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Expression of the Second Isoform of Gonadotropin-Releasing Hormone (Chicken GnRH-II Type) in the First Trimester Human Placenta

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임신초기 사람의 태반조직에서 GnRH-II mRNA와 Peptide의 발현

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ABSTRACT : Gonadotropin-releasing hormone (GnRH) has been known to play a role in the regulation of hCG secretion by human placenta. Recently, a gene encoding the second form of GnRH (GnRH-II) was identified in human. Herein, we demonstrate that GnRH-II is expressed in human placenta and assess GnRH-II expression by nested RT-PCR and immunohistochemistry in human placenta during the first trimester. We found that two alternatively spliced transcripts of GnRH-II mRNA were expressed in human placental tissues of first trimester and the shorter variant had a 21-bp deletion in GnRH-associated peptide (GAP). Immunoreactive GnRH-II was localized in both cytotrophoblastic and syncytiotrophoblastic cytoplasm. The immunostaining intensity was stronger in cytotrophoblast. Villous stromal cells also showed GnRH-II immunoreactiviry. The results of our study report that the second isoform of GnRH (GnRH-II) is expressed in the first trimester human placenta and we suggest that GnRH-II may also play a regulatory role in maintenance of early pregnancy and hCG secretion in human placenta.

Key words : Human GnRH-II, Chicken GnRH-II type, Placenta, RT-PCR, Immunohistochemistry.

요 약 : GnRH는 10개의 아미노산으로 구성된 호르몬으로서 생식기능을 조절, 관장하는 중요한 역할을 담당하는 것으로 알려져 있다. 특히 임신 중에는 태반에서 hCG의 분비를 조절하는 중요한 역할을 한다. 최근 사람의 두 번째 GnRH 유전자가 발견되었으며 그 10개의 아미노산 서열은 닭에서 두 번째로 발견된 GnRH (chicken GnRH-II)와 동일한 것으로 확인되었다. 이제까지 사람에서의 두 번째 GnRH (GnRH-II)의 발현은 중뇌와 신장에서 보고된 바 있으며, 본 연구자들에 의해서 처음으로 사람의 자궁내막에서의 발현이 보고되었다 (Cheon et al., 2001). 이에 본 연구에서는 임신초기의 태반조직에서 GnRH-II의 mRNA와 Peptide가 발현되는가를 조사하였다. 본 연구결과를 통해 태반에서 발현되는 GnRH-II mRNA는 두 가지 형태라는 것이 확인되었으며, 특히 GAP 부위에 21개의 뉴클레오티드 결실을 갖는 작은 전사체는 조직 특이적인 alternative splicing 기작에 의하여 태반조직에서만 특이적으로 발현되는 것으로 확인되었다. 면역화학염색법을 이용하여 GnRH-II peptide의 발현을 조사한 결과, 세포영양막과 융합영양막의 세포질에서 모두 발현되는 것으로 확인되었으며, 특히 세포영양막에서 더 많은 양이 발현되었다. 이상의 결과는 임신초기 태반에서 기존의 GnRH (GnRH-I)이외에도 다른 아미노산 서열의 GnRH-II가 발현된다는 사실을 말해주며 이는 GnRH-II 역시 태반조직에서 임신의 유지 및 생식기능의 조절에 관여할 가능성을 시사한다 하겠다.

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is a hypothalamic decapeptide, plays a key role in the regulation of reproductive functions. GnRH acts on the gonadotrophs of the anterior pituitary to stimulate the synthesis and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Conn & Crowley, 1991). In addition to this hypothalamic-pituitary-gonadal axis, local expression and biological function of GnRH has been reported in reproductive organs such as the ovary (Aten et al., 1987), testes (Bhasin et al., 1983), myometrium (Chegini et al., 1987), endometrium (Pahwa et al., 1991) and placenta (Lee

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et al., 1981). The extrahypothalamic GnRH is immunologically, biologically and chemically identical to the hypothalamic GnRH (Gibbons et al., 1975; Khodr & Siler-Khodr, 1980; Imai et al., 1992). GnRH receptor mRNA and protein also localized in human preimplantation embryo, endometrium (Raga et al., 1998; Casañ et al., 1999) and placental trophoblasts (Wolfahrt et al., 1998). GnRH has been proposed to be important in the differential regulation of placental glycoprotein release (Petraglia et al., 1990). GnRH mediated the release of human chorionic gonadotropin (hCG) from human placenta of different gestational ages and stimulated hCG secretion from cultured trophoblasts by a receptor-mediated process (Merz et al., 1991; Barnea et al., 1992; Szilagyi et al., 1992). Moreover, intravenous administration of GnRH increased serum hCG levels in normal pregnant women during the first and second trimester (Iwashita et al., 1993). Taken together, these studies support the hypothesis that GnRH plays a regulatory role in placental hCG secretion by autocrine/paracrine loop (Gohar et al., 1996; Wolfahrt et al., 1998).

In 1998 human GnRH-II, a gene encoding the second isoform of GnRH was cloned in human thalamus. GnRH-II gene is located on chromosome 20p13, distinct from the mammalian GnRH (GnRH-I) on 8p21~8p11.2. The amino acid sequence of human GnRH-II (also referred to as chicken GnRH-II type) is pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2, which differs from the mammalian sequence at the fifth, seventh and eighth amino acids (White et al., 1998). In vertebrates, the most ubiquitous variant of GnRH isoforms is commonly called chicken GnRH-II (cGnRH-II), which is identical to human GnRH-II (King & Millar, 1997; Sealfon et al., 1997). Expression of GnRH-II has been found in human brain stem (midbrain, pons and medulla oblongata) and kidney (White et al., 1998; Chen et al., 1998). Our previous study reported for the first time that human GnRH-II was expressed in cycling endometrium (Cheon et al., 2001). Placental existence of cGnRH-II isoform was ascertained and the stability, receptor binding, bioactivity and production of cGnRH-II were studied (Siler-Khodr & Grayson, 2001). The biological and physiological role of human GnRH-II has not been studied well. However, numerous data suggested that cGnRH-II is another key molecule to regulate reproductive function in vertebrates (Lescheid et al., 1997; King & Millar, 1997).

In this report we evaluated the presence of GnRH-II mRNA and peptide in human first trimester placenta and the existence of reproductive organ (endometrium, placenta and ovary) specific transcript of GnRH-II by alternative splicing mechanism.

MATERIALS AND METHODS

1. Tissue collection

For total RNA extraction, the human placental tissues were collected from 6 women at various gestational ages of the first trimester; 6 wk (n=3), 7 wk (n=1) and 9 wk (n=2). We were permitted to use human materials by the Ethical Committee of Samsung Cheil Hospital. Placental tissues were obtained immediately following therapeutic abortion in 6, 7 wk and missed abortion at 9 wk. The tissues were washed with 0.01 M phosphate buffered-saline, pH 7.4 (PBS) to remove excess blood and immediately frozen in liquid nitrogen and stored at −70℃ until use.

For immunohistochemistry, placental tissues were obtained following therapeutic abortion at 5 wk (n=5), 6 wk (n=3), 7 wk (n=1), 8 wk (n=2) and 9 wk (n=2). The samples were fixed with 10% formalin (Sigma, St. Louis, MO, USA).

2. Reverse transcription (RT)

Total RNA from 6 placental tissues was isolated by the single step acid guanidium thiocyanate phenol/chloroform method (Chomczynski & Sacchi, 1987) using TRIzol reagent (Gibco BRL, Grand Island, NY, USA). The total RNA was quantified by measuring the absorbance at 260 nm using the spectrophotometer, GENESYS 5 (Spectronic Instruments, Rochester, NY, USA).

Two μg of total RNA were mixed with 100 pmol oligo-dT15 and diluted with DEPC-treated water in a final volume of 20 μl, denatured at 65℃ for 10 min. The mixture was rapidly cooled in wet ice and reverse transcription was performed in a final volume of 40 μl under the following reaction conditions; 50 mM Tris-HCl (pH 8.5), 8 mM $MgCl_2$, 30 mM KCl, 1 mM each of dATP, dTTP, dGTP, dCTP, 40 U RNase inhibitor, 40 U M-MuLV (Boehringer-Mannheim, Germany). The reaction mixture was incubated for 60 min at 37℃ and 5 min at 95℃ in the DNA Thermal cycler (Perkin-Elmer, Norwalk, CA, USA).

3. Polymerase chain reaction (PCR) for GAPDH and hCG-β

Two μl of each RT products was used for PCR amplification with each primer set (Table 1). We amplified GAPDH cDNA as a housekeeping gene control. Expression of hCG-β mRNA was assessed to confirm the viability of placental tissues used for this study. Two μl aliquots of each RT product were diluted to a final volume of 20 μl; 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM each of dATP, dTTP, dGTP, dCTP, 2 pmol of sense and antisense strand primer and 0.5 U Taq polymerase (Boehringer-Mannheim, Germany). After an initial denaturation of 2 min at 92℃, 25 cycles of amplification for GAPDH (35cycles for hCG-β) were performed with 40 sec denaturation at 92℃, 1 min annealing at 58℃ (66℃ for hCG-β) and 1 min extension at 72℃ in a Robo cycler gradient 96 (Stratagene, La Jolla, CA, USA). The last cycle had an elongation time of 10 min at 72℃.

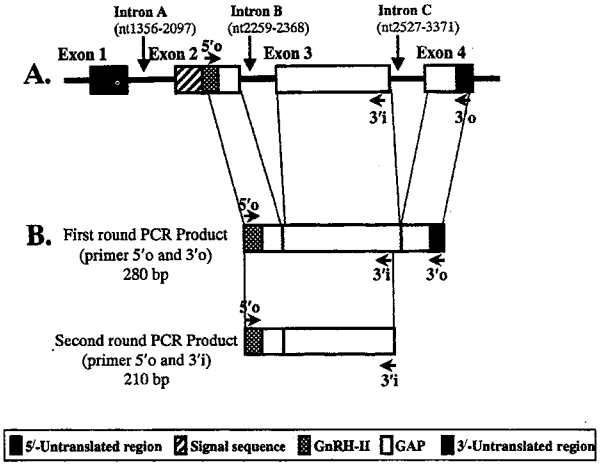

Fig. 1. Schematic diagrams of human GnRH-II gene structure and nested RT-PCR products. (A) The genomic sequence is quoted from the previous study (White et al., 1998; accession no. AF036329). Exons are represented as boxes and introns as bold lines. (B) The size of RT-PCR products by each primer sets are indicated.

4. Nested PCR for human GnRH-I and GnRH-II

For PCR amplification of human GnRH-I and GnRH-II cDNA, one sense and two antisense strand primer sets were designed and used for nested PCR as shown in Table 1. The schematic diagrams of genomic GnRH-II structure and nested -PCR amplification strategies are shown in Fig. 1.

For the first round PCR, 2 μl of each RT product were diluted to a final volume of 20 μl in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM each of dATP, dTTP, dGTP, dCTP, 1 pmol each of sense and outer antisense strand primer (primer 5'o and 3'o) and 0.5 U Taq polymerase. After an initial denaturation of 2 min at 92℃, 35 cycles of amplification for GnRH-I were performed with 40 sec denaturation at 92℃, 1 min annealing at 55℃ (62℃ for GnRH-II) and 1 min extension at 72℃ in a Robo cycler gradient 96. The last cycle had an elongation time of 10 min at 72℃. PCR products were stored at −20℃ until the second round PCR.

For the second round PCR, 2 μl of each the first PCR

Table 1. Primers used for PCR

cDNA	Size of amplified fragment	Position of primers on cDNA	3′/5′- end*	Sequence of oligonucleotide
hCG-β	298bp	192~ 211	5	5′- GCTACTGCCCCACCATGACC - 3′
		468~ 489	3	5′-ATTCTACTGACTTGGTGCGTG - 3′
GnRH	246bp	85~ 104	5′ o	5′- TGGAAGGCTGCTCCAGCCAG - 3′
		446~ 465	3′ o	5′- ACAACACAGCACTTTATTAT - 3′
		311~ 330	3′ i	5′- TCCTTCTGGCCCAATGGATT - 3′
GnRH-II	210bp	130~ 147	5′ o	5′- TCCCATGGCTGGTACCCT - 3′
		391~ 409	3′ o	5′- CTTTATTGGAGGATGGCGG - 3′
		320~ 339	3′ i	5′- CTTCCTGTGAAGGGACCACT - 3′
GAPDH	452bp	586~ 605	5′	5′- ACCACAGTCCATGCCATCAC - 3′
		1018~1037	3′	5′- TCCACCACCCTGTTGCTGTA - 3′

hCG : human chorionic gonadotrophin; GnRH: gonadotrophin releasing hormone; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.
* 5′ o/3′ o/3′ i : outer and inner primers for the nested PCR.

