발생과 생식 제 4권 제 1호, 87~93 (2000)
Dev. Reprod. Vol. 4, No. 1, 87~93 (2000)

Differential Expression of Glycoprotein Hormones in Equine Placenta and Pituitary

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말 태반과 뇌하수체에서 당단백질 호르몬의 특이적인 발현

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ABSTRACT: Equine chorionic gonadotropin (eCG) consists of highly glycosylated noncovalently linked α- and β-subunits and belongs to the glycoprotein hormone family that includes lutropin (LH), follitropin (FSH), and thyrotropin (TSH). eCG is a unique member of the gonadotropin family because it elicits response characteristics of both FSH and LH in other species than the horse. eCG is synthesized and secreted by trophoblastic cells of the endometial cups between 40 and 130 days of gestation. In the present study, mRNA expression ratio of eCG, eLH and eFSH α- and β-subunints was investigated in the placenta and pituitary. mRNA was extracted from equine placenta on day 70 of gestation and from pituitary of male horse (27 month-old). When the expression of both subunit mRNAs of eCG in the equine placenta was compared by Northern blotting, the expression of the β-subunit mRNA was relatively greater than that of the α-subunit. And mRNA expression of α-, LH β- and FSH β-subunits was analysed in the equine pituitary. An α-subunit was revealed with a size of approximately 0.8 kb. FSH β-subunit mRNA also was detected out 1.8 kb. It is the same size of the FSH β-subunit mRNA cloned. The intensity of α-subunit mRNA was greater than that of the β-subunit, suggesting that the expression of α-subunit was dominant in the equine anterior pituitary.

Thus, the subunit mRNA levels seem to be independently regulated and their imbalance may account for differences in the quantities of α- and β-subunits in the equine placenta and pituitary.

Key words : eCG, Equine, Placenta, Pituitary, Differential expression.

요 약: eCG는 LH, FSH 및 TSH 와 같이 당단백질 호르몬에 속하고, 당쇄가 많이 첨가된 α와 β subunits의 비공유결합으로 구성되어 있고, 말에서 보다 다른 동물에서 FSH와 LH의 이중 생리활성을 나타내는 아주 특이한 성선 자극 호르몬이다. eCG는 임신 40~130일 사이에 말의 자궁내막배의 영양막세포에서 합성·분비된다. 따라서 본 연구에서는 eCG, eLH 및 eFSH의 각각 subunits mRNA발현을 태반과 뇌하수체에서 분석하였다. mRNA의 추출은 임신 70일의 태반과 27개월된 숫컷 말의 뇌하수체에서 분리하였다. 말 태반을 이용한 eCG mRNA 발현의 Northern blotting 분석결과 β subunit가 α subunit보다 아주 많이 발현되었으며, 또한 뇌하수체에서 α-, LH β-, FSH β-subunits의 분석결과 α subunit는 약 0.8 kb, FSH β subunit는 1.8 kb의 크기로 발현되었는데, 이러한 FSH β subunit는 cloning 되어진 cDNA의 크기와 일치한다. 뇌하수체 전엽에서는 α subunit가 LH β subunit와 FSH β subunit보다 현저히 많이 발현된다는 사실이 밝혀졌다. 따라서, 태반과 뇌하수체에서 발현되는 각각 subunit의 mRNA는 독립적으로 조절되어 결과적으로 발현량에 차이가 나타난다고 시사되어진다.

INTRODUCTION

Equine chorionic gonadotropin (eCG), known as pregnancy mare serum gonadotropin (PMSG), has a number of interesting and unique characteristics of gonadotropin family since it appears to be a single molecule that possesses both luteinizing hormone (LH)- and follicle-stimulating hormone (FSH)-like activities in other species than the horse (Min et al., 1994, 1996a). This dual activity of eCG in heterologous species is of fundamental interest to the study of the structure-function relationships of gonadotropins and their receptors.

With a given species, the α-subunits are identical, whereas the β-subunit is specific to each hormone (Pierce & Parson, 1981). Both subunits are required for these glycoprotein

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hormones and combination of the subunits is essential for the expression of their biological activity. Cells from the chorionic girdle of the equine trophoblast invade the maternal endometrium on day 36 of gestation and eCG is synthesized and secreted by trophoblastic cells of the endometrial cups between 40 and 130 days of gestation (Murphy & Martinuk, 1991) (Fig. I).

The difference between eCG and eLH lies in the structure of their glycoresidues, which are both sialylated and sulfated in LH and sialylated CG (Fig. 2). The carbohydrate content of eCG, over 40% (w/w), is the highest of the glycoprotein family, including LH, FSH, TSH and hCG (Christakos & Bahl, 1979). The α-subunit of eCG has two N-glycosylation sites (Asn 56 and Asn 82) and its β-subunit has one (Asn 13). In addition to these N-glycosylation sites, the β-subunits of human and baboon CGs are heavily glycosylated C-terminal extensions, which increase the average lengths of these β-subnuits from 120 to 145 amino acids (Pierce & Parsons, 1981; Crawford et al., 1986). Analysis of a purified preparation of eCG revealed that its β-subunit consists of 149 amino acids (Sugino et al.,

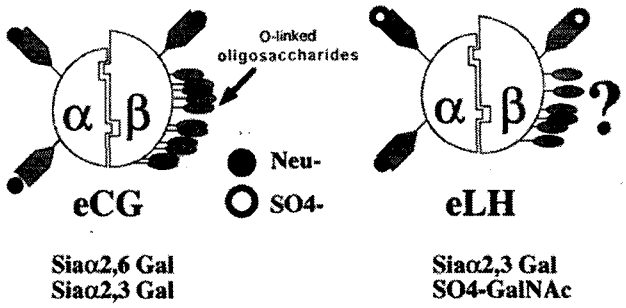

Fig. 2. Oligosaccharide structures of eCG and eLH. The α-subunit of eCG has two N-glycosylation sites (Asn 56 and 82), and its β-subunit has one (Asn 13). There seem to be at least 11 O-glycosylation sites on the extended C-termianal region of the eCG β-subunit. The β-subunit of eLH has at least 6 O-glycosylation sites in the C-terminal. The eCG β-subunit contained only sialylated oligosaccharides (Siaα2,3 and 2,6 Gal), whereas the eLH β-subunit contained both sialylated and sulfated carbohydrate moieties (Siaα2,3 SO4-GalNAc).

