—Research Note—

Expression of Uterine Sensitization-Associated Gene-1 (USAG-1) in the Mouse Uterus During the Peri-Implantation Period

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Abstract. Rat uterine sensitization-associated gene-1 (USAG-1) mRNA is expressed in the uterus during the peri-implantation period, and its mRNA expression in uterine epithelial cells is highest on day 5 of pregnancy. On the other hand, since changes in USAG-1 mRNA expression in the mouse uterus are not seen during the estrous cycle, USAG-1 expression might be specifically regulated by embryonic factors rather than by the maternal environment. However, the expression pattern and function of USAG-1 in the mouse uterus have not been determined. Thus, we examined the tissue-specific USAG-1 mRNA expression in the uteri of ICR mice during peri-implantation using real-time quantitative PCR. Uterine tissues, such as the myometrium, luminal epithelium, and stroma, were collected by laser capture microdissection at 3.5–6.5 dpc. USAG-1 mRNA was expressed in the uteri of pregnant mice from 3.5 dpc to 6.5 dpc, and the highest level of expression was seen at 4.5 dpc ($P < 0.01$). Significantly high USAG-1 mRNA expression was detected in the luminal epithelium at 4.5 dpc ($P < 0.05$). The stroma and myometrium exhibited unchanged expression levels of USAG-1 mRNA at 3.5–5.5 dpc. USAG-1 mRNA was undetectable in blastocysts and implanting embryos. Expression of USAG-1 mRNA appears to be associated with blastocyst implantation to the luminal epithelium, suggesting that physiological or biochemical contact of the blastocyst to the uterus is required for USAG-1 expression.

Key words: Implantation, Mouse, mRNA, Uterine sensitization-associated gene-1 (USAG-1), Uterus (J. Reprod. Dev. 53: 931–936, 2007)

For successful establishment of pregnancy, blastocyst development must proceed beyond the attachment stage and result in endometrial decidualization and subsequent placental formation. Estrogen and progesterone regulate the events that lead to the receptivity and sensitization of the endometrium for the decidual cell reaction [1]. In rodents and humans, implantation is

initiated with an apposition between the trophoblast and apical surface of the luminal epithelium. This is followed rapidly by adhesion of the conceptus to the luminal surface and then penetration through the luminal epithelium to the underlying stroma, which initiates the endometrial decidualization reaction [2, 3]. Uterine sensitization-associated gene-1 (USAG-1), a gene of unknown function during conceptus development, is upregulated in the sensitized endometrium of the rat uterus during the peri-implantation period [4].

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This molecule is expressed as an antagonist to bone morphogenetic protein (BMP) in teeth [5] and the kidney renal tubules [6] during late embryogenesis. The homology of rat USAG-1 cDNA to those of mouse and human is 96% and 91%, respectively [7]. We previously identified USAG-1 mRNA expression in mouse uteri during the estrous cycle [8]. In contrast to the temporally regulated expressions of COX-1, COX-2, EGF, LIF, and other growth factors/cytokines, USAG-1 mRNA expression did not change during the estrous cycle [8]. However, the expression pattern and function of USAG-1 in the mouse uterus during the peri-implantation period have not been studied. Thus, we examined tissue-specific expression of USAG-1 mRNA in pregnant uteri and blastocyst stage embryos following dissection of various uterine tissues through laser microdissection.

Materials and Methods

Animals

ICR and (C57BL/6J × C3H/HeN)F1 mice at 8–10 weeks of age were purchased from a commercial supplier (CLEA Japan, Tokyo, Japan) and maintained in the animal facility of the National Research Center for Protozoan Diseases at Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan. All animals were housed in

polycarbonate cages and maintained in a specific pathogen-free environment in light-controlled (lights on from 07:00 to 19:00) and air-conditioned rooms (temperature of 24 ± 1 °C, humidity of $50 \pm 10\%$). They had access to standard laboratory chow (CE-2; CLEA Japan) and water *ad libitum*. The animals used in this study were cared for and used under the Guiding Principles for the Care and Use of Research Animals set forth by the Obihiro University of Agriculture and Veterinary Medicine.

Natural mating and tissue and embryo collection

Female mice were mated with fertile ICR males or vasectomized B6C3F1 males. The morning a vaginal plug was discovered was designated 0.5 days post coitum (dpc). Plug-positive females were sacrificed by cervical dislocation at 3.5–6.5 dpc. Five animals were sacrificed each day. The uteri of the animals were excised immediately immersed into liquid nitrogen, and stored at -80 °C until use. For total RNA extraction from blastocysts, 200 blastocysts were collected from the uteri of naturally mated females at 3.5 dpc, immediately immersed in liquid nitrogen, and stored at -80 °C until use.

Laser capture microdissection (LCM) of uterine tissues

Frozen uterine tissue from pregnant and pseudopregnant animals were embedded in OCT

Fig. 1. Laser capture microdissection (LCM) of uterine tissues. LCM was performed using an AS LMD laser microdissection system (Leica) to separately collect attached blastocyst, myometrium, luminal epithelium and stromal tissue. The collected tissues were subjected to real-time quantitative PCR for detection of USAG-1 mRNA expression. A: before LCM. B: after LCM. 1. Luminal epithelium. 2. Stroma. 3. Muscle.

