Expression of gonadotropin-releasing hormone (GnRH) gene in human uterine endometrial tissue

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Many peripheral reproductive tissues have been found to contain gonadotrophin-releasing hormone (GnRH) and express the GnRH gene at low levels, presumably because the hormone functions in a paracrine/autocrine fashion. This study was designed to investigate and characterize GnRH gene expression in human endometrial tissue at different stages of the endometrial cycle. Reverse transcriptase–polymerase chain reaction (RT–PCR) analysis together with Southern blot assay demonstrated that human endometrial tissue expresses the proGnRH gene. RNA samples from endometrial tissue were analysed with two pairs of oligonucleotide primers. Both gave a doublet 870 bases apart at the expected sizes, indicating that both the upstream and downstream transcriptional start sites of the GnRH gene are used in endometrial tissue and that transcripts with and without intron 1 were produced. Our data also demonstrated that utilization of the two promoters varies with the stage of the endometrial cycle. The largest difference came from the mRNA transcribed from the downstream promoter and without intron 1. This mRNA was expressed at a very low level during the proliferative phase and dramatically increased almost 10-fold (P < 0.01) during the early secretory phase, and subsequently decreased 5-fold during the late secretory stage. The presence of GnRH mRNA in the endometrium, as well as the differential expression of the GnRH gene during the early secretory phase provides physiological evidence that human GnRH may play a paracrine/autocrine function in the human uterus. Key words: endometrial cycle/human GnRH gene/mRNA levels/uterine tissue

Introduction

Gonadotrophin-releasing hormone (GnRH) plays an integral role in the regulation of reproductive processes. It is released into the pituitary portal vessels from axon terminals in the median eminence. Subsequently, GnRH acts on the gonadotrophs of the anterior pituitary gland to stimulate the synthesis and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH).

Initially, it was believed that GnRH was produced exclusively by hypothalamic neurons, although the peptide was observed in a variety of reproductive tissues. Using nucleic acid probes, it has finally been recognized that GnRH can be produced by non-neural tissues as well (Dong et al., 1993, 1997). An increasing number of reports have described the presence of immunoreactive GnRH in human reproductive tissues, such as the ovary (Aten et al., 1987), testes (Bhasin et al., 1983), and mammary gland (Seppälä and Wahlström, 1980). Furthermore, GnRH receptor-binding activity has been demonstrated in the ovary (Koves et al., 1989; Latouche et al., 1989), placenta (Currie et al., 1989) and mammary gland (Eidne et al., 1987). These data, together with the demonstration of GnRH mRNA (Dong et al., 1993, 1997) and GnRH receptor mRNA (Peng et al., 1994) in reproductive tissues, strongly indicate that GnRH and GnRH receptors are produced locally in these same tissues, and that GnRH acts as a local regulatory factor in various reproductive tissues.

Our previous studies (Dong et al., 1993) demonstrated that the human GnRH gene has two transcription start sites, with the second one 5’ to the previously described hypothalamic transcription start site (Radovick et al., 1990). The upstream transcription start site, however, appeared to be significantly utilized only in reproductive tissues such as placenta, ovary and mammary gland (Dong et al., 1993, 1997). Although GnRH has been reported to be present in most reproductive tissues, whether the GnRH gene is actually expressed in human endometrial tissue is still unknown. Recently, Chegini et al. (1996) reported that the GnRH and GnRH receptor genes are expressed in human uterine myometrium tissue. High affinity binding and direct antiproliferative effects of GnRH analogues have also been found in human endometrial cancer cell lines (Gallagher et al., 1991; Emons et al., 1993). Furthermore, the GnRH receptor and its mRNA have been identified in endometrial carcinoma (Imai et al., 1994), suggesting that GnRH has a local function in the human uterus. This study was designed to investigate whether: (i) the GnRH gene is expressed in the...
human endometrial tissue; and (ii) GnRH gene expression varies during the endometrial cycle.

Materials and methods

Isolation of RNA

The human endometrial tissues were collected from patients at various stages of the menstrual cycle by endometrial biopsy using an endometrial suction curette ( Pipelle; Unimar Inc, Wilton, CT, USA). The utilization of human samples from endometrial biopsy is approved by the Institutional Review Board of Mount Sinai Medical School. The endometrial tissues were collected from a total of 23 healthy women of reproductive age (22–35 years old) with regular cycles. Hormonal profiles of the women and histological dating of the tissue samples were not carried out, but all the women had apparently normal menstrual cycles. The tissues were immediately frozen in liquid nitrogen and were stored at −80°C until used. The human term placental tissues and muscle tissue were generously provided by Drs Amalia Kelly and Grant Ko respectively, and used as positive and negative controls. Total RNA from these tissues was isolated by the guanidinium thiocyanate cesium chloride centrifugation method (Cathala et al., 1983). The total RNA was quantified by measuring the absorbance at 260 nm. The integrity of the RNA obtained and absence of chromosomal DNA was verified by agarose gel electrophoresis.