product were diluted with 18 μl of distilled-deionized water. From the diluted PCR product, 2 μl aliquot was mixed to a final volume of 20 μl with sense and inner antisense strand primer (5'o and 3'i) as previously described. After an initial denaturation of 2 min at 92℃, 40 cycles of amplification were performed as described above. Products were stored at −20℃ until electrophoresis.

The first and the second round PCR products were electrophoresed on 2% agarose gel (Sigma, St. Louis, MO, USA) and stained with ethidium bromide. The gels were visualized and photographed under UV light using BIO-PROFIL Image analysis software (Vilber Lourmat, Marne La Vallee Cedex, France).

5. Subcloning and sequencing of GnRH-II PCR product

The second round PCR product of GnRH-II was ligated to pCR4-TOPO vector and transformed into TOPO 10 *E. coli* K12 competent cells (Invitorgen, Carlsbad, CA, USA). Recombinant colonies were chosen and the plasmid containing the cDNA insert was purified using the high pure plasmid isolation kit (Boehringer-Mannheim, Germany). The purified plasmid was digested with EcoR I endonuclease to confirm the presence of the insert. DNA sequencing analysis was carried out with the T7 sequenase version 2.0 DNA sequencing kit (Amersham, Cleveland, OH, USA) using dideoxy chain-termination method.

6. Immunohistochemistry

All placental samples were fixed with 10% formalin and paraffin-embedded. Serial sections (4 μm) were attached on poly-lysine coated slide (Menzel-glaser, Germany). Slides were cleared in xylene for 30 min and rehydrated in ethanol. For antigen unmasking, slides were boiled for 3 min in 10 mM sodium citrate buffer (pH 6.0) using microwave. This step was repeated three times. The sections were incubated with 0.3% hydrogen peroxide for 15 min to inhibit endogenous peroxidase activity. After an additional wash, the slides were incubated for 1 hr in a blocking solution of PBS containing 5% normal goat serum and 1% Triton-X100 to reduce non-specific binding. Anti-GnRH-I antibody (Chemicon, Temecula, CA, USA) and anti-chicken GnRH-II antibody (a generous gift from Dr. I. S. Parhar in Nippon Medical School, Tokyo, Japan) were used in this study. The slides were incubated with each antisera (1:300 dilution for GnRH-I and 1:100 for GnRH-II) at 4℃ overnight. After washing twice with PBS for 10 min, the slides were incubated with a biotinylated anti-rabbit IgG (DAKO, Carpinteria, CA, USA) for 40 min, washed with PBS twice and incubated with horseradish peroxidase conjugated streptavidin (DAKO) for 40 min. The slides were washed twice with PBS for 10 min and incubated with a 3,3-diaminobenzidine solution (DAKO liquid DAB substrate/chromogen system) for 5 min. Finally, the slides were counterstained with a Mayer's hematoxylin solution and dehydrated in ethanol. Sections were cleared in xylene and mounted with Permount (Fisher Chemicals, Springfield, NJ, USA). All slides were examined by a Nikon OPTIPHOT-2 microscope and photographed by a Nikon UFX -DX II camera under 400X magnification. The red precipitates indicated positive staining by each primary antibody.

RESULTS

1. Expression of the GnRH-II mRNA in human placental tissues

We examined 6 placental samples of the first trimester to detect GnRH-II mRNA. All the samples expressed two variants of GnRH-II mRNA. Also all the samples expressed mRNA of GAPDH, GnRH-I and hCG-β. The representatives of RT-PCR results are shown in Fig. 2. After nested RT-PCR amplification of GnRH-II, a 210-bp of expected size band (upper) and an additional smaller band of 189-bp (lower) were amplified. Interestingly, the intensity of lower band was stronger than that of upper band in this study. We could already find these two variants of GnRH-II mRNA in human endometrium (Cheon et al., 2001) and ovary (unpublished data).

To verify the nested RT-PCR result, two spliced variants of the second round PCR products were sequenced. We found that

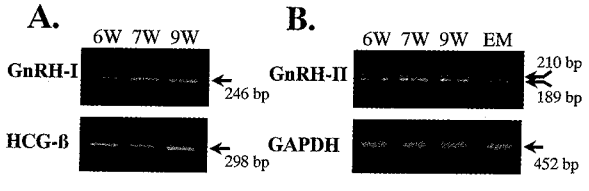

Fig. 2. RT-PCR results of GnRH-I, GnRH-II, hCG-β and GAPDH in human first trimester placental tissues. (A) Representatives of nested RT-PCR product for GnRH-I and hCG-β (B) GnRH-II and GAPDH. EM; human endometrium.

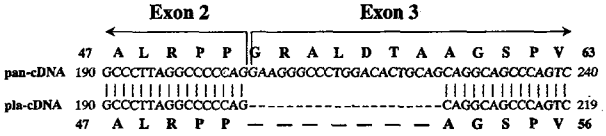

Fig. 3. Sequence comparison of GAP in two alternatively spliced transcripts of GnRH-II. The placenta-specific short transcript described in the present study is designated pla-cDNA, identical to endometrium-specific variant of our previous report (Cheon et al., 2001; accession no. AF190640) and that previously described is designated pan-cDNA (White et al., 1998; accession no. AF036330). Nucleotides are numbered from 5′ to 3′ , beginning with exon 1 and the deleted 21 nucleotides and 7 amino acids are presented as dashes. Predicted amino acid residues are numbered with the first residue (Met) in the open reading frame and indicated bold letters.

additional small variant has a 21-bp deletion in GAP and the predicted length of GAP is decreased from 77 to 70 amino acids (Fig. 3). All together, these data showed that human placental tissues expressed two alternatively spliced variants of GnRH-II mRNA during the first trimester.

2. Immunohistochemical localization of the GnRH-I and GnRH-II in human placental villi

Immunoreactive GnRH-II was detected in all placental tissues of the first trimester we examined. GnRH-II peptide was localized in cytoplasm of both cytotrophoblast and syncytiotrophoblast but stronger immunostaining intensity was shown in cytotrophoblast. (Fig. 4B, C). In compared with the distribution pattern of GnRH-II, cellular localization of GnRH-I was similar to that of GnRH-II (Fig. 4A). Villous stromal cells (Hofbauer cell and mesenchymal cells) also showed immunoreactivity of both GnRH-I and GnRH-II. The tissue histology was shown by hematoxylin and eosin staining (Fig. 4D). There was no significant immunoreactivity of placental villi without primary antibody (data not shown).

