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Expression and Possible Role of 20 α -Hydroxysteroid Dehydrogenase in the Placenta of the Goat

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Abstract. 20 α -Hydroxysteroid dehydrogenase (20 α -HSD) catalyzes the conversion of progesterone to its inactive form 20 α -dihydroprogesterone (20 α -OHP). 20 α -HSD is expressed in the murine placenta, suggesting a role, yet unidentified, played by this enzyme during the course of pregnancy. To elucidate the possible roles of 20 α -HSD during pregnancy, 20 α -HSD gene expression in the placenta was examined by Northern blot analysis, and progestin (progesterone and 20 α -OHP) concentrations in the maternal and fetal sera and the amniotic fluid were measured by radioimmunoassay in pregnant Shiba goats. The expression of 20 α -HSD mRNA was observed in the placenta and the intercaruncular part of the uterus during mid to late pregnancy. Analysis by in situ hybridization revealed that 20 α -HSD mRNA was mainly localized in the endometrial epithelium of the caruncle side of the placenta. Considerable enzyme activity of 20 α -HSD was also detected in the cytosolic fraction of the placenta and intercaruncular part of the uterus. Although concentrations of progesterone and 20 α -OHP in the maternal serum showed similar profiles, progesterone levels in the fetal serum stayed extremely low throughout the pregnancy. The 20 α -OHP concentration in the fetal serum was always higher than that in the maternal serum. In the amniotic fluid, the concentrations of both progesterone and 20 α -OHP remained at very low levels throughout the pregnancy. These results support the notion that 20 α -HSD protects the fetus from the cytotoxic effects of progesterone, and thereby maintains the normal development of the fetus.

Key words: 20 α -Hydroxysteroid dehydrogenase, Progesterone, 20 α -Dihydroprogesterone, Placenta, Goat

(J. Reprod. Dev. 51: 265–272, 2005)

20 α -Hydroxysteroid dehydrogenase (20 α -HSD; EC.1.1.1.149) is an enzyme that catalyzes the conversion of progesterone to its inactive form, 20 α -dihydroprogesterone (20 α -OHP). 20 α -HSD is expressed in the corpus luteum of rats and mice, and is recognized to be involved in the process of functional luteolysis during their characteristically

short estrous cycles [1, 2]. In these rodent species, 20 α -HSD activity in the corpus luteum is suppressed by prolactin or placental lactogens during pregnancy [3–5] and abruptly increases prior to parturition, which is associated with a decrease in progesterone levels and a reciprocal increase in 20 α -OHP levels [6, 7]. Interestingly, 20 α -HSD has also been localized in the placenta of rats [8], mice [9], goats [10] and humans [11, 12], though the physiological role of 20 α -HSD in the

Accepted for publication: December 27, 2004

Published online: February 10, 2005

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placenta remains to be elucidated.

Progesterone is essential for preparing the body for pregnancy and, if pregnancy occurs, maintaining it by preparing the endometrium for possible implantation and inhibiting uterine contractions until birth. On the other hand, it is known that progesterone exerts inhibitory effects on the proliferation of several cell types (reviewed in [13]). For example, 20 α -HSD in T lymphocytes is considered to promote cell proliferation by exempting them from the cytostatic action of progesterone [14]. It is also noteworthy to mention that 20 α -HSD activity is present in some neoplastic tissues [15]. Furthermore, it has been shown that, if progesterone is injected in large quantities into the amniotic sacs of fetuses or subcutaneously to pregnant mice, it causes fetal death [16]. These observations suggest that progesterone at high levels has some adverse effects on the development of fetuses, while it is mandatory for maternal tissues to maintain pregnancy. Accordingly, 20 α -HSD in the placenta may be involved in reducing the cytotoxic effects of progesterone the developing fetuses.

The cDNAs for both rat [17] and mouse 20 α -HSD [9] and the genomic DNA for mouse 20 α -HSD [18] have been cloned in our laboratory, and we also recently cloned goat 20 α -HSD cDNA, which has 74% and 76% homology to rat and mouse 20 α -HSD cDNAs, respectively [10]. We have also shown that bacterially expressed protein using cloned goat 20 α -HSD cDNA indeed possesses 20 α -HSD activity [10]. In addition, we detected expression of 20 α -HSD mRNA in the goat placenta, but not in the corpus luteum, liver, spleen or adrenal gland, at day 90 of pregnancy by means of RT-PCR [10]. To determine the possible physiological roles of 20 α -HSD in the maintenance of pregnancy in the goat, 20 α -HSD gene expression in the placenta together with changes in progestin concentrations in the maternal and fetal sera and amniotic fluid during pregnancy of the goat were investigated in the present study.

