ovulation ($p < 0.05$). The densitometry ratios of FasL: β-actin tended to be stronger in the isthmus and the ampulla after ovulation but did not vary significantly. The results indicated for the first time that FasL mRNA is present in the porcine oviduct and might be involved in a Fas/FasL system that mediates the survival of spermatozoa, oocytes and early embryo development.

Keywords : Fas Ligand, oviduct, immune privileged, pig

[1]**Department of Anatomy, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, 10330**

[2]**Laboratory of Animal Reproduction, Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, Japan**

[*]**Corresponding author**

[1]ภาควิชากายวิภาคศาสตร์ คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย กรุงเทพฯ 10330

[2]ห้องทดลองระบบสืบพันธุ์ของสัตว์ บัณฑิตวิทยาลัยแห่งเกษตรศาสตร์ มหาวิทยาลัยโทโฮกุ เมืองเซ็นได ประเทศญี่ปุ่น 981-8555

[*]ผู้รับผิดชอบบทความ

บทคัดย่อ

ไพศาล เทียนไทย[1*] ยูมิ โฮชิโน[2]

การแสดงออกของ Fas Ligand ในท่อนำไข่สุกร

การปรากฏของ Fas/FasL เป็นกลไกสำคัญที่ระบุถึงสถานภาพที่เรียกว่า immune privilege ของอวัยวะต่างๆ เช่น อัณฑะ กระจกตา และรก วัตถุประสงค์ของรายงานวิจัยนี้เพื่อศึกษา FasL mRNA จะปรากฏในเยื่อบุของท่อนำไข่สุกรหรือไม่ ทำการเก็บตัวอย่างเยื่อบุท่อนำไข่ของสุกรแต่ละส่วน คือ รอยต่อระหว่างปีกมดลูกกับท่อนำไข่ อิสมัสและแอมพูลล่า ในระยะก่อนและหลังตกไข่ เพื่อตรวจหาการแสดงออกของ FasL mRNA โดยใช้วิธี semiquantitative RT-PCR พบว่า FasL mRNA สามารถตรวจพบได้ในเยื่อบุท่อนำไข่ในส่วนเป็นบริเวณที่กักเก็บตัวอสุจิ (รอยต่อของปีกมดลูกกับท่อนำไข่และอิสมัสที่อยู่ใกล้ชิดกัน) รวมทั้งในท่อนำไข่ส่วนแอมพูลล่า ทั้งในระยะก่อนและหลังตกไข่ โดยระดับการแสดงออกที่ปรากฏในส่วนรอยต่อของปีกมดลูกกับท่อนำไข่มีความแตกต่างอย่างมีนัยสำคัญ ($p < 0.05$) เมื่อเปรียบเทียบกับท่อนำไข่ส่วนอื่นในระยะก่อนการตกไข่ นอกจากนี้ระดับความเข้มของ FasL mRNA ที่ปรากฏในอิสมัสและแอมพูลล่ามีแนวโน้มสูงขึ้นในระยะหลังการตกไข่ แต่ระดับความเข้มนี้ไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ ผลการศึกษานี้บ่งชี้เป็นครั้งแรกว่า FasL mRNA ปรากฏในท่อนำไข่ของสุกรและอาจเกี่ยวข้องกับการทำงานของ Fas/FasL ซึ่งมีผลกระทบในการรักษาการอยู่รอดของตัวอสุจิ โอโอไซต์และตัวอ่อนในระยะแรกที่อยู่ภายในท่อนำไข่

คำสำคัญ : Fas Ligand ท่อนำไข่ immune-privileged สุกร

Introduction

The oviduct plays an important role in fertilization and early embryonic development by providing a suitable environment that facilitates sperm transport, sperm capacitation, oocyte transport and oocyte maturation (Hunter, 1988). Both spermatozoa and oocytes are in contact with the oviductal epithelium and luminal fluid (Rodriguez-Martinez et al., 2001). During pre-ovulation sperm transport in the female pig, a sperm reservoir is established at the utero-tubal junction (UTJ) and the adjacent isthmus where viability and fertilising ability are maintained for up to 30-48 hours (Hunter, 1981). After ovulation, the simultaneous movements of male and female gametes to the fertilization site, i.e. the ampullary-isthmic junction, occur and the subsequent passage of the embryo into the uterus, at a precise developmental stage, is essential to allow implantation (Buhi et al., 1997). It is interesting to see that spermatozoa and fertilized oocytes, carrying foreign antigens, escape detection by the mucosal immune system of the oviduct. It can be postulated that, this organ may serve as an immune privileged site, like the testis (Koji et al., 2001), cornea (Bellgrau et al., 1995) and placenta (Kauma et al., 1999) where cells expressing foreign proteins escape rejection.