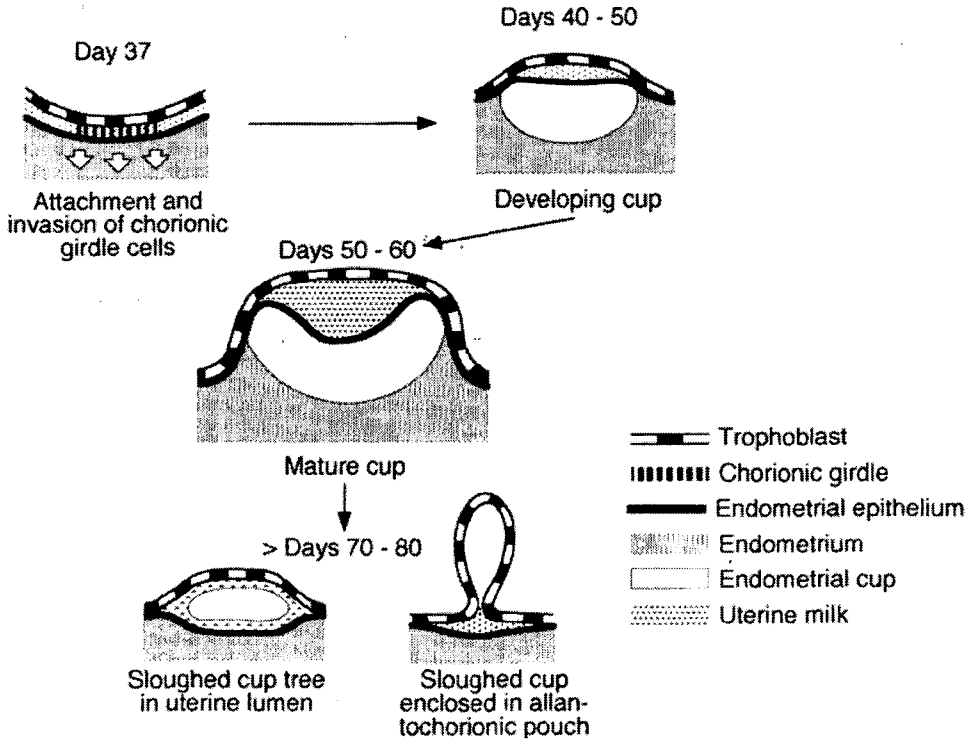

Fig. 1. Morphogenesis and demise of the endometrial cups in the mare. The source of the gonadotropin is the specialized trophoblast cells of the chorionic girdle, which attach to, invade, and phagocytose the maternal epithelium. This invasion begins on day 36 of pregnancy. These patches of girdle cells form the endometrial cups. Girdle cells aggressively migrate through the uterine epithelium into the endometrial stroma where they form distinct nodules and highly differentiated tissue type buried within the endometrial stroma. These nodules are called "endometrial cups".

1987), which was confirmed by the molecular cloning of its cDNA described previously (Min et al., 1994, 1996a). Ther seem to be at least four to six, or even as many as 11, O-glycosylation sites on the extended C-terminal region of the eCG β-subunit (Bousfield et al., 1992; Min et al., 1996a). In horse, a single gene encodes both eCG and eLH β subunits (Min et al., 1994).

We previously studied the regulation of rat and equine placental function at different stages of pregnancy and identified pregnancy-stage specific placental functions which include secretion of growth modulators called placental lactogens (Hirosawa et al., 1994; Shiota et al., 1997), eCG, equine relaxin (Min et al., 1994, 1996a,b, 1997), rat CG (rCG) (Shinozaki et al., 1997), leukemia inhibitory factor receptor (Aikawa et al., 1997), and LH/CG receptor functions (Min et al., 1998; Min, 1999, 2000).

The balance of α- and β-subunit gene expression in the equine is unknown. In the human placenta, free α-subunit is observed in the serum during the trimester and increases progressively during pregnancy (Ozturk et al., 1988a,b). In the horse, free eCG β-subunit has been detected in both serum and urine, while the free eCG α-subunit is undetectable (Couture et al., 1993), suggesting that synthesis of the eCG β-subunit is in excess in the equine placenta. In the present study, the expression ratio of α- and β-subunit mRNA was investigated in the equine placenta on day 70 of gestation and pituitary.

MATERIALS AND METHODS

1. Animal and Tissue

Equine placental tissue (endometrial cups) was obtained at laparotomy under general anesthesia from a horse (3 years old) on day 70 of gestation (Fig. 3), and processed for the preparation of the Northern blot analysis. Equine pituitary tissue was also obtained from a male horse (27 month-old). The tissue was kept at -80℃ until extraction of RNA.

2. Total RNA, First-strand cDNA Synthesis and PCR Amplification

Extraction of RNA, first-strand cDNA synthesis, PCR amplification, and Northern blotting were done as described previously (Min et al., 1994). The total RNAs were extracted according to the method previously described (Min et al., 1996b). First strand cDNA was synthesized by AMV reverse transcriptase. Synthetic oligonucleotide primers were purchased from Grainer Japan (Tokyo, Japan). The primers for the eFSH β-subunit (the mixed sense primer; 5'-CCA GGA TGA AGT CNG TCC AGT-3', the antisense primer; 5'-GTA CAC ACA GAC ATC TTG GAT-3') were designed on the basis of the nucleotide sequence informations of human (Watkins et al., 1987), rat (Maurer, 1987), and bovine (Esch et al., 1986) FSH β-subunits. PCR was carried out using 2.5 units of pfu polymerase (Stratagene, CA, USA) in Quick Thermo-II (Nippon Genetics, Tokyo, Japan). The PCR fragments amplified were ligated into the Sma I digested site of pUC119. Sequence data were analyzed using MacMolly Tetra computer software (Soft Gene, Berlin, Germany).

Fig. 3. Equine placental tissue. The endometrial cups were obtained at laparotomy under general anesthesia from mare (3-year-old) on day 70 of gestation.