Reverse transcriptase–polymerase chain reaction (RT–PCR)

RT–PCR was used to determine whether the human endometrial tissue expresses the GnRH gene as well as which gene transcriptional start site was actually used. RT–PCR with two sets of DNA oligonucleotide primers, were used to examine the different GnRH mRNA species transcribed from the two promoters. The first pair (A, AATTTCACAAAACACACAATGGT, and E, CGTGGTGCAATTGGCTGGTTT) was designed to identify transcripts initiated from the upstream start site (Figure 1C). The second pair of primers (B, AGTACTCAACCTACTTCAAG, and E, CGTGGTGCA TTCG- A AGCGTTGGGTTCT) was designed to identify transcripts initiated from both upstream and downstream start sites (Figure 1C).

Total RNA (10 µg) was hybridized with oligo(dt), 12-18 in RT buffer (BRL, Gaithersburg, MD, USA), and the reaction was carried out with M-MLV reverse transcriptase (BRL) for 2 h at 37°C. The reaction was terminated by heating for 15 min at 68°C, and the reaction mixture was diluted to a final volume of 100 µl. An aliquot of 10 µl of the reverse transcription reaction was diluted to a final volume of 50 µl in 10 mM Tris–HCl pH 8.4, 2.5 mM MgCl₂, 250 µM (dATP, dGTP, dCTP and dTTP), 0.5 µg each of sense and antisense strand primer and 2.5 IU Taq polymerase (Perkin-Elmer Cetus, Foster City, CA, USA). The polymerase amplification was carried out for 30 cycles using a 94°C denaturing cycle (1 min), a 55°C annealing cycle (30 s) and a 72°C extension cycle for 2 min followed by a final extension for 10 min. The PCR products were then visualized by electrophoresis in an agarose gel with ethidium bromide.

Quantitative RT–PCR

[35S]dCTP (1 µM) was used in each reaction in the RT–PCR quantification studies (Dong et al., 1993). For the standard curve, the RT–PCR products amplified from the total RNA of JEG cells (a human placental tumour cell line, ATCC) by primers B/D (Figure 2) were gel purified, quantified by measuring the absorbance at 260 nm, and used as a DNA template for quantification. The RT–PCR products were separated on a 4% polyacrylamide gel, and radioactivity was quantified using a Molecular Dynamic Phosphorimager (Image Quant software program). The level of light emission (proportional to radioactivity) was plotted against the known amount of standard DNA to generate a regression line from which the content of the specific PCR product in each sample was computed. The same first strand cDNA was used for PCR analysis with a pair of primers which are complementary to the 3’ end of the human cyclophilin gene. The data from the PCR analysis of this housekeeping gene were used as internal controls for the quantitative RT–PCR.

Statistical analysis

All assays were repeated three times. Results are expressed as mean ± SEM. The data were evaluated by analysis of variance.

Results

Expression of the GnRH gene in human endometrial tissue

Our previous studies (Dong et al., 1993) demonstrated that the human GnRH gene has two transcription start sites, with a second one 5’ to the previously described hypothalamic transcription start site (Radovick et al., 1990). When primers A/E were used, the 979 and 1849 bp fragments were observed in the endometrial tissue and the placenta. When primer B/E was used, RT–PCR analysis of the first strand cDNA derived from the RNA isolated from human endometrial tissue yielded 256 and 1126 bp fragments (Figure 1A), however, these transcripts could also have been derived from the upstream transcriptional start site. The quantitative analysis described in the next section shows that the transcripts obtained using the B primer were always present in greater abundance, both with and without the first intron, than those obtained from primer A. This study suggests that both transcription start sites are used in human endometrial tissue. RNA samples from endometrial tissue and placenta assayed with primer pairs A/E and B/E gave doublets 870 bases apart at the expected sizes, indicating that the upstream and downstream promoters both produce transcripts with and without the first intron. Southern blot analysis showed that those RT–PCR products hybridized to the human GnRH cDNA probe (Figure 1B). When reverse transcriptase was omitted or when cDNA from human muscle tissue was used, neither pair of primers produced any amplification products by PCR. Together, both RT–PCR analysis and Southern blot assay indicated that human endