Expression of the second isoform of gonadotrophin-releasing hormone (GnRH-II) in human endometrium throughout the menstrual cycle

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We examined the expression of the protein and mRNA of the newly cloned isoform of human gonadotrophin-releasing hormone (GnRH-II) in the normal human endometrium during the menstrual cycle. Nested RT–PCR and sequence analysis revealed that two spliced variants of GnRH-II mRNA were expressed during the entire menstrual cycle, with the shorter transcript having a 21 nucleotide deletion in the region coding for GnRH-associated peptide. Using immunohistochemistry, we identified immunoreactive GnRH-II in both stromal and glandular epithelial cells during the entire menstrual phase. The immunostaining intensity was stronger during the early and mid-secretory phase compared with the proliferative and late-secretory phase. A large amount of immunoreactive GnRH-II was localized in the apical pole of the glandular lumen. Our results show that the second isoform of GnRH (GnRH-II) is expressed in the human endometrium during the entire menstrual phase. We also suggest that an increased expression of endometrial GnRH-II peptide, noted during the early and mid-secretory phase, may play an important role in human embryo implantation.

Key words: chicken GnRH-II/endometrium/immunohistochemistry/nested RT–PCR/spliced variant

Introduction

Gonadotrophin-releasing hormone (GnRH) is a decapeptide that plays a key role in the regulation of reproductive functions, by stimulating the synthesis and release of the pituitary gonadotrophins, which in turn promote steroidogenesis and gametogenesis (Conn and Crowley, 1991). The presence of GnRH has been reported in a variety of reproductive organs such as the ovary (Aten et al., 1987), testes (Bhasin et al., 1983), placenta (Siler-Khodr and Khodr, 1979), myometrium (Chegini et al., 1996) and endometrium (Pahwa et al., 1991). The extrahypothalamic GnRH is immunologically, biologically and chemically identical to the hypothalamic GnRH (Gibbons et al., 1975; Khodr and Siler-Khodr 1980; Imai et al., 1992). Moreover, the mRNA and protein expression of GnRH and its receptor in the human preimplantation embryo and endometrium have been reported, indicating the importance of GnRH in implantation (Raga et al., 1998; Casañ et al., 1999). It is evident that at least two or often three isoforms of GnRH are present in more than 80 vertebrate species. The most ubiquitous variant of GnRH is commonly called chicken GnRH-II, and is identical to human GnRH-II (King and Millar, 1997; Sealfon et al., 1997). In the primates, a low dose of synthetic GnRH-II stimulates LH release in adult rhesus monkeys when administered during the mid-luteal phase but not mid-follicular phase. GnRH-II also has a high binding affinity for the GnRH receptor both in primate and human (Davidson et al., 1996; Lescheid et al., 1997). In humans, a recent study reported that a gene encoding the second form of GnRH (GnRH-II) was cloned from brain tissue. The amino acid sequence of human GnRH-II (also referred to as chicken GnRH-II) is pGlu-His-Trp-Ser-Gly-Trp-Tyr-Pro-Gly-NH₂, which differs from the mammalian sequence at the fifth, seventh and eighth amino acids (White et al., 1998). Up to now, the expression of GnRH-II in humans has been found only in brain stem (midbrain, pons and medulla oblongata) and kidney (Chen et al., 1998; White et al., 1998). We postulated that GnRH-II may also be expressed in the reproductive tissues. The aim of this study is to investigate the presence of GnRH-II mRNA and peptide in human endometrium.
Materials and methods

Tissue collection
For total RNA extraction, the human endometrial tissues were collected from six women with proven fertility (aged 32-39 years). The sampling and use of human samples from endometrial biopsies was approved by the Ethical Committee of Samsung Cheil Hospital. Endometrial tissues were obtained by Novak curette at various stages of the menstrual cycle: proliferative (n = 2), ovulatory (n = 1) and mid-secretory phase (n = 3). The tissues were immediately frozen in liquid nitrogen and stored at −70°C until use. For immunohistochemistry, endometrial samples were obtained from 23 healthy women (aged 28–39 years) with apparently normal menstrual cycles; proliferative (n = 4), early secretory (n = 8), mid-secretory (n = 6) and late secretory phase (n = 5). Patients with endometriosis and pelvic inflammatory disease were excluded from the study. The tissues were taken with the use of a Novak curette or Z-sampler (BEI Medical Systems, Chatsworth, CA, USA). The stage of menstrual cycle was established by the last menstrual period, transvaginal ultrasonographic finding and LH surge. Histological dating (Noyes et al., 1950) was used to verify the menstrual dating.

RNA preparation and reverse transcription (RT)
Total RNA from six endometrial tissue samples was isolated by the single step acid guanidinium thiocyanate phenol-chloroform method (Chomczynski and 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). 2 µg of total RNA was mixed with 100 pmol oligo-dT 15 and diluted with diethyl pyrocarbonate-treated water in a final volume of 20 µl, denatured at 65°C for 10 min. The mixture was rapidly cooled in ice and the RT was performed in a final volume of 40 µl under the following reaction conditions: 50 mmol/l Tris–HCl (pH 8.5), 8 mmol/l MgCl2, 30 mmol/l KCl, 1 µmol/l each of dATP, dTTP, dGTP, dCTP, 40 U RNase inhibitor, 40 U Molony-murine leukaemia virus (M-MuLV) reverse transcriptase (Boehringer, Mannheim, Germany). The reaction mixture was incubated for 60 min at 37°C and 5 min at 95°C in the DNA thermal cycler (Perkin-Elmer, Norwalk, CT, USA).

Polymerase chain reaction (PCR) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
The GAPDH cDNA was amplified as a housekeeping gene with the sense strand primer 5'-ACCACACGTCCATGCCATCAC-3' and the antisense strand primer 5'-TCCACCACTTGTGCTGA-3' (Moore et al., 1998). 2 µl of each RT product was diluted to a final volume of 20 µl in 10 mmol/l Tris–HCl (pH 8.3), 50 mmol/l KCl, 1.5 mmol/l MgCl2, 0.1 mmol/l each of dATP, dTTP, dGTP, dCTP, 1 pmol each of sense and antisense strand primer and 0.5 U Taq DNA polymerase (Boehringer). After an initial denaturation of 2 min at 92°C, 25 cycles of amplification were performed with 40 s denaturation at 92°C, 1 min annealing at 58°C and 1 min extension at 72°C in a Robocycler gradient 96 (Stratagene, La Jolla, CA, USA). The last cycle had an elongation time of 10 min at 72°C.

Nested PCR for human GnRH-II
For PCR amplification of human GnRH-II cDNA, one sense strand primer A (5'-TCCATGGCTGGTACCCT-3' at position 2183/2200) and two antisense strand primers (outer primer B 5'-CTTATT-GGAGGATGGCCG-3' at position 3399/3417 and inner primer C 5'-CTTCTGTGAAGG-GACACT-3' at position 2483/2502) were designed as shown in Figure 1 using the Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3 www.cgi). The sequences of cDNA and genome for human GnRH-II (White et al., 1998) were obtained from the GenBank Database of the National Center for Biotechnology Information (NCBI) of the National Institutes of Health (http://www.ncbi.nlm.nih.gov/ cgi-bin/Genbank). For the first round PCR, 2 µl of each RT product was diluted to a final volume of 20 µl in 10 mmol/l Tris–HCl (pH 8.3), 50 mmol/l KCl, 1.5 mmol/l MgCl2, 0.1 mmol/l each of dATP, dTTP, dGTP, dCTP, 1 pmol each of sense and outer antisense strand primer (primer A and B) and 0.5 U Taq DNA polymerase. After an initial denaturation of 2 min at 92°C, 40 cycles of amplification were performed with 40 s denaturation at 92°C, 1 min annealing at 62°C and 1 min extension at 72°C in a Robo cycler gradient 96. The last cycle had an elongation time of 10 min at 72°C. PCR products were stored at −20°C until the second round PCR. For the second round PCR, 2 µl of each first PCR product was diluted with 18 µl of distilled-deionized water. From the diluted PCR product, a 2 µl aliquot was mixed to a final volume of 20 µl with sense and inner antisense strand primer (primer A and C). The other components and their final concentrations in the second round PCR mixture were the same as those of the first round PCR mixture. After an initial denaturation of 2 min at 92°C, 40 cycles of amplification were performed with 40 s denaturation at 92°C, 1 min annealing at 62°C and 1 min extension at 72°C in a Robo cycler gradient 96. The last cycle had an elongation time of 10 min at 72°C. 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).

Subcloning and sequencing of nested PCR product
The short variant of the second round PCR product was ligated to pCR4-TOPO vector and used to transform TOPO 10 E.coli K12 competent cells (Invitrogen, Carlsbad, CA, USA). Recombinant colonies were chosen and the plasmid containing the cDNA insert was purified using the High Pure Plasmid Isolation Kit (Boehringer). The purified plasmid was digested with EcoRI 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 the dyeodeoxy chain-termination method.

Immunohistochemistry
All endometrial samples were washed with 10 mmol/l phosphate buffered-saline, pH 7.4 (PBS) to remove excess blood and fixed with 10% formalin (Sigma) for 16–18 h and sunk down in PBS with 20% sucrose overnight. Fixed tissues were embedded and frozen in OCT compound, Tissue-Tek (Sakura, Torrance, CA, USA). Serial sections (8–10 µm) were obtained by cryostat, Leica CM 1900 (Leica, Nussloch, Germany), attached on the gelatin-coated slide and fixed on the slide at 58°C for 4–6 h using a slide warmer. We used abdominal muscle for the negative tissue controls. All the slides were stored at −70°C until use. To determine the presence of GnRH-II in human endometrium, slides were washed with PBS for 10 min and incubated with 0.3% hydrogen peroxide for 15 min to inhibit endogenous peroxidase activity. After an additional wash, the slides were incubated for 1 h in a blocking solution of PBS containing 5% normal goat serum and 1% Triton-X100 to reduce non-specific binding. The slides were incubated with rabbit antiserum (1/2000 dilution: aCH6, a generous gift from Dr Okuzawa, Mie, Japan) against chicken GnRH-II (Okuzawa et al., 1990; Parhar et al., 1996; Senthilkumaran et al., 1999) at 4°C overnight. For a negative control without specific antibody, the endometrial samples were incubated with blocking solution instead of specific antisera. The specificity of
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Figure 1. Schematic diagrams of nested reverse transcription-polymerase chain reaction (RT–PCR) primer sites on the human GnRH-II. (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. GAP is the region encoding for the GnRH-associated peptide. The signal sequence is the specific localization signal. (B) The sizes of RT–PCR products are indicated. (C) The sequences of one sense (primer A) strand and two antisense strand primers (primer B, C) are noted.

Results

Expression of GnRH-II mRNA in human endometrial tissues

All of the samples expressed both variants of GnRH-II mRNA. To examine the expression of GnRH-II mRNA in human endometrial tissues, three primers were designed as shown in Figure 1, and nested RT–PCR was performed. Six endometrial samples of fertile women at various stages of the menstrual cycle were examined for GAPDH and GnRH-II mRNA expression. After nested RT–PCR amplification, a 210 bp band of expected size and an additional smaller band (189 bp) were amplified. Representatives of the nested RT–PCR results are shown in Figure 2. The intensity of the small band was stronger than that of the expected band in all samples.

To verify the nested RT–PCR results, the second round PCR products were ligated to pCR4-TOPO vector and sequencing analysis was performed. We found that the smaller variant has a 21 nucleotide deletion in the region coding for the GnRH-associated peptide (GAP) and the predicted length of GAP is decreased from 77 to 70 amino acids (Figure 3). All together, both nested RT–PCR and sequencing analysis indicated that human endometrium expressed two spliced variants of GnRH-II mRNA during the entire menstrual cycle.

Expression of immunoreactive GnRH-II in human endometrial tissues

Immunoreactive GnRH-II was localized in the glandular and stromal cells of all endometrial samples examined in this study (Figure 4A-D). There was no significant staining in the...
respectively). There were no significant differences in the mean H-score between glandular and stromal cells during each phase of menstrual cycle were analysed. In glandular epithelial cells, the mean H-scores of early and mid-secretory phases (3.5 ± 0.1 and 3.3 ± 0.3 respectively) were significantly higher (P < 0.05) than those of proliferative and late secretory phases (2.1 ± 0.4 and 2.6 ± 0.3 respectively). Also in the stromal cells, the mean H-scores of early and mid-secretory phases (3.0 ± 0.2 and 3.1 ± 0.3 respectively) were significantly higher (P < 0.05) than those of proliferative and late secretory phases (1.6 ± 0.1 and 2.1 ± 0.1 respectively). There were no significant differences in the mean H-score between glandular and stromal cells during each of the menstrual phases (Figure 5).