Table 1. Primer/probe sequence used for real-time quantitative PCR

Transcript	Primer/probe sequence (5' to 3')	GenBank accession number
USAG-1	F GAGGCAGGCATTTCAGTAGCA R TGTATTTGGTGGACCGCAGTT FAM-TCGAAACAGTCGAGTTCA-MGB	NM_025312
Beta-Actin	F GCTCTGGCTCCTAGCACCAT R GCCACCGATCCACACAGAGT FAM-ATCAAGATCATTGCTCCTC-MGB	NM_007393
18S rRNA	VIC-ABI (Part No. 4308329) F ABI (Cat No. 430448604022) R ABI (Cat No. 430449004024)	
GAPDH	VIC-ABI (Part No. 4308313) F ABI (Cat No. 0412021) R ABI (Cat No. 430410906026)	

compound (Tissue-Tech, Sakura, Tokyo, Japan). Serial cryostat sections were cut to 12–16 μm in thickness and mounted on autoclaved polyethylene naphthalate (PEN) foil on a glass slide (Leica, Wetzlar, Germany). The slides were fixed in 70% ethanol : acetic acid (19:1) for 3 min and briefly rinsed with RNase-free DEPC-treated water for 1 min. They were then stained with 0.05% toluidine blue (Merk, Darmstadt, Germany) dissolved in RNase-free DEPC-treated water for 1 min at room temperature and washed twice with RNase-free DEPC-treated water for 1 min each. The slides were then air-dried for 5 min and stored at -80°C .

LCM was performed using an AS LMD laser microdissection system (Leica) to collect the attached blastocyst, myometrium, luminal epithelium, and stromal tissues separately [9]. PEN foil slides were mounted on an AS LMD laser microdissection system with the section facing downwards. After adjusting the intensity, aperture, and cutting velocity, the pulsed UV laser beam was carefully directed along the borders of the cell layer (Fig. 1). A 20x objective was used for the laser microdissection system along with the following settings: intensity was set to 45, aperture was set to 10, speed was set to 6, and offset was set to 39. The separated tissues were transferred by gravity alone into a microcentrifuge tube cap containing TRI reagent (Sigma, St. Louis, MO, USA) that was placed directly underneath the sections.

Real-time quantitative PCR

Total RNA was extracted from frozen reproductive tissues and blastocysts by means of a TRI Reagent Kit (Sigma) according to the

manufacturer's protocol. The extracted total RNA was then subjected to real-time PCR analysis. Primers and a TaqMan probe for the USAG-1 gene and beta-actin [8] were designed using the primer design software Primer Express version 1.5 (Applied Biosystems, Foster City, CA, USA). Primers and a TaqMan probe for 18S rRNA and GAPDH were purchased from a commercial supplier (Applied Biosystems). The GenBank Accession numbers of all the cDNA sequences are summarized in Table 1. Quantification of all gene transcripts was carried out with an ABI Prism 7900 HT (Applied Biosystems). Templates for real-time PCR were obtained by reverse transcriptase reaction using total RNA. For the RT-PCR reaction, a TaqMan One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems) was used with 20 μl /tube as follows: the template (20 ng) was mixed with 2 $\times$ Master Mix without UNG, 40 $\times$ MultiScribe and RNase Inhibitor Mix, 200 nM TaqMan Probe, and 900 nM of each primer. The reaction conditions were 1 cycle at 48 $^\circ\text{C}$ for 30 min, 1 cycle at 95 $^\circ\text{C}$ for 10 min, and then 45 cycles of the amplification step (95 $^\circ\text{C}$ for 15 seconds and 60 $^\circ\text{C}$ for 1 min). The gene expression levels of USAG-1 were calculated as gene expression rates, as reported previously [10]. Briefly, the amounts of USAG-1, 18S rRNA, GAPDH and beta-actin mRNA in the samples were estimated with standard curves representing the log of the input amount (the log starting with cDNA molecules) as the X axis and the threshold cycle as the Y axis. A relative standard curve (SC) for real-time PCR was used as a common set of samples that linked the experimental PCR plates together and permitted overall analysis of the

Fig. 2. Expression pattern of USAG-1 mRNA in the uteri of pregnant and pseudopregnant mice. Total RNA was extracted from the uteri of pregnant and pseudopregnant mice and subjected to real-time PCR analysis. The results are shown as the mean $\pm$ SE. Asterisks above the error bar indicate significant difference between the experimental groups. *: $P < 0.05$.

Fig. 3. Expression pattern of USAG-1 mRNA in the uterine tissues, blastocysts, and implanting embryos of pregnant mice. An LCM was used to separately collect uterine tissue samples from pregnant mice, and then total RNA was extracted from the tissue samples and subjected to real-time PCR analysis. The results are shown as the mean $\pm$ SE. Asterisks indicate significant differences between the experimental groups. *: $P < 0.05$. The expression level of USAG-1 mRNA in the luminal epithelium at 4.5 dpc was significantly higher than that at 6.5 dpc ($P < 0.05$).

samples. Preparation and utilization of this SC as a quality control of the efficiency of amplification of the PCR plate is described elsewhere [11]. The gene expression rate was obtained by normalizing the amount of USAG-1 with that of a housekeeping gene. Kidney mRNA was used as the standard.