Materials and Methods

Experimental animals and sampling

Female Shiba goats (*Capra hircus*) of 2 to 4 years of age that have been maintained for experimental purposes as a closed colony at the experimental

farm of The University of Tokyo were used in this study. The experiments were conducted according to the Guidelines for the Care and Use of Laboratory Animals, Graduate School of Agriculture and Life Sciences, The University of Tokyo. All of the goats used were confirmed to have regular estrous cycles. The day of estrous was determined by behavioral estrous signs and the female goats were mated with a fertile male on that day, which was designated day 0 of pregnancy.

Pregnant uteri were collected on days 40 (n=4; as representatives of early pregnancy) and 90 (n=4; as representative of mid pregnancy) of pregnancy under general anesthesia, which consisted of the intravenous injection of ketamine (150–200 mg) followed by inhalation of isoflurane and oxygen. The end-tidal isoflurane concentration was controlled between 1 to 2%. Pregnant uteri on days 130 (n=4; as representatives of late pregnancy) and 145 (n=3; as representatives of term of pregnancy) of pregnancy were collected after sacrificing the animals by bleeding from the jugular artery following deep ketamine anesthesia. The placenta (both caruncle and cotyledon) and intercaruncular uterine tissue were dissected from the pregnant uteri, frozen immediately in liquid nitrogen, and stored at –80 C until further use. Sera and amniotic fluids were obtained from pregnant goats and fetuses, respectively, on days 40, 90, 130 and 145 of pregnancy and stored at –20 C.

Northern blot analysis

Total RNA was extracted from the placenta and intercaruncular part of the uterus on days 40, 90, 130 and 145 of pregnancy using TRIzol reagent (Life Technologies Inc., Rockville, MD) according to the manufacture's instructions. DNA fragments of about 1 kilobase, containing the entire coding region of the goat 20 α -HSD [10], were removed from the pGEM^R-T vector (Promega, Madison, WI) by Pst I and Sph I digestion and purified with a QIA quick gel extraction kit (QIAGEN Inc, Valencia, CA). An aliquot of the above sample was used as a template to prepare the probe. The probe was prepared by ³²P labeling using a random primer extension labeling system (Roche Diagnostics GmbH, Mannheim, Germany). Briefly, 9 μ l (50 ng) of DNA solution were heated at 100 C for 5 min and immediately placed on ice for 2 min to denature the template. After that, 2 μ l of reaction mixture containing random primers, 3 μ l of dNTP

mix (without dCTP), 5 μ l of [α -³²P]-dCTP (3000 Ci/mmol) and 1 μ l of DNA polymerase were added to the DNA solution, mixed well and incubated at 37 C for 2 hours. After 2 hours of incubation, 80 μ l of TE buffer (Tris EDTA buffer) were added to the reaction mixture, and the labeled probe was purified using ProbeQuantTMG-50 micro columns (Amersham Biosciences, Piscataway, NJ). Northern blot analysis using 20 μ g total RNA/lane was performed as described previously [19]. After electrophoresis on agarose gel, RNA samples were transferred to a membrane (Biodyne; Pall Corp., Pensacola, FL) using a capillary elution method. After 2 hours of prehybridization in the hybridization solution (6 $\times$ SSC, 5 $\times$ Denhardt's, 0.5% SDS, 0.1 mg/ml salmon sperm DNA, 50% formamide), the probe solution was added and incubated at 42 C overnight. The membrane was exposed to X ray film (Kodak, Rochester, NY) and kept at -80 C for 2 days; the X ray film was developed and analyzed using an NIH image analyzer.

