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<td>Author(s)</td>
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Immunohistochemical localization of the estrogen receptor alpha (ERα) and progesterone receptor (PR) in the uterus of sika deer (Cervus nippon) during pregnancy

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Abstract
Information on steroid hormone receptor distribution in the uterus is essential to understand the roles of their ligands in pregnancy. This study examined the spatio-temporal localization of estrogen receptor alpha (ERα) and progesterone receptor (PR) in the uterus of sika deer (Cervus nippon) to determine the estrogen and progesterone action site during pregnancy. Ovaries and uteri were collected from 21 pregnant sika deer with single fetus and two corpora lutea, ranging from Day 20 to Day 207 of pregnancy. In addition, genital organs were also collected from three sika deer whose gestational status was unknown: one female had only one developing corpus luteum: ≤ Day 4 (metestrus) and two females had two corpora lutea, one of which was at the developing stage equivalent to diestrus or early pregnancy: > Day 7 (diestrus). Staining of ERα and PR was clear in all cell types during metestrus. During diestrus, the presence of ERα was also clear in deep glandular epithelium, stroma and myometrium, whereas it was suppressed in luminal epithelium and shallow glandular epithelium. Staining of PR was suppressed in luminal epithelium but was detectable in other cell types. Staining of ERα in all cell types and PR in luminal epithelium and glandular epithelium became undetectable by Day 28. PR was presented in stroma and myometrium throughout pregnancy. The distribution pattern of ERα and PR was different during diestrus from that in a ruminant. This could be attributed to estrogen secretion from the maturing and ovulating follicles in the presence of developed corpus luteum.

Key words: pregnancy, sika deer, steroid hormone receptor, uterus

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Introduction

Sika deer (Cervus nippon) is a seasonal breeder, mating in autumn and fawning in early summer after 224-230 days' gestation. Ovarian steroid hormones should play a central role in the regulation of uterine function to establish and maintain pregnancy. Information on the steroid hormone receptor distribution in the uterus is, thus, essential to understand when and where steroid hormones (i.e., estrogen and progesterin) are required in the uterus during the estrous cycle and pregnancy.

Spatio-temporal distribution of estrogen receptor (ER) and progesterone receptor (PR) has been investigated in various domestic species, such as sheep, cattle, pigs, and horses, and has provided insights into uterine physiology. In ruminants, including cervid species, luteolysis, which is triggered by secretion of prostaglandin F2α (PGF2α) from the uterus, must be prevented to establish pregnancy. As PGF2α secretion is triggered by oxytocin through an up-regulation of oxytocin receptor (OTR) which has been up-regulated by estrogen, the distribution pattern of ER is thought to be important in the onset of PGF2α secretion in sheep. On the other hand, progesterone, the hormone of pregnancy, is essential to create a uterine environment that supports the development of the conceptus to term. It also influences uterine histotroph secretion, presumably aiding embryo survival to maintain pregnancy in sheep.

This study was conducted to characterize the spatio-temporal distribution of endometrial and myometrial ER alpha (ERα) and PR protein during the gestation period to understand the physiological roles of estrogen and progesterone. It is known that over 80% of pregnant sika deer possess two steroidogenic corpora lutea (CLs), known to be formed at a different period, even though they have a single fetus. Therefore, uterine samples from sika deer with two CLs and single fetus were exclusively used in this study except for a single sample (No. 1, Table 1), which represents the time between two ovulations.

Materials and Methods

Animals and uterine samples: Ovaries and uteri were collected in the wild from 21 pregnant sika deer (No.4-24, Table 1) and three additional females whose gestational status could not be determined (No.1-3, Table 1). The animals were shot legally on the island of Hokkaido, Japan, in January 2004, November 2005, January to May 2006 and November 2007 to January 2008. All pregnant females (No.4-24) had a single fetus and two CLs in the ovaries. Among three additional females (No.1-3), female No.1 had only one developing CL with a cavity and clot in it, indicating that she was most likely within four days after estrus (metestrus). Females No.2 and 3 had two CLs, one fully developed and the other at the developing stage. They were assumed to be seven days after estrus or later (diestrus), since development of CL takes about seven days in cattle and sheep. Since we failed to recover an embryo from these three animals, they were thus considered to be non-pregnant in this study. In sheep, it is known that there is no difference in uterine steroid hormone receptor distribution between pregnant and non-pregnant animals until Day 11. However it started to be different on Day 13: the period corresponded to the time of interferon tau (INFτ), the factor of maternal recognition of pregnancy, secretion by an embryo. Since INFτ is known to be secreted by the embryo from Day 14 in cervid species, uterine steroid hormone receptor distribution is assumed to be the same until Day 14 regardless of the gestational status. Therefore, three non-pregnant animals are used to estimate the steroid hormone receptor distribution in early pregnancy.

Estimation of fetal age: Fetal weights were measured with an electronic balance or spring scale, and crown-rump lengths were measured with a caliper. Fetal age was estimated by crown-rump...
length up to 60 days (≈ crown-rump length 80.6 mm) of pregnancy (age = −2.08 + 14.15LnX, X = crown-rump length) and fetuses over 60 days old (CRL > 80.6 mm) were estimated by fetal weight according to Suzuki et al. (age = (\sqrt{X} + 2.73)/0.091, X = fetal body weight). The fetal ages ranged from Day 20 to day 207. Since the gestational length of sika deer is reported to be about 224-230 days, samples covered almost the whole range of gestation periods.

**Immunohistochemistry:** Uteri were fixed within one hour after killing and preserved in 10% phosphate-buffered formalin for 3-18 months before embedding. Fixed tissues from the dorsal part of uterine horn in which the fetus was present were used as the sample for immunohistochemistry. In three animals (No.1-3), in which the presence of an embryo was not confirmed, tissue samples were obtained from the uterine horns ipsilateral to the ovary with the CL. After fixation, the specimens were dehydrated in an ethanol series and embedded in paraffin. Tissues were cut into 5-µm-thick paraffin sections and mounted on silane-coated slide glass (MAS, S9226, Matsunami, Osaka, Japan).

To reveal the presence and distribution of ERα and PR in the uteri, the avidin-biotin immunoperoxidase technique (Vectastain® ABC kit; Vector Laboratories, Inc., Burlingame, U.K.) was used. After deparaffinization through an ethanol gradient and rehydration in phosphate-buffered salines, the sections were incubated briefly in an antigen-unmasking solution, Target Retrieval Solution (Dako Cytomation, Carpinteria, CA, USA) for 40 min at 99°C. The sections were then incubated in 3% hydrogen peroxide/methanol solution for 10 min to block the endogenous peroxidase activity, and treatment with blocking serum was applied to each section to prevent nonspecific reactions. Primary antibodies were added to sections overnight at 4°C for incubation. The rabbit polyclonal antibody (PA1-309, Affinity Bioreagents, Golden, CO, U.S.A.) to the synthetic peptide corresponds to amino acid residues 21-32 from human ERα which were completely conserved in several species, including the cervid species (accession X98007) and cross react with human, porcine, and rat ERα was used as primary antibody to ERα. Also the mouse monoclonal antibody (Ab-8, Neo Markers, Fremont, CA, U.S.A.) to human PR which cross reacts with human, horse, sheep and pig PR was used as primary antibody to PR. Negative controls were treated with normal rabbit serum or normal mouse serum. Colorization was performed by 3, 3’-diaminobenzidine-H2O2 solution (0.02% w/v) for 5 to 10 min. Steroid receptor staining intensities were scored visually referring to Spencer and Bazer (- absent, ± scattering, + weak, ++ moderate, or +++ strong) in the luminal epithelium (LE), glandular epithelium (GE), endometrial stroma (ST) and myometrium (MYO). In addition, GE and ST were distinguished into shallow and deep parts when there was a difference in staining. We classify the area adjacent to epithelium as shallow and the area adjacent to myometrium as deep region.

**Results**

**Estrogen receptor localization**

Immunoreactive ERα were detected predominantly in the nuclei of uterine cells. Results of ERα staining in uteri are summarized in Table 1. In the uterus of No.1 (≤ Day 4), strong staining of ERα was observed in the deep GE, ST, and MYO, while the LE and shallow GE showed moderate staining (Fig. 1A, B). In deep GE, ST and MYO of uteri of female No. 2 and 3, ERα staining was similar to that in uterus of No. 1 (Fig. 1D), while the staining in the LE and shallow GE was weaker in No. 2 than in No. 1 (Fig. 1C). Staining of ERα in LE became undetectable by Day 20 (No. 4), and remained undetectable up to Day 207 (No. 24). In the deep GE, ST and MYO, weak to moderate ERα staining were observed on Day 20 (No. 4, Fig. 1E), but the staining became undetectable by Day 28 (No. 7) and thereafter (Fig. 1F). The staining became detectable during late pregnancy again (Day
No appreciable staining was detected in uterine tissue sections incubated with normal rabbit serum substituted for primary antibody (Fig. 1H).

**Progesterone receptor localization**

Immunostaining for PR was localized predominantly in the nuclei of uterine cell. Results of PR staining in uteri are summarized in Table 1. The staining of PR in LE was only detected in No. 1 (≤ Day 4, Fig. 2A) and No. 4 (Day 20). In the GE, the staining of PR was detectable in No. 1-3 (Fig. 2A-D) and No. 4 (Day 20, Fig. 2E), and became undetectable by Day 25 (No. 6). In contrast, moderate to strong staining was detectable in the ST and MYO in all samples (Fig. 2F). From Day 164 to Day 207 (No. 21-24), they were consistently detected as strong (Fig. 2G). No appreciable staining was observed in sections incubated with normal mouse serum in place of primary antibody (Fig. 2H).

**Discussion**

This is the first study to describe the distribution of steroid hormone receptors in the uterus of cervid species. The results indicate that distribution of uterine ERα and PR is differentially regulated in both a spatial and temporal manner dur-

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ERα, estrogen receptor alpha; PR, progesterone receptor; LE, luminal epithelium; GE, glandular epithelium; ST, stroma; MYO, myometrium; CL, corpus luteum;

“Fetal ages were estimated by crown-rump length up to 60 days according to Yanagawa et al.52, and by fetal body weight thereafter according to Suzuki et al.50. Days for No.1-3 are estimated days after estrus.

- Absent; ±, Scattering; +, Weak; ++, Moderate; ++++, Strong
Fig. 1. Immunohistochemical localization of estrogen receptor alpha in the uterus of sika deer.
(A) Endometrium of female with one CL (No. 1). (B) Myometrium of female with one CL (No. 1). (C) Endometrium of female with two CLs, developed and developing CL (No. 3). (D) Myometrium of female with two CLs, developed and developing CL (No. 3). (E) Myometrium of female on Day 20 of pregnancy (No. 4). (F) Endometrium and myometrium of female in mid-pregnancy (No. 12). (G) Myometrium of female in late pregnancy (No. 21). (H) Negative control. LE, luminal epithelium; GE, glandular epithelium; ST, stroma; MYO, myometrium. Scale bar = 100 μm.
ing pregnancy. The distribution pattern of ER and PR differed between the endometrium and myometrium, and also among cell types in the endometrium.

In the uterus with one developing CL assumed to be within several days after estrus (No. 1), the staining of ER and PR were strong or moderate in all cell types. This is similar to that of a cyclic ruminant. The strong or moderate staining of ER and PR may be due to the influence of a high concentration of circulating estrogen from the graafian follicle at the estrus, since estrogen is known to up-regulate the ER and PR.

Although the present results show a trend in which ER staining decreases with days after estrus is similar to the change of ER in sheep, a domestic species in which steroid receptor distribution has been well documented, the time and extent of decline may differ between deer and sheep. In sheep, staining of ER in all cell types decreased dramatically by Day 11 of the estrous cycle or pregnancy and remained weak thereafter, when animals are pregnant, while staining of ER delayed to decrease in sika deer and was detectable as late as Day 25 in the ST. The difference in the time of ER suppression between sheep and deer could be attributed to estrogen secretion by the follicle which is maturing and ovulating after formation of the first CL (>Day 7). This hypothesis could be verified when ER staining in pregnant deer, which does not form a second CL, decreased around Day 11 as in sheep. Alternatively, the delay in suppression of ER staining in shallow GE, ST and MYO up to Day 25 may suggest a potential role of estrogen in these cell types during early pregnancy in this species.