Fas Ligand (FasL) and its receptor Fas are known to play an important role in the regulation of immune responses. FasL is a type II membrane related to the tumor necrosis factor (TNF) family (Nagata, 1994). FasL can be either membrane bound (42-48 kDa form) or released as 26 kDa in a soluble form. FasL has been detected in the endometrium (Yamashita et al., 1999) and placenta (Kauma et al., 1999) of human. The Fas/FasL system has been demonstrated to be the mediator for immune privilege (Griffith et al., 1995). It has been hypothesized that the expression of FasL confers immune privilege by inducing Fas-mediated apoptosis in lymphocytes which infiltrate FasL-bearing tissues, thus preventing potential inflammatory damage in these vulnerable sites (Newell

and Desbarats, 1999). The detection of a Fas/FasL system in the pre- and post-ovulatory oviduct could be a very elegant mechanism for local immune tolerance that might eliminate cytotoxic T lymphocytes, preventing them from attacking spermatozoa, oocytes and early developing embryo. The present study was undertaken to determine whether FasL mRNA is expressed in the porcine oviductal epithelium during pre- and post-ovulatory periods, using a semiquantitative reverse transcription-polymerase chain reaction (RT-PCR).

Materials and methods

Experimental animals

Multiparous cross-bred (Swedish Yorkshire (Swedish Landrace) sows (n = 10), were recruited for these experiments from a commercial farm on the day of weaning, and individually penned at the Department of Obstetrics and Gynecology, Swedish University of Agricultural Sciences. The sows received water *ad libitum* and a standard ration, according to the Swedish breeding stock standard for dry sows. A fertile boar was always penned in the vicinity. Experienced personnel checked the sows twice daily for behavioral estrus and all sows remained non-inseminated. The experimental design has previously been reviewed and approved by the Ethical Committee for Experimentation with Animals, Uppsala, Sweden.

Collection of oviductal tissue

The ovaries were periodically explored for the presence of follicles by transrectal ultrasonography, as described by Mburu et al. (1995). The animals were slaughtered during two different stages of standing estrus, i.e. pre-ovulation (8-10 h before expected ovulation, n = 5) and post-ovulation (10-12 h past spontaneous ovulation, n = 5). The oviducts from each period were retrieved and divided into the uterotubal junction (UTJ), the isthmus, and the ampulla sections. All segments were promptly deep-frozen in liquid nitrogen (LN_2) until the separation of RNA.

RNA extraction and semiquantitative RT-PCR

The lining epithelium from the UTJ, isthmus and ampulla segments was scraped using the blunt side of a scalpel blade. RNA was isolated from these epithelial cells according to the instructions supplied with the RNeasy mini kit (QIAGEN GmbH, Hilden, Germany). The extracted RNA was electrophoresed on a 1% formaldehyde agarose gel for quality checking and kept at -20°C until needed. RT-PCR was performed using Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech, Piscataway, NJ) that were optimized to allow the first-strand of cDNA synthesis and PCR reactions to proceed sequentially, as a single-step reaction, using a PCR thermal cycler TP2000 (TaKaRa, Kyoto). The primers used were designed for porcine FasL (Sigma-Genosys Ltd., Pampisford Cambridgeshire, UK). The sequences of the sense and antisense of FasL primers were as follows: sense 5'-CAG CCA AAG GCA TAC AGA ATC ATC T-3' and antisense 5'-CTC AGG GGC TGG TTG CAG TAC-3' (Genebank accession number AY033634). For β-actin, which was used both as an internal control and to calculate the relative abundance of FasL, the sense primer was 5'-GAC CCA GAT CAT GTT TGA GAC C-3' and the antisense was 5'-ATC TCC TTC TGC ATC CTG TCA G-3' (Genebank accession number X03672).

The aliquot of RNA (100 ng) was reverse-transcribed as previously described (Tienthai et al., 2003). PCR amplification proceeded after inactivation of the reverse transcriptase by heating for 5 min at 95°C. PCR cycling for β-actin and FasL was carried out with 45 cycles of 30-sec denaturation at 95°C, 30-sec annealing at 50°C, and 30-sec extension at 72°C. To detect genomic DNA contamination, RNA was subjected to RT-PCR, without reverse transcriptase, using β-actin primer pairs. The intensity of the objective bands was quantified by densitometric scanning, using NIH Image Version 1.62 free software (NIH, Bethesda, MD). The relative abundance

of FasL was normalized against that of β-actin by establishing a ratio of FasL: β-actin.

Statistical analyses

The densitometry ratio for FasL: β-actin used to determine the expression of FasL mRNA was examined by using one-way factorial ANOVA. Differences between means were determined by a student's t-test and *p* values < 0.05 were considered statistically significant.

Results and discussion

The expression of FasL mRNA was examined by semiquantitative RT-PCR after extraction of RNA from the oviductal epithelium of the UTJ, the isthmus and the ampulla sections during pre- and post-ovulatory periods. An expected 270 bp band was detected in the samples from all segments of the pig oviduct in both periods (Fig. 1). Both pre-ovulation and post-ovulation, the FasL mRNA was expressed more intensely in the UTJ than in the isthmus and the ampulla. The densitometry ratios of FasL:(-actin for the UTJ and the other segments of the porcine oviduct differed significantly ($p<0.05$) during the pre-ovulatory period (Fig. 2). No statistical differences were detected in gene expression among the samples during post-ovulation, however, FasL expression was higher in the isthmus and the ampulla compared to these segments examined during pre-ovulation.

The local expression of FasL in the testis (Koji et al., 2001), the cornea (Bellgrau et al., 1995) and the placenta (Kauma et al., 1999) was suggested, in part, to induce immune privilege by promoting apoptosis of activated Fas-bearing lymphocytes. The function of Fas/FasL signaling occurrs when activated inflammatory lymphocytes enter the immune privilege sites; the cells bearing Fas undergo apoptosis due to their binding with FasL (Griffith and Ferguson, 1997). Thus, the oviduct would be come an immune privileged organ (Cardenas et al., 1998) similar to the cornea, the testis and the placenta.

The main functions of the pig oviduct involve in sperm transport, oocyte pick-up, oocyte maturation, fertilization and early embryo development and happen for a few days during the estrous cycle (Hunter, 1988). As described earlier, the UTJ acts as the sperm reservoir in the pig oviduct to maintain sperm fertilizable ability during pre-ovulation (Hunter, 1981). A possible aid in this regulation could be the FasL presence in the epithelium of the sperm reservoir. The higher expression of FasL in the isthmus and the ampulla after ovulation might be for the preparation of ovulated oocytes, fertilized oocytes and early embryos which are moving down to the uterus. Spermatozoa and zygote contain antigens that might be regarded as foreign by the female immune system. In pigs, the spermatozoa in the uterine lumen after insemination trigger an invasion of polymorphonuclear leukocytes and a phagocytosis process occurs (Woelders and Matthijs, 2001). Interestingly, a leukocytic attack was not seen in the pig's oviduct (Rodriguez-Martinez et al., 1990). One of the reasons that spermatozoa escape from the leukocytes in the UTJ and the isthmus is the presence of a mucous-like luminal fluid (Johansson et al., 2000) in which spermatozoa are immersed during standing estrus. This mucus contains hyaluronan (Tienthai et al., 2000) which has ability to maintain sperm viability and enables spermatozoa to avoid recognition by cytotoxic lymphocytes (Rodriguez-Martinez et al., 2001). However, the oviduct might also have a mechanism that requires the receptor and the ligand to induce an apoptosis of the cells involved in the local immune response to foreign proteins. The expression of FasL in this study may suggest that the Fas/FasL system induces Fas-mediated apoptosis in cytotoxic T lymphocytes and other Fas expressed leukocytes in the pig oviduct. The evidence might support the hypothesis that FasL can protect spermatozoa and early embryos from these cells.

In *in vitro* fertilization (IVF), oviductal epithelial cells have been used to reduce the polyspermic phenomenon (Romar et al., 2001), and improve the quality of the fertilizing oocyte until the blastocyst stage