In situ hybridization

Histological analysis by in situ hybridization of 20 α -HSD mRNA expression was performed using the placental tissue from day 130 of pregnancy. The tissues were fixed in 4% paraformaldehyde in phosphate buffered-saline (PBS) followed by immersion in 30% sucrose in PBS, then they were sectioned at 10 μ m thickness using a cryostat at -20 C. The sections were mounted on slides and stored at -80 C. The entire coding region of the 20 α -HSD cDNA in pGEM^R-T vector was used as a template for the preparation of the cRNA probe. After linearizing the plasmid with Sal I (for synthesis of the sense probe using T7 promoter) or Nco I (for synthesis of the antisense probe using SP6 promoter), digoxigenin (DIG)-labeled sense and antisense cRNA probes were synthesized using T7 and SP6 RNA polymerases with a DIG RNA labeling kit (SP6/T7) (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Briefly, 1 μ l (1 μ g) of template cDNA, 2 μ l of 10 $\times$ NTP labeling mix (10 mM NTP), 2 μ l of 10 $\times$ transcription buffer, 20 U of RNase inhibitor, 40 U SP6 or T7 RNA polymerase and 12.5 μ l of RNase free water were mixed well and incubated at 37 C for 3 hours. After that, the probes were stored at -80 C until use. In situ

hybridization was performed according to the method described previously [9]. Probe solutions were applied to the slide sections. The slides were placed in a covered moisture box moistened with 2 $\times$ SSC and kept at 50 C overnight. After hybridization, the sections were washed with 2 $\times$ SSC at 50 C for 10 min, 50% formamide/2 $\times$ SSC solution at 50 C for 20 min, twice, and 0.2 $\times$ SSC at 50 C for 20 min to remove any extra probe material. Then, the sections were subjected to immunological detection for the DIG-labeled probe.

Analysis of enzyme activity

The activity of 20 α -HSD was measured in the placenta and the intercaruncular part of the uterus on day 130 of pregnancy. This stage was chosen since it was previously reported as the stage of the highest expression of 20 α -HSD mRNA. To prepare the placental and uterine cytosol, samples were homogenized with 5 mM potassium phosphate buffer (containing 1 mM EDTA, 10% glycerol and 1 mM DTT, pH 7.0) and centrifuged at 4 C for 90 min at 105,000 $\times$ g. After centrifugation, supernatants (cytosol) were collected and stored at -80 C. The protein concentrations of the supernatants were measured by the Bradford method using bovine serum albumin (BSA) as a standard. Five hundred microliters of the substrate solution (0.1 M Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, 300 μ M NADP and 60 μ M 20 α -OHP) were incubated at 37 C for 3 min and the enzymatic reaction was initiated by adding 25 μ l of cytosol. Changes in the absorbance at 340 nm were measured using a spectrophotometer. The initial velocity of NADPH production was calculated, and the enzyme activity was expressed as the formation of nmol NADPH/min/mg of cytosolic protein.

Hormone assay

The serum and amniotic fluid samples (500 μ l) were diluted to 1 ml with distilled water and extracted twice with 4 ml of diethyl ether. The extracts were subjected to radioimmunoassay for progesterone and 20 α -OHP using specific antibodies, as described previously [20]. The range of linearity was between 50-1600 pg for both progesterone and 20 α -OHP. The intraassay coefficients of variation were 7.9% for progesterone and 9.0% for 20 α -OHP.

Results

Expression of 20 α -HSD mRNA in the placenta and uterus

Changes in the 20 α -HSD mRNA expression in the placenta and intercaruncular part of the uterus on days 40, 90, 130 and 145 of pregnancy are shown in Fig. 1A and 1B, respectively. 20 α -HSD mRNA expression was low or at a minimum in the placenta on day 40 of pregnancy, but it had increased by day 90 and remained high until the term of pregnancy (Fig. 1A). 20 α -HSD mRNA was not detected in the interplacental part of the uterus on day 40 of pregnancy, whereas low expression was detected on day 90 and strong signals were present on days 130 and 145 (Fig. 1B).

Localization of 20 α -HSD mRNA in the placenta

To determine the cell types responsible for the 20 α -HSD mRNA expression in the placenta, in situ hybridization analysis was performed on the placenta obtained on day 130 of pregnancy. As shown in Fig. 2, 20 α -HSD mRNA was mainly localized in the endometrial epithelium on the caruncle side of the placenta.