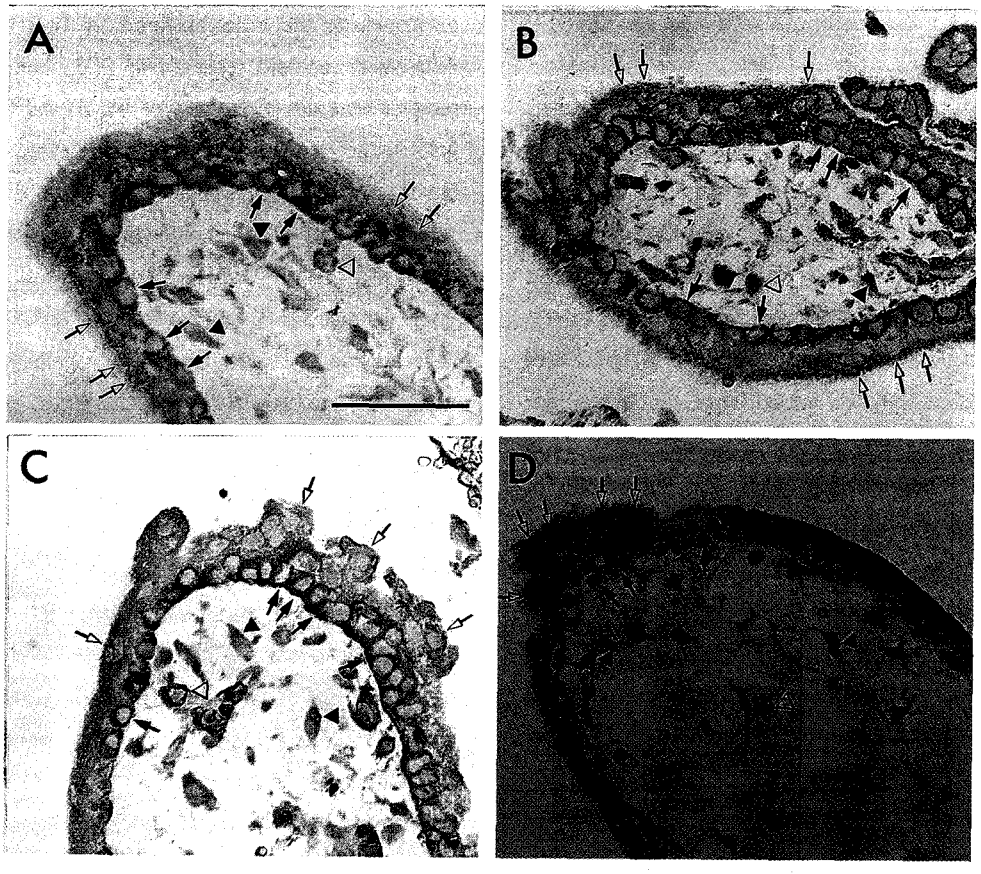

Fig. 4. Localization of human GnRH-I and GnRH-II peptide in the first trimester human placental villi. (A) GnRH-I at 9wk, (B) GnRH-II at 6wk, (C) GnRH-II at 9wk and (D) H/E staining at 9wk. Cytotrophoblast (▶), Syncytiotrophoblast (▷), Hofbauer cell (▷), mesenchymal cell (▶), Scale bar; 50 μm (400X magnification).

DISCUSSION

In human, GnRH-II gene was cloned recently in brain and the mRNA expression was reported in brain and kidney (White et al., 1998). Placental secretion of cGnRH-II was found by a highly specific RIA for cGnRH-II (Siler-Khodr & Grayson, 2001). Previously, we reported for the first time that this GnRH-II mRNA and peptide were expressed in human endometrium during normal menstrual cycle. Cellular distribution of GnRH-II peptide was identical to that of GnRH-I, localized in both glandular epithelium and stromal cells (Cheon et al., 2001). In first trimester placental trophoblast, a large amount of pro-GnRH mRNA are present in cytotrophoblast, a major source of GnRH-I secretion (Duello et al., 1993). We also showed the same pattern of GnRH-I localization in this report. Moreover, GnRH-II peptide also localized dominantly in cytotrophoblast during the first trimester. So, we suggest that placental cytotrophoblast is not only a major source of GnRH-I but also GnRH-II during pregnancy.

In the first trimester placental tissues, we were able to detect placenta-specific transcript of GnRH-II with 21-bp deletion in GAP by alternative splicing as well as a predicted full-length mRNA identical to that of brain and kidney. Moreover, this shorter transcript was predominantly expressed in first trimester placenta. Previously, others demonstrated that 5′ end of exon 3 is a variable region by alternative splicing and a 21-bp inserted variant was expressed only in human brain. In this longer transcript, the predicted length of GAP is increased from 77 to 84 amino acids (White et al., 1998). As our results of sequencing analysis, placental specific short transcript has a 21-bp deletion of 5′ end of exon 3. In this shorter transcript, the predicted length of GAP is decreased from 77 to 70 amino acids. We have already reported that human GnRH-II was expressed in human endometrium and endometrium-specific spliced variant of GnRH-II mRNA (identical to placental shorter variant) also existed. (Cheon et al., 2001). In addition, we also found these two spliced variants in human ovary (unpublished data). So we can suggest that reproductive organ-specific splicing machinery of GnRH-II exists and this shorter GAP protein may have a unique role in reproductive organs.