Lower staining of ERα in the LE and shallow GE than in the other part of the uterus is comparable to sheep. Since OTR appearance is regulated by estrogen, down-regulation of ER may have the advantage of preventing the luteolytic mechanism via OTR down-regulation as oxytocin stimulates the synthesis of luteolytic PGF2α secretion. Down-regulation of ER in LE and shallow GE may be important since PGF2α is thought to be secreted from LE and shallow GE because of the specific localization of cyclooxygenase-2, an important enzyme for PGF2α synthesis in these cell types in sheep. The mechanism of ER appearance which is regulated differently between cell types is not clear, but down-regulation of ER in LE and shallow GE might be due to the effect of INF. INF produced by the conceptus and involved in the maternal pregnancy recognition is known to act in a paracrine fashion on LE and shallow GE to suppress transcription of ER and OTR genes, thereby preventing development of the endometrial luteolytic mechanism in sheep. In red deer, INF may have been responsible for the reduced staining of ERα in LE and GE, which was similar to that in sheep, although the gestational status (i.e., the presence of an embryo) of No. 2 and 3 was unknown.

Staining of PR in uteri of No. 2 and 3 was relatively low in LE. Moreover, staining in LE and GE declined after Day 20 and became undetectable by Day 25. Decline of PR in LE and GE may be due to decline of PR up-regulation by estrogen since ERα appearance was suppressed at this period, or to prolonged exposure to progesterone action since progesterone has negative effects on PR appearance. On the other hand, high staining of PR in ST and MYO was detected during early pregnancy (Day 20-25) and might be important to establish and maintain pregnancy.

During Day 28 to Day 127 (No. 7-20), ERα staining diminished in all cell types, possibly due to a high level of circulating progesterone as it down-regulated ER appearance and to prevent PGF2α secretion and myometrial contraction for the maintenance of pregnancy. On the other hand, the reactivity of PR remained high in ST and MYO, consistent with that observed in sheep and cattle. The PR in ST may be required for proliferation of the endometrium which is stimulated by stromal cell-derived growth factors induced by progesterone. Presence of PR in MYO throughout pregnancy might also indicate a direct influ-
Fig. 2. Immunohistochemical localization of progesterone receptor in the uterus of sika deer.
(A) Endometrium of female with one CL (No. 1). (B) Myometrium of female with one CL (No. 1). (C) Endometrium of female with two CLs, developed and developing CL (No. 3). (D) Myometrium of female with two CLs, developed and developing CL (No. 3). (E) Myometrium of female Day 20 of pregnancy (No. 4). (F) Myometrium of female in mid-pregnancy (No. 16). (G) Myometrium of female in late pregnancy (No. 21). (H) Negative control. LE, luminal epithelium; GE, glandular epithelium; ST, stroma; MYO, myometrium. Scale bar = 100 µm
ence of progesterone on contractile activity of this layer, finally resulting in myometrial quiescence.

In late pregnancy, from Day 164 and later (No. 21-24), ERα staining was detected weakly in all uterine cell types except for LE. It may be due to secretion of estrogen toward parturition as reported in domestic animals. Increase in estrogen secretion toward term is also reported in cervid species; red deer, reindeer and white-tail deer. The presence of ERα in LE and GE is crucial in late pregnancy since ER up-regulation may increase OTR appearance, which stimulates production of PGF2α, a luteolytic factor. Also, the presence of ERα in ST might play an important role in luteolysis since estrogen induces epithelial proliferation through ER-positive ST. The presence of ER in MYO may be important because oxytocin, which promotes myometrial contractility, coacted with PGF2α during late pregnancy and parturition. Interestingly, the timing of ERα up-regulation, which may be caused by estrogen, was about eight weeks prepartum in sika deer while estrogen secretion has been reported to increase about six weeks prepartum in other cervid species. Paired samples of uteri and peripheral blood of sika deer of known gestational age are needed to confirm the timing in increase of estrogen and up-regulation of ER in sika deer.

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