3. ^{32}P-labeled Probes for Northern Blot Analysis

For Northern blot analysis, fragments of eCG α- (387 bp) and β- (524 bp) subunit cDNAs cloned previously, which was cleaved by *Eco* RI and *Xho* I, were labeled with [α-^{32}P] dCTP (3,000 Ci/mmol) (New England Nuclear, Boston, USA) using random primer reagent as described previosly (Min et al., 1994). The eCG β-subunit was also used as probe for eLH β-subunit, and then probe for eFSH β-subunit was used the cDNA fragment (466 bp) cloned by primers. The total RNA (20 μg) was resuspended in electrophoresis buffer (20 mM MOPS pH 7.0, 5 mM sodium acetate, and 1 mM EDTA) containing 50% formamide and 6.5% formaldehyde, and heated at 65℃ for 15

min. The RNA was electrophoresis on 1.0% agarose/formamide gel and then transferred to a nylon membrane. The blots were hybridized at 42℃ for overnight with each [α-^{32}P] dCTP-labeled probes. After washing, the hybridized membranes were scanned by a Fuji BAS 2,000 Bioimaging Analyzer (Fuji Film, Co. Ltd. Tokyo, Japan).

RESULTS AND DISCUSSION

The expression of glycoprotein horomone mRNAs in the equine placenta and pituitary was investigated by using Northern blot analysis. A mRNA analysis prepared from equine placenta revealed a transcript of the predicted size. An intense mRNA ratio of α to β at first trimester placenta was about 1: 5 (Fig. 4). It is well documented that the human placenta secretes free α-subunits (Ozturk et al., 1988a,b). This can be explained by the imbalanced synthesis of α-and β-subunits. Since accumulation and secretion of a net excess α-subunit have been observed in the pituitary gland as well as in the placenta (Edmonds et al., 1975), sharing of α-subunits in the glycoprotein hormone family may require the excess expression of α-subunit rather than of each individual β-subunit. However, secretion of free α-subunit from equine placenta has not been clearly demonstrated. Interestingly, Couture et al.(1993) showed that the free eCG β-subunit exists in serum and urine, whereas the free eCG α-subunit was undetectable. In our results, the expression of α-subunit in the equine placenta was lower than that of the eCG β-subunit, suggesting that the biosynthesis of eCG β is dominant and secretion of free eCG α-subunit is reduced in the equine placenta at day 70 of gestation.

The expressions of α-, LH β- and FSH β -subunits were analysed using mRNA extracted from equine pituitary. The α -subunit was revealed with a size of approximately 0.8 kb, FSH β-subunit mRNA was approximately 1.8 kb (Fig. 5). It is the same size of the FSH β gene cloned. The intensity of the α -subunit mRNA was greater than that of the β-subunit, suggesting that the expression of α-subunit was dominant in the equine anterior pituitary. The control of α-subunit gene expression has been examined in considerable detail (Delegeane et al., 1987; Jameson et al., 1988; Bokar et al., 1989). A practical matter responsible for much of the focus on a gene

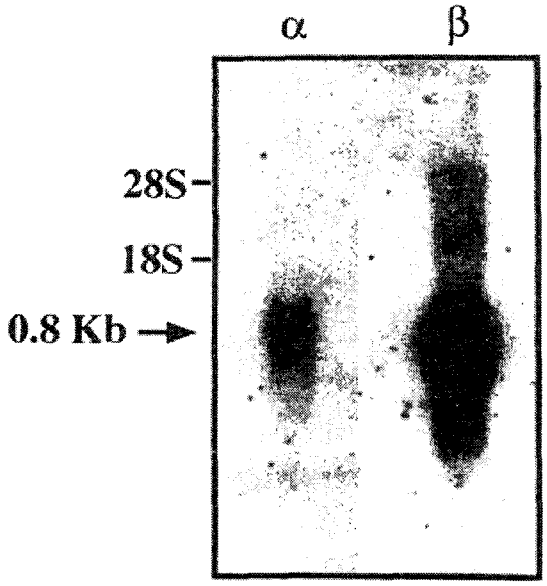

Fig. 4. Northern blot analyses of eCG α- and β-subunits mRNAs in placenta. Total RNA preparation (20 μg) extracted from equine placenta (day 70 of gestation) were electrophoresed on 1.0% agarose/formamide gel, transferred to a nylon membrane, and hybridized with [^{32}P]-labeled eCG α- and β-subunit cDNAs. The mobilities of 28S and 18S are shown on the left side and the arrow indicates eCG α- and β-subunit mRNAs of 0.8 kb.