It has been reported that there is a distinct efficacy for each isoforms of GnRH on a single cognate GnRH receptor. Both cGnRH-II and catfish GnRH, ([His5, Asn8]-GnRH, cfGnRH), two endogenous GnRH forms in the catfish, were able to mediate phosphatidylinositol hydrolysis in human embryonic kidney (HEK) 293 cells transfected with cfGnRH receptor and cGnRH-II has a 70-fold high potency than cfGnRH. Moreover, cGnRH-II induced cAMP production as well as phosphatidylinositol hydrolysis in HEK 293 cells, while cfGnRH only led phosphatidylinositol hydrolysis (Tensen et al., 1997). This observation indicated a possibility of distinct signal transduction pathway of GnRHs via same GnRH receptor. The cGnRH-II also has a high binding affinity for mammalian GnRH receptor in both primate and human (Davidson et al., 1996). In this point of view, GnRH-I and GnRH-II may bind to one GnRH receptor and play a similar and unique role in human placenta.

On the other hand, there are some evidences in the existence of GnRH receptor subtypes in vertebrates. The extracellular loop 3 (EC3) domain of mammalian GnRH receptor plays a role in distinguishing the different forms of GnRH. In the basis of different amino acid sequence of EC3, three groups of GnRH receptor were designated as type IA, IB and type II from lizard, goldfish, zebrafish and human (Troskie et al., 1998). In goldfish, previous studies demonstrated significant differences between salmon GnRH ([Trp7, Leu8]-GnRH, sGnRH) and cGnRH-II ([His5, Trp7, Tyr8]-GnRH), induced desensitization of gonadotropin hormone-II (GTH-II) release in terms of dependence on concentration and pulse frequency (Habibi, 1991). Eventually, two GnRH receptors (GfA and GfB) were isolated and cloned in goldfish. Although the receptors shared 71% amino acid identity, there were remarkable differences in their ligand selectivity by distinguishing the position 8 of GnRHs. GfA showed a greater preference for cGnRH-II and a lesser preference for the other natural GnRHs than did GfB (Illing et al., 1999). All together, it is possible that another GnRH receptor (prefer to cGnRH-II) may exist in the mammals including human.

There are few studies about reproductive function of GnRH-II in human. In primate, a low dose of synthetic cGnRH-II stimulates LH release in adult rhesus monkeys when administered during the mid-luteal phase but not mid-follicular phase (Lescheid et al., 1997). Our previous study reported that during early and mid-secretory phase, GnRH-II peptide was increased in endometrial glandular epithelial and stromal cells. This cycle

-dependant change of GnRH-II is similar to that of GnRH-I and we suggested that GnRH-II may play a role in the embryonic development and implantation as well as GnRH-I (Cheon et al., 2001). In the recent studies, it has been reported that GnRH-I and GnRH-II could increase hCG secretion in cytotrophoblastic cells and placental explant culture but GnRH-I was more effective than GnRH-II on hCG synthesis and secretion. In addition, GnRH-I, but not GnRH-II, down-regulated hCG secretion when incubated for 96 h (Islami et al., 2001). The effect of cGnRH-II on placental hCG secretion was also revealed by Siler-Khodr and his colleague. Using a placental perifusion system and a highly specific RIA for cGnRH-II, the pulsatile release of cGnRH-II from the early human placenta was demonstrated. Interestingly, both cGnRH-II and mammalian GnRH were rapidly degraded by placental enzymes, yet the chicken-II isoform is six times more stable (Siler-Khodr & Grayson, 2001). These studies showed that GnRH-II has a similar and different functions compared with GnRH-I in the reproductive organ and their difference could be explained by several possibilities, different pathways and half life of GnRH degradation, different binding affinities to same GnRH receptor, or even by different types of GnRH receptors.

It is difficult to predict exactly how human GnRH-II plays a role in human placenta and affects maintenance of early pregnancy. However, the presence of GnRH-II as well as GnRH-I may indicate a more complex event of maintenance of early pregnancy. Furthermore, biochemical and physiological studies of GnRH-II may be helpful in understanding this, and the possible existence of unknown GnRH receptor is also valuable to explain this event.

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