Polyclonal Antibody to a 37-kDa Recombinant Protein Derived from Bovine 20α-Hydroxysteroid Dehydrogenase

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ABSTRACT

We prepared the polyclonal antibody anti-20 α-hydroxysteroid dehydrogenase (anti-20 α-HSD) against the recombinant full-length protein bovine 20 α-HSD in *Escherichia coli*. The specificity of anti-20 α-HSD was demonstrated using Chinese hamster ovary (CHO) cells transfected with recombinant bovine 20 α-HSD and bovine placental tissues. According to western blot analysis, anti-20 α-HSD specifically recognizes the 37-kDa protein bovine 20 α-HSD. The protein is not present in untransfected CHO cells. Anti-20 α-HSD also recognizes a specific protein in the ovaries and placenta of other animals. Immunostaining was used to detect expression of bovine 20 α-HSD protein in the cultured luteal cells during the estrous cycle later.

(Key words : Polyclonal antibody, Bovine, 20 α-HSD, Ovary, Placenta)

INTRODUCTION

The recent cloning and expression of the bovine 20 α-hydroxysteroid dehydrogenase (HSD) cDNA revealed that this enzyme is a member of the aldo-keto reductase (AKR) family (Naidansuren *et al.*, 2011). A group of AKRs known as HSDs play critical roles in modulating and regulating of steroid hormones (Jez *et al.*, 1997; Penning, 2003) such as androgens, estrogens, and progestins, and as such are considered important targets for drug design (Penning *et al.*, 2004). Progesterone production in the rodent corpus luteum (CL) is regulated by hormones, including prolactin (PRL) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), through their luteotropic and luteolytic functions, respectively (Stocco *et al.*, 2001a,b).

These drug targets play a major role in progesterone metabolism and maintaining pregnancy (Lambert *et al.*, 1995; Zhang *et al.*, 2000) via formation of progestin, the inactive form of progesterone (20 α-hydroxyprogesterone). Accumulation of excess progestin can ultimately lead to premature birth and infant mortality (Lewis *et al.*, 2004; Piekorz *et al.*, 2005). 20 α-HSD also plays a crucial role in terminating pregnancy and initiating parturition (Seo *et al.*, 2011; Seong *et al.*, 2002). Accordingly, 20 α-HSD in the placenta may be involved in reducing the cytotoxic effects of progesterone in the developing fetus. Several 20 α-HSD isoforms have been identified. Seong *et al.* (1992, 2002) purified 20 α-HSD and found two distinct 20 α-HSD molecules (HSD-1 and HSD-2). Notably, 20 α-HSD has also been found in the placenta of rats (Shiota *et al.*, 1993), mice (Ishida *et al.*, 1999), goats (Jayasekara *et al.*, 2004), bovine (Naidansuren *et al.*, 2011), deer (Naidansuren *et al.*, 2012), monkeys (Nanjidsuren *et al.*, 2011), pigs (Seo *et al.*, 2011) and humans (Nakajima *et al.*, 2003; Sticker *et al.*, 1981; Zhang *et al.*, 2000), although the physiological role of 20 α-HSD in the placenta remains to be elucidated.

A tissue distribution study in ruminant animals revealed that 20 α-HSD is expressed in the placenta and ovaries. Goat 20 α-HSD mRNA was primarily localized to the endometrial epithelium on the caruncle side of the placenta (Jayasekara *et al.*, 2005). We recently reported the molecular characterization of the bovine, porcine, deer, and monkey 20 α-HSD gene (Naidansuren *et al.*, 2011, 2012; Nanjidsuren *et al.*, 2011; Seo *et al.*, 2011). In particular, we sequenced bovine 20 α-HSD cDNA, analyzed sequence homology, and produced the recombinant protein in Chinese hamster ovary (CHO) cells.

In this study, we developed a specific antibody that can be used to examine the structure, synthesis and processing of bovine 20 α-HSD. An antibody against recombinant 20 α-HSD protein produced in *Escherichia coli* can be used for 20 α-HSD biochemical characterization analysis due to its ability to recognize 20 α-HSD from several species using western blot analysis or immunohistochemistry.

* This work was supported by a research grant from the Academic Research Foundation of Hankyong National University for a scholarly exchange program in 2011).

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MATERIALS AND METHODS

Materials

The cloning vector pCR2.1 and expression vectors pcDNA3 and pcDNA4/HisMax were purchased from Invitrogen (Carlsbad, CA, USA). Restriction enzymes and the DNA ligation kit were purchased from Takara (Tokyo, Japan). PRO-PREP protein extraction solution was obtained from Intron Biotechnology (Seoul, Korea). A Lumi-Light Western blot kit was bought from Roche (Basel, Switzerland). Horse serum, goat serum, an avidin-biotin-HRP (ABC) detection kit, 3,30-diaminobenzidine (DAB), hematoxylin and methyl green were purchased from Vector Laboratories (Burlingame, CA, USA). Anti-rabbit immunoglobulins and Alexa 488 secondary antibody were purchased from Dako Cytomation (Glostrup, Denmark). Competent *E. coli* cells were from Yeastern Biotech Co. Ltd. (Seoul, Korea). Serum-free CHO-S-SFM II and Lipofectamine 2000 reagents were purchased from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from Hyclone Laboratories (Logan, UT, USA). Oligonucleotides were synthesized by Genotech (Daejeon, Korea). All other chemicals were obtained from local suppliers.

Tissues

Placental tissues were obtained from cesarean delivery on day 100 of gestation. The placentas were collected and stored at −80℃ until use. All experiments were conducted according to the Guidelines for the Care and Use of Animals, Hankyong National University.

Preparation of Bovine 20α-HSD cDNA

PCR primers were designed based on the bovine 20 α-HSD cDNA sequence as previously reported (Naidansuren *et al.*, 2011). Total RNA was extracted using the Trizol reagent (Invitrogen) and cDNA synthesis was performed using the SuperScript First-Strand Synthesis System according to the manufacturer's instructions. Specific primers (sense: 5'-CAG AAT CTC ATA TGG ATC CCA AAT ACC AG-3' and antisense: 5'-GTC TCT CGA GTC AGT ATT CTT CGG-3') were synthesized from the nucleotide sequence of the cloned 20a-HSD gene. Next, PCR was carried out using 2.5 units of *Pfu* polymerase in a Quick Thermo-II PCR machine and included 30 cycles of denaturation (91℃ for 1 min), annealing (37℃ for 1 min), and extension (72℃ for 2 min). PCR products were analyzed using electrophoresis in Tris-acetate buffer containing EDTA. The PCR products were ligated into the pCR2.1 vector, and sequence data were analyzed using computer software (DNASIS).

Cells and Transfections

CHO-KI cells were cultured in Ham's F12 growth media containing penicillin (50 U/mL), streptomycin (50 mg/mL), glutamine (2 mM), and 10% FCS] and incubated at 37℃ in 5% CO_2. Cultured CHOK1 cells were transfected with the expression vectors using the liposome transfection method as described previously (Min *et al.*, 2004). After 4∼6 h of transfection, 250 mL 20% FBS was added to the wells, and the cells were maintained at 37℃ in a CO_2 incubator for 24 h. On the following day, transfected cells were washed twice, and then 500 mL serum-free medium was added and the cells were incubated at 37 ℃ for 48 h. The culture medium was removed, and cells were collected into a tube. The cells were centrifuged at 13,000 ×g for 10 min, and the cell debris was recovered.

Production of Polyclonal Antibody

Ab Frontier (Seoul, Korea) manufactured the antibody according to a previously reported method (Naidansuren *et al.*, 2011). Briefly, the pRSET vector containing bovine 20α-HSD full-length cDNA was constructed and transformed into *E. coli*. Three colonies were selected for production of recombinant protein. The protein was purified using DEAE column chromatography. Next, 5 mg protein was coupled to 5 mg maleimide-activated keyhole limpet hemocyanin according to the manufacturer's instructions. The conjugated protein was emulsified with an equal volume of Freund's complete adjuvant and subcutaneously (s.c.) injected into rabbits. One month after initial injection, a 200 mg protein booster shot in an equal volume of incomplete Freund's adjuvant was administered. Booster injections were repeated at 2-week intervals. Total blood was collected and tested using western blot analysis for the detection of recombinant bovine 20α-HSD prepared from CHO-K1 cells. Only one rabbit injected with the recombinant protein developed antibodies against 20α-HSD. Finally, IgG fractions were prepared from the rabbit immune sera using protein-A chromatography.