20 α -HSD enzyme activity in the placenta and uterus

20 α -HSD enzyme activity was determined in the cytosolic fraction of the placenta and the interplacental part of the uterus on day 130 of pregnancy. The enzyme activity was expressed in terms of the formation of nmol NADPH/min/mg of cytosolic protein. The enzyme activity in the placenta was 5.65 ± 3.49 (mean $\pm$ SEM, $n=4$), and that in the uterus was 0.95 ± 0.53 (mean $\pm$ SEM, $n=4$).

Progesterone and 20 α -OHP levels in the maternal and fetal sera and amniotic fluids

Changes in progesterone and 20 α -OHP levels in the maternal and fetal sera and amniotic fluids on days 40, 90, 130 and 145 of pregnancy are shown in Fig. 3A, 3B, and 3C, respectively. Since blood samples from fetuses on day 40 of pregnancy could not be obtained, the values in the fetal serum for this day were not available. In the maternal serum, the highest progesterone concentration was observed on day 90 of pregnancy, and thereafter the concentration gradually decreased toward the term of pregnancy. 20 α -OHP concentrations in the maternal serum changed in a similar way to

Fig. 1. Northern blot analysis of 20 α -HSD mRNA expression in the placenta (A) and intercaruncular part of the uterus (B) on days 40, 90, 130 and 145 of pregnancy in goats. Representative blots of 4 separate experiments are shown.

progesterone, though the highest concentration was observed on day 130 of pregnancy. In the fetal serum, progesterone concentrations were extremely low throughout the pregnancy as compared to those in the maternal serum. Conversely, 20 α -OHP concentrations in the fetal serum were higher than those in the maternal serum at all stages examined throughout pregnancy in this study. In the amniotic fluids, concentrations of both progesterone and 20 α -OHP were kept low throughout pregnancy.

Discussion

The present study demonstrated that the placenta and intercaruncular part of the uterus of the goat expressed 20 α -HSD mRNA during pregnancy. This is consistent, at least partially, with previous reports showing the presence of mRNA [9, 10, 12, 21, 22], protein [8] and the activity [8, 11] of 20 α -HSD in the placenta and/or uterus of

Fig. 2. Localization of 20 α -HSD mRNA expression in the endometrial epithelium on the caruncle side of the placenta by *in situ* hybridization. A, antisense probe; B, sense probe; C, an enlargement of the boxed area in A. EE, endothelial epithelium.

several mammalian species. The expression of 20 α -HSD in the intrauterine tissue in goats was especially prominent in the latter half of pregnancy, when the maternal serum progesterone levels were high. There are progesterone and glucocorticoid response elements in common in the promoter region of the 20 α -HSD gene of both rats [23] and mice [19]. Although it has been suggested that progesterone inhibits the expression of the 20 α -HSD gene through glucocorticoid receptors in the rat corpus luteum [24], certain levels of progesterone appear to be able to stimulate 20 α -HSD expression in human endometrial stromal cells [12]. Since progesterone receptors have been shown to be present in human decidual cells [25] and in sheep endometrial stromal cells [26], the increase in progesterone concentrations beyond a certain level may be a prerequisite for the induction of 20 α -HSD gene expression in the placenta and uterus during pregnancy. On the other hand, although the maternal serum progesterone levels decreased at the term of pregnancy, the 20 α -HSD mRNA expression level remained high in the intrauterine tissues. In goats, prostaglandin (PG) F_{2 α} levels increase in the uterine venous blood before parturition [27]. It has been shown that PGF_{2 α} induces 20 α -HSD gene expression in the rat

ovary [28], and that the promoter of the human 20 α -HSD gene is activated by PGF_{2 α} [21]. These observations suggest that high 20 α -HSD mRNA expression in the placenta at the term of pregnancy is due to the local effect of PGF_{2 α} on the 20 α -HSD gene expression in the uterus. However, since the 5'-flanking region of the goat 20 α -HSD gene has not yet been characterized, further studies are needed to clarify the regulation of 20 α -HSD expression in the goat.

The present study has shown that 20 α -HSD mRNA was mainly localized in the endometrial epithelium on the caruncle side of the placenta on day 130 of pregnancy. In the rat, expression of the 20 α -HSD protein was immunohistochemically demonstrated in decidual cells, trophoblastic giant cells, spongiotrophoblasts and visceral yolk sac [8]. In the mouse, *in situ* hybridization analysis revealed that 20 α -HSD mRNA was localized in the endometrial epithelial cells, maternal placental endothelial cells and the fetal epidermal cells during pregnancy [9]. In addition, 20 α -HSD mRNA was also localized in the peripheral maternal venous plexus of the mouse placenta, in which the fetal chorionic epithelium is bathed in maternal blood. In contrast to rodent placenta, the placenta of ruminants is a non-invasive type and

Fig. 3. Progesterone and 20α-OHP concentrations in the maternal serum (A), fetal serum (B) and amniotic fluids (C) during pregnancy. Values are means $\pm$ SEM (n=4).

the maternal endometrial epithelium contributes to form the placenta, and thereby functions as one of the barriers between the maternal and fetal blood.

This difference in the microscopic structure and function of the placenta between rodents and ruminants may be related to the differences in the histological distribution of 20α-HSD between them.

Since 20α-HSD mRNA expression was observed in the placenta and intercaruncular part of the uterus, the enzyme activity was also analyzed in the cytosolic fraction on day 130 of pregnancy. Considerable activity was detected in the goat placenta, which was almost comparable to that detected in the rat placenta, though at a much lower level than that in the rat ovary during the luteolytic phase [8]. This observation confirms that not only mRNA expression, but also enzyme activity is present in the goat placenta and it indicates the presence of translation of the mRNA message to the active protein. Although the expression level of mRNA of 20α-HSD in the intercaruncular part of the uterus was almost the same as that in the placenta, the enzyme activity in the uterus was less than one fifth of that in the placenta. The reason for this difference currently remains to be resolved, but there may be some differences in the post-transcriptional regulation of 20α-HSD enzyme activity between the endometrial epithelium of the caruncle and of the intercaruncular part of the uterus.

In the present study, changes in 20α-OHP as well as progesterone concentrations in the maternal serum, fetal serum and amniotic fluid during the pregnancy of the goat were determined. The maternal serum 20α-OHP levels increased from the early to mid pregnancy, remained high at late pregnancy, and decreased at the term, a similar pattern to that reported by Sawada *et al.* [29]. This indicates that 20α-HSD is expressed throughout pregnancy in the goat. 20α-OHP concentrations in the fetal serum were even higher than those in the maternal serum at all the stages of pregnancy examined, while the fetal progesterone levels stayed extremely low at all the stages examined. These observations suggest that 20α-HSD expressed in the placenta indeed leads to the conversion of progesterone to 20α-OHP, with a resultant decrease in progesterone levels in the fetal serum. This conversion may also take place within the fetuses, since it has been shown that 20α-HSD activity in fetal lamb blood continues to increase throughout pregnancy and rapidly disappears from the newborn [30]. Both progesterone and 20α-OHP concentrations were further decreased in the

amniotic fluid. This is consistent with the report by Shiota *et al.* [8], who showed that, in the rat, the progesterone concentration in the amniotic fluid was only 3–4% of that in the maternal serum, though they did not measure the 20 α -OHP concentration. Thus, the progesterone concentration is much lower, not only within the fetus itself, but also in its surrounding environment compared with that in the mother. The decrease in the 20 α -OHP concentration also suggests that 20 α -OHP is further metabolized in and/or around the fetus.

In summary, the present study has demonstrated that 20 α -HSD mRNA expression and its enzyme activity are present in the endometrial epithelium on the caruncle side of the placenta and the intercaruncular part of the uterus during the pregnancy of goats. Progesterone levels in the fetal serum and surrounding amniotic fluids were extremely low, while the 20 α -OHP level in the fetal

serum was higher than that in the maternal serum during pregnancy. These observations suggest that the placental 20 α -HSD regulates the progesterone concentration in the fetus and surrounding amniotic fluid, thereby protecting the fetus from the cytotoxic effects of progesterone and maintaining normal pregnancy. Since expression of 20 α -HSD in the placenta has been reported in many mammalian species, 20 α -HSD may be commonly involved in maintaining pregnancy among mammals, though it plays a specific role in luteolysis in rodent ovaries.

Acknowledgements

This study was supported in part by Research for the Future Program, the Japan Society for the Promotion of Science (JSPS-RFTF 97L00904).

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