Discussion

We have shown for the first time the mRNA and protein expression of the second isoform of GnRH (GnRH-II) in the human endometrium. Furthermore, the endometrium-specific splice variant of GnRH-II mRNA was shown in this study. The function of GnRH has been highly conserved during 500 million years of vertebrate evolution (Kasten et al., 1996). Today, a dozen isoforms of GnRH are known in the vertebrates and these decapeptides are conserved by 10–50% as compared to the amino acid sequence of mammalian GnRH (GnRH-I) (Sherwood et al., 1993). It has been reported that at least two or often three isoforms of GnRH are present in most vertebrates. The common isoform of GnRH is the so-called chicken GnRH-II (King and Millar, 1997; Sealfon et al., 1998). Distribution of the GnRH-II peptide was reported in the brains of amphibia (Conlon et al., 1993), musk shrew, one of the most primitive mammals (Dellovade et al., 1993) and primates, the stump-tailed and rhesus monkeys (Lescheid et al., 1997). For humans, the GnRH-II gene was recently cloned from the thalamus and mRNA was identified in the brain and kidney (White et al., 1998). This decapeptide was identical to chicken GnRH-II. Immunoreactive GnRH-II has also been found in the periaqueductal area as well as oculomotor and red nuclei of the midbrain (Chen et al., 1998). Similarly, we have been able to localize immunoreactive GnRH-II in these nuclei of the midbrain obtained from the abortus (unpublished data).

In our study, we were able to detect endometrium-specific transcript of GnRH-II with 21 nucleotide deletion in GAP, in addition to the mRNA previously reported in human brain and kidney. White and colleagues have demonstrated that alternative splicing occurs at the 5' end of exon 3 and a 21 nucleotide inserted variant is expressed only in human brain (White et al., 1998). Our nested RT–PCR and sequencing analysis results show that the endometrium-specific, shorter transcript has a 21 nucleotide deletion of the 5' end of exon 3, implying tissue-specific processing of GnRH-II transcripts.
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Figure 4. Localization of human GnRH-II peptide in the human endometrium. (A) Proliferative, (B) early secretory, (C) mid-secretory and (D) late secretory phase endometrium, (E) mid-secretory endometrium without primary antibody and (F) abdominal muscle with primary antibody as a negative tissue control. GnRH-II immunoreactivity was found in both stroma and glandular cells and the staining intensity was increased during the early and mid-secretory phases. Original magnification ×200. Scale bar = 100 µm.

Figure 5. Mean (± SEM) immunohistochemistry (H-SCORE) of GnRH-II in human endometrial glandular and stromal cells throughout the menstrual cycle. The immunoreactive GnRH-II was significantly increased in glandular epithelial and stromal cells during early and mid-secretory phase. *P < 0.05 between two phases. The number in the vertical bar denotes the number of the samples.

In this shorter transcript, the predicted length of GAP is decreased from 77 to 70 amino acids.

Immunoreactive GnRH-II peptide was also localized in the human endometrium throughout the menstrual phase. Both stromal and glandular cells expressed GnRH-II and the expression was increased during the early and mid-secretory phase. GnRH-I and the GnRH receptor are reported to be expressed in human endometrium in a cycle-dependent manner (Casañ...
et al., 1998; Raga et al., 1998) and also in preimplantation embryos (Casañ et al., 1999). Immunoreactive GnRH-I was also localized in stromal and glandular cells and the amount of immunoreactive GnRH-I was increased in both cells during the secretory phase (Casañ et al., 1998). In our findings, the localization and quantitative expression pattern of the GnRH-II peptide was similar to that of GnRH-I throughout the menstrual cycle (Casañ et al., 1998) and a large amount of immunoreactive GnRH-II was detected in the stroma and glands during early and mid-secretory phase. During the late secretory phase, the GnRH-II peptide was accumulated in the apical pole of the glandular lumen by apocrine secretion. The above-mentioned previous studies demonstrated that GnRH-I has a possible role in early embryonic development and implantation. Furthermore, there is an embryonic-maternal dialogue, in which the embryo and the endometrium induce changes in each other to promote embryonic development and endometrial receptivity (Tazuke and Guidice, 1996). Altogether, GnRH-II as well as GnRH-I may play a role in the embryonic development and implantation by their high-affinity binding to the GnRH receptor or another signal transduction pathway via an unknown GnRH receptor, possibly a novel receptor specific for GnRH-II.

In summary, our study demonstrates the presence of GnRH-II mRNA and peptide in human endometrium. The presence of GnRH-II as well as GnRH-I and the GnRH receptor may indicate a more complex series of events in the human endometrium. Furthermore the increased GnRH-II peptide during early and mid-secretory phase indicates their possible role in embryo implantation.

Acknowledgements

The authors wish to thank Gyun Jee Song for her technical advice and Dr Jeong-Wook Kim for his useful discussion of the manuscripts.

References