Statistical analysis

All data are expressed as the mean $\pm$ standard error (SE). Statistical comparisons of the relative mRNA expressions of each gene between experimental groups were analyzed by two-way analysis of variance (ANOVA) followed by a *post hoc* test using StatView 4.5 (Abac Concepts, Berkeley, CA, USA). In all statistical tests, differences were considered significant when P was < 0.05 .

Results

Expression profiling of housekeeping genes in the pregnant uteri of the mice

The geNorm software (<http://medgen.ugent.be/jvdesomp/genom>) was used to select the most appropriate housekeeping gene as an internal control [12]. The internal control gene-stability

measure (M) was calculated using geNorm. Since the M values of beta-actin, 18S rRNA, and GAPDH in the pregnant uteri were 4.134, 4.823 and 7.527, respectively, beta-actin was selected as the internal control gene for further experiments.

Expression pattern of USAG-1 mRNA in the uteri of the pregnant and pseudopregnant mice during the peri-implantation period

As shown in Fig. 2, USAG-1 mRNA expression in the uteri of the pregnant mice dramatically increased from 3.5 dpc to 4.5 dpc ($P < 0.05$) and subsequently declined at 5.5 and 6.5 dpc ($P < 0.05$). Although the differences were not statistically significant, the expression level of USAG-1 mRNA in the pregnant uterus was higher than that in the pseudopregnant uterus at 4.5 dpc. This difference may have been caused by the presence or absence of embryos in the reproductive tract.

Expression of USAG-1 mRNA in the myometrium, stroma, and luminal epithelium tissue of pregnant uteri during the peri-implantation period and in blastocysts and implanting embryos

The combination of LCM and real-time quantitative PCR revealed that the significant increase in USAG-1 mRNA seen in the pregnant

uterus at 4.5 dpc was localized mainly to the luminal epithelium (Fig. 3). On the other hand, the uterine stroma and myometrium exhibited minimal changes in USAG-1 mRNA expression at 3.5–5.5 dpc. As shown in Fig. 3, USAG-1 mRNA expression was undetectable in blastocysts collected from the uterus at 3.5 dpc and implanted embryos at 4.5, 5.5 and 6.5 dpc.

Discussion

USAG-1 expression in the reproductive tract was first found in the rat [4]. Induction of USAG-1 mRNA is restricted to the uterine glandular epithelial cells of the pregnant and pseudopregnant uteri on Day 5 [4]. Given the remarkably tight localization of its expression, USAG-1 might be involved in the onset of endometrial receptivity for implantation. It has been demonstrated that the expression of implantation-associated genes is influenced by ovarian hormones such as estrogen and progesterone [1]. We have previously examined the expression pattern of 16 implantation-associated genes, including USAG-1, during the estrous cycle in mice [8]. Expression of the majority of genes involved in the implantation process is temporally regulated during the estrous cycle [8]. However, interestingly, USAG-1 mRNA expression does not change during the estrous cycle in mice [8]. The results from these reports suggest that USAG-1 expression could be regulated by an embryonic signal(s) rather than an ovarian hormone(s).

The present study revealed that USAG-1 mRNA was expressed in both pregnant and pseudopregnant uteri throughout the peri-implantation period, i.e., from 3.5 dpc to 6.5 dpc, and was upregulated in pregnant animals on 4.5 dpc (Fig. 2). Furthermore, USAG-1 mRNA was localized exclusively in the luminal epithelium of

the pregnant uteri at 4.5 dpc (Fig. 3). The expression of USAG-1 in blastocysts at 3.5 dpc and implanting embryos at 4.5 and 5.5 dpc was not detected by analysis using real-time quantitative PCR (Fig. 3). Furthermore, USAG-1 expression was not upregulated in pseudopregnant uteri during the peri-implantation period. These results indicate that the presence of embryos and/or a factor(s) from the embryos were an essential for upregulation of USAG-1 expression in the luminal epithelium at implantation in the mice. Stimuli from implanting embryos may influence uterine endometrial tissues and subsequently upregulate USAG-1 gene expression. We believe that blastocysts make their first molecular contact with the luminal epithelium for establishment of implantation and decidualization in conjunction with the expression of USAG-1. It is known that Mif is released from the luminal epithelium and superficial glandular epithelium by IFN-tau, which is produced by trophoblast cells in the cow [13], and that the epithelial cell-derived secreted adhesion proteins are regulated by progesterone and/or IFN-tau in sheep [14]. Although mice deficient in USAG-1 appear to be fertile [15] and the physiological function/s of USAG-1 for uterine receptivity and the process of implantation are still unclear, further studies using of gene targeting should clarify the role of USAG-1 in mammalian implantation.

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