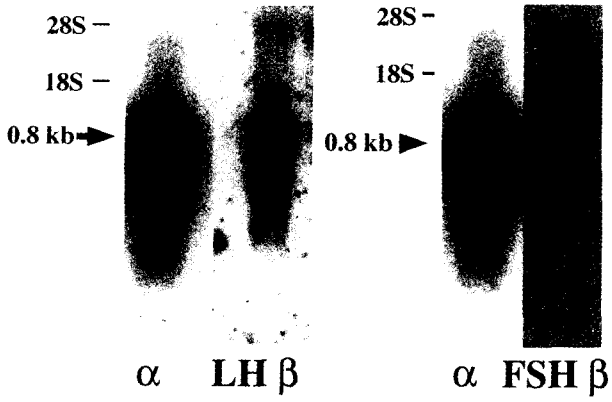

Fig. 5. Northern blot analyses of α-subunit, eLH β- and eFSH β -subunits mRNAs in pituitary. Total RNAs (20 μg) extracted from equine pituitary (27 month-old, male) were electrophoresed on agarose/formamide gel, and hybridized with [^{32}P]-labeled equine common α- and LH β-subunit cDNAs (left panel) and also FSH β -subunit (right panel).

expression has been derived human choriocarcinoma cell lines that express the endogenous gene and the absence of established pituitary cell lines in the gonadotrope lineage. These observations have raised several important questions including what is

responsible for the cell-type-specific expression of a gene and why the gene is expressed to placenta in primates and equids but not in other mammals.

Pituitary expression of the α-subunit gene occurs in all mammals, but placental expression is limited to primates and equids (Min et al., 1994). Humans and higher primates, including gorilla and pygmy chimpanzee, contain tandem cAMP response element (CRE)-like element, whereas Old World monkeys (baboon and rhesus monkey) contain only one CRE. Nevertheless, they all synthesize CG, suggesting that one CRE is sufficient for enhancer activity (Soares et al., 1993). The bovine α-subunit is expressed endogenously in bovine pituitary but not placenta, this result further underscores the importance of a functional CRE to the combinatorial code necessary for placenta-specific expression of the α-subunit gene. Equine chorionic girdle cells, the progenitors of horse placenta, synthesize and secrete eCG. It is not clear how the horse accomplishes this, as the equine α-subunit gene promoter contains a nonfunctinal sequence rather then a functional CRE sequence (Soares et al., 1993). One possibility is that the horse achieves placental expression through a completely different combinatorial code.

Boothby et al.(1983) reported the presence of twice as much hCG α mRNA as hCG β mRNA. In contrast, the amount of eCG β mRNA was higher than that of eCG α mRNA in the equine placenta. Thus, the subunit mRNA levels seem to be independently regualted and their imbalance may account for differences in the quantities of α- and β-subunits in the placenta. Animals, including horses, have a single copy of the cAMP response element (CRE)-like element in the 5'-flanking region of the α-subunit gene (Fenstermaker et al., 1990), while the human α-subunit gene has the repeated copies of the CRE. Inspection of the genomic DNA sequence in the 5'-flanking region that contains the α-gene' CRE revealed interesting features (Steger et al., 1991).

The expression of subunit mRNA seem to be independently regulated and their balance may account for differences in the quantities of α- and β-subunits in the placenta and pituitary, and it is thought that CRE controls the expresison of equine α -subunit. Thus, eCG is a distinct molecule from the view points of its biological function and glycoresidue structures. Recombinant eCG and FSH including the mutants will be useful tools for analyzing of the structure-function relationships of gonadotropins in the horse as well as other species.

ACKNOWLEDGEMENT

I wish to thanks Dr. Kunio Shiota of the Lab. of Cellular Biochemistry, Dept. of Veterinary Medical Science, University of Tokyo, for his invaluable suggestions as well as constant contributions.

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