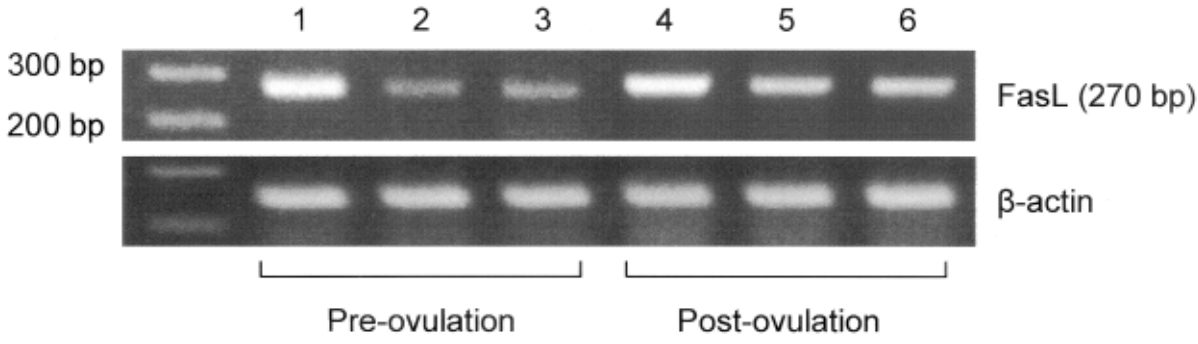

Figure 1. Relative expression of FasL mRNA in the epithelium of the pig oviduct during pre-and post-ovulatory periods. The expression of FasL and β-actin (internal positive control) is depicted. Lane 1 and 4 = UTJ (uterotubal junction), lane 2 and 5 = isthus, lane 3 and 6 = ampulla.

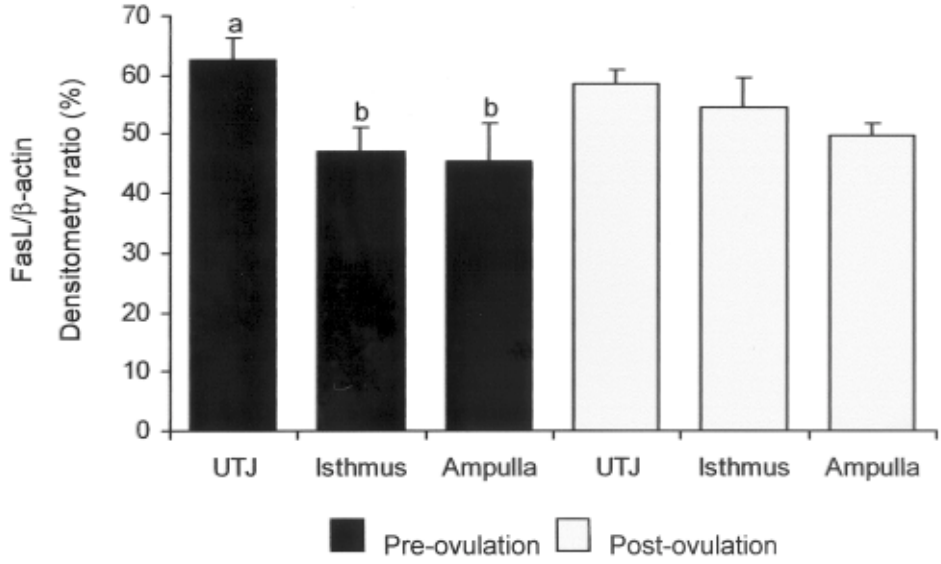

Figure 2. Representative densitometry histograms showing the ratio (%) of FasL: β-actin for the various segments of the porcine oviduct during pre- and post-ovulation. Values from bars with different labels[a,b] differ significantly ($p < 0.05$).

(Kidson et al., 2003). Xu et al. (2000) demonstrated that coculture with human, oviductal, epithelial cells enhances mouse embryo development by reducing apoptosis. Basically, the apoptotic cells were first detected at the two-cell to morula stage during embryo culture due to the lack of endogenous or exogenous survival factors in the cultured medium (Brison and Schultz, 1997). These findings suggest that the oviductal epithelial cells may provide survival factors which reduce or suppress apoptosis. Previous studies indicated that transforming growth factor-α, expressed by human, oviductal epithelium (Morishige et al., 1993), is known to suppress apoptosis in mouse embryo (Brison and Schultz, 1997). Further, the catalase that suppresses apoptosis of both spermatozoa and oocyte by preventing the formation of reactive oxygen has been found in bovine and porcineoviductal fluid (Lapointe et al., 1998). Since the mechanism by which the oviductal epithelium reduces the apoptotic process is still unclear, the expression of FasL in the pig oviduct might be considered as a Fas/FasL system which could be the main mechanism for preventing apoptosis in spermatozoa, oocytes and early embryos.

In conclusion, our results document for the first time that the expression of FasL has been detected in the porcine oviduct. However, further studies relating to Fas/FasL signaling and its influence on the survival of spermatozoa and fertilized oocytes in the pig oviduct are still needed.

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