Bovine CL Cell Culture

Bovine CL cells from the ovary were cultured at the CH2 stage. CL tissues were washed twice with 1× PBS, cut into 0.5 cm sections, and placed in Dulbecco's modified Eagle's medium (DMEM). The tissues were then cultured with 0.2% collagenase at 37℃ in a shaking incubator for 2 h. The culture medium was filtered through a cell strainer and centrifuged at 1,000 ×g for 5 min, after which the supernatant was collected. Finally, cells were counted using a cytometer, and 2×10^6 cells were seeded onto a cell culture plate. The cells were collected at 96 h and at 120 h. These samples were

subjected to western blot analysis.

Western Blot Analysis

Total proteins were extracted using PRO-PREP protein extraction solution. Approximately 10~20 mg of placental tissues was used. Tissues were minced and transferred to tubes. The samples were homogenized in 60 mL PRO-PREP solution, and cell lysis was induced by incubating the cells on ice for 30 min. Finally, the samples were centrifuged at 105,000 ×g at 4~8℃ for 5 min, and the supernatants were transferred to fresh 1.5 mL tubes. Protein concentration was measured using the Bradford method (Bradford, 1976). Samples were separated using SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane using a semi-dry electroblotter apparatus. After blotting, the membrane was blocked with 1% blocking reagent for 1 h followed by incubation with a 1:1,500 dilution of a polyclonal 20a-HSD antibody for 1 h. The membrane was washed to remove unbound antibody, and then incubated in a 1:2,000 dilution of a secondary antibody linked to anti-rabbit IgG-peroxidase for 30 min. The membrane was then incubated for 5 min with 2 mL Lumi-Light substrate solution, following which the solution was discarded. The membrane was placed on plastic wrap, covered with another layer of plastic wrap, and exposed to X-ray films for 1~10 min.

RESULTS

Polyclonal antibodies were raised against a rec-protein corresponding to residues 1~323 of bovine 20α-HSD. We amplified full length 20α-HSD cDNA [967 (bp)] in the open reading frame, which encodes for a putative protein of 323 amino acids (Fig. 1A). Five clones were subjected to sequencing analysis to determine sequence error. Three clones contained the proper nucleotide sequence. We then sub-cloned the fragment into the pRSET vector under control of the T7 promoter

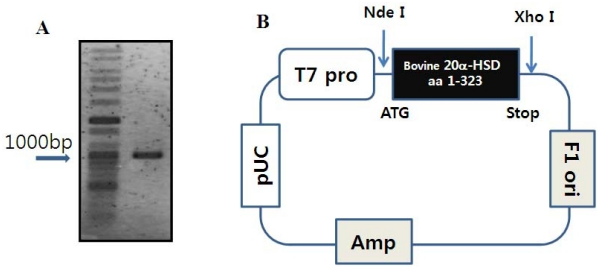

Fig. 1. Construction of expression vector in *E. coli*. (A) We amplified full length 20α-HSD cDNA (967 bp) in the open reading frame that encodes a putative protein of 323 amino acids. (B) Expression vector was also sub-cloned into the pREST under the T7 promoter.

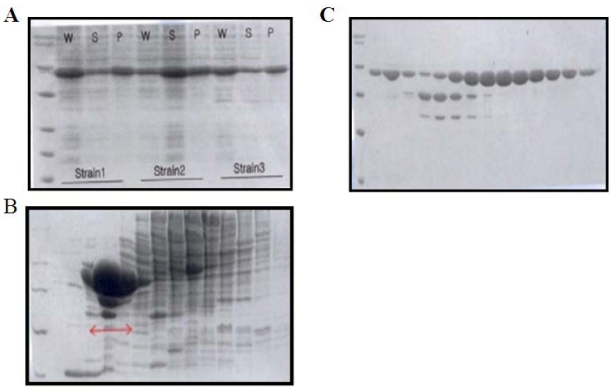

Fig. 2. Expression of bovine 20α-HSD protein in *E. coli*. (A) The pREST vector was transfected into *E. coli*. After culture, whole cell, soluble form and IB form were subjected to SDS-PAGE. (B) Purification by 1st DEAE column chromatography. (C) Purification by 2nd DEAE column chromatograph.

(Fig. 1B).

The pRSET vector was transfected into *E. coli*. Three colonies were chosen for production of the rec-protein. After culture, the whole cells, soluble form, and IB form of three strains were subjected to SDS-PAGE (Fig. 2A). Rec-20*a*-HSD protein was detected in all strains. Particularly, strain 2 showed the highest production in the whole cells. We then purified the strain 2 sample using 1st DEAE column chromatography (Fig. 2B). The collected fraction was purified using 2nd DEAE column chromatography (Fig. 2C).

Next, 5 mg protein was coupled to 5 mg maleimide-activated keyhole limpet hemocyanin according to the manufacturer's instructions. The conjugated protein was injected into 3 rabbits. After the 1st bleeding, the rabbits were administered an equal volume of the protein and incomplete Freund's adjuvant. Before total blood was collected, anti-serum and pre-immune serum were tested using the ELISA method. Both serums were diluted at ratios of 1:100, 1:500, 1:1,000, 1:5,000, 1:10,000, 1:50,000, and 1:100,000 times. The results from the ELISA are shown in Fig. 3. Anti-serum for 20α-HSD was detected as high activity to the serum dilution times. One rabbit responded to the rec-protein, and its serum was purified to produce a specific antibody against bovine 20α-HSD.

We next examined the ability of anti-serum to recognize the bovine 20α-HSD using western blots of placentas prepared during pregnancy and before parturition. The data presented in Fig. 4 show that anti-serum specifically recognized a 37-kDa band from the placenta sample. However, no additional 20α-HSD band was detected in the control sample. In other experiments, we showed that the placenta was separated into caruncle and cotyledon on day 100 of pregnancy. Protein expression level was assessed in the normal placenta (Fig. 4A), and was robustly expressed on the cotyledon

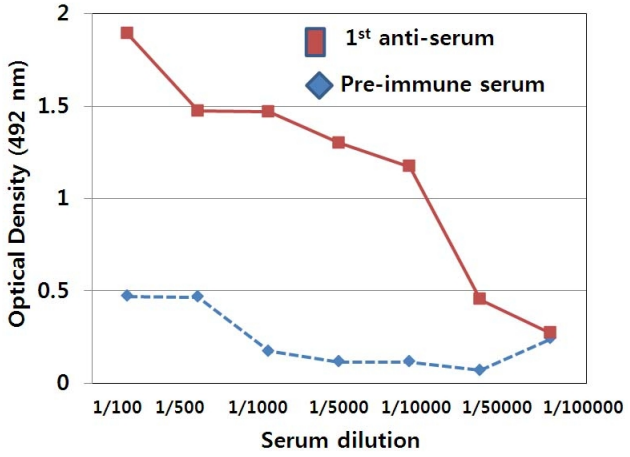

Fig. 3. Test of anti-serum after 1st bleeding. Anti-serum and pre-immune serum were collected and tested with ELISA method. Both serum were diluted as shown in Material and Method. Anti-serum for 20α-HSD was detected as high activity to the serum dilution times. One of the rabbits responded to the rec-protein.

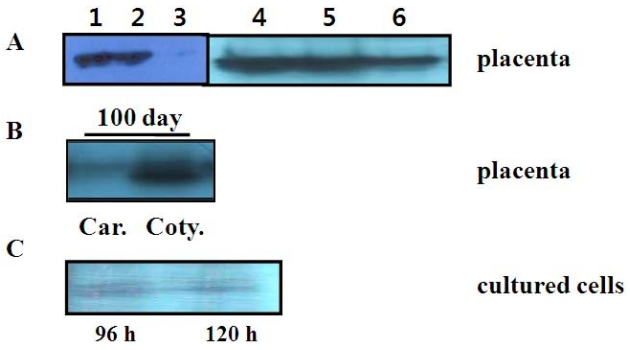

Fig. 4. Western blots analysis of bovine placenta during pregnancy and cultured CL cells. (A) Placenta obtained from random (1~2 lane, 4~6 lanes: placenta; 3 lane: negative control), (B) Placentome was collected on day 100 of pregnancy, (C) The corpus luteal cells of the CH_2 stage were cultured until 120 h. The cell lysates were collected and adjusted to western blot.

side of the placenta on day 280 of pregnancy (data now shown). Little expression was observed on the caruncle side of the placenta on day 100 of pregnancy (Fig. 4B). In an additional attempt to characterize 20α-HSD, we used anti-serum to detect 20α-HSD protein in lysate prepared from CL cells. As shown in Fig. 4C, 20α-HSD protein was first observed at 96 h after culture and increased very little up to 120 h after culture.

DISCUSSION

We raised a polyclonal antibody (anti-bovine 20α-HSD) against the rec-protein corresponding to residues 1~323 of bovine 20α-HSD. The availability of this antibody had allowed us to assess the structure and expression level of bovine 20α-HSD. Western blot analysis, which can be used to detect the mature protein, revealed the same moleucar weight protein in the placenta and in cultured cells. The data obtained using this approach suggests that bovine 20α-HSD is a 37-kDa protein that is highly expressed in the bovine placenta during pregnancy. Thus, the deduced size of bovine 20α-HSD expressed in *E. coli* and CHO cells is similar to that of the polypeptide chain predicted from cDNA cloning.

In rats, 20α-HSD levels were low early in pregnancy, undetected between days 5~19, and increased rapidly and abruptly between days 19~21. When levels of the 37-kDa protein were examined in the corpora lutea undergoing luteolysis on day 21 of pregnancy in rats and in hypophysectomized rats, higher expression of protein was observed in hypophysectomized rats (Albarracin *et al.*, 1994). To determine whether the 37- kDa antiserum could neutralize 20α-HSD activity, Albarracin *et al.* (1994) incubated protein samples with the IgG fraction from either the 37-kDa antiserum or normal rabbit serum before the assay. Their results suggested that preincubation with 37-kDa antiserum did not inhibit 20α-HSD activity. Recently, we suggested that 20α-HSD protein expression could only be detected in ovaries in the CL3, CL2, and CL1 stages throughout the estrous cycle. This protein was also robustly expressed on the cotyledon side of the placenta just before parturition (Purevjargal *et al.*, 2001). Additionally, bovine 20α-HSD protein was intensely localized to large luteal cells during the late estrous cycle. We also showed that 20α-HSD expression increased during the late estrous cycle, and nearly mirrored the mRNA expression pattern. This observation is consistent with previous reports demonstrating the presence of 20α-HSD in the placenta (Seong *et al.*, 2002; Shiota *et al.*, 1993).

We also reported that a high level of this protein was detected in the placenta but not in the fetal skin tissue of deer (Naidansure *et al.*, 2012). Recombinant 20α-HSD produced in mammalian cells and in bacterial systems showed a molecular weight of approximately 37 kDa. The deer 20α-HSD protein signal was specifically localized to the basal region of the primary chorionic villi and chorionic stem villus of the placenta during early pregnancy. A previous study examining pig AKR1C1 using western blot analysis suggested that protein expression was detected at approximately 37 kDa; this study used a specific anti-bovine 20α-HSD antibody (Seo *et al.*, 2011). AKR1C1 protein was detected in all ovaries during the estrous cycle. The highest protein expression was detected on day 0 of the estrous cycle. The level of the protein was remarkably decreased in the ovary on day 15 of the estrous cycle. On day 30 on pregnancy, the protein was detected in the uterus at a size of approximately 37 kDa. Our pre-

vious studies on examining monkey 20α-HSD allowed comparisons of 20α-HSD expression in the placenta, oviduct, and ovary (Nanjidsuren *et al.*, 2011). The 20α-HSD protein was more highly expressed in the ovary than in other tissues. Interestingly, 3 protein bands were detected in the placenta. Monkey 20α-HSD was primarily localized to isthmus cells of the oviduct and in syncytiotrophoblasts cells of the placenta during preparturition. The data presented here show that the antibody produced in this study may be useful for examining protein production in the bovine placenta and ovary during the estrous cycle/pregnancy. Bovine placenta was shown to express significant amounts of 20α-HSD, which was similar to the levels observed in goat (Jayasekara *et al.*, 2004) and in deer placental tissues (Naidansuren *et al.*, 2012). Additionally, this antibody can be used to examine tissues of other species (pig, monkey, and deer).

In summary, the data presented here clearly show that we have developed a specific antibody to bovine 20α-HSD. This antibody is useful for examining the structure as well as the synthesis and processing of 20α-HSD in transfected cells. Although we have attempted to use this antibody to detect 20α-HSD in western blots of normal placental tissues and cultured luteal cells, it can also be used for other tissues (ovary) and in biochemical characterization of 20*a*-HSD. Further studies are necessary to determine the functional significance of bovine 20 a-HSD during pregnancy and parturition.

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(Received: 25 May 2012/ Accepted: 21 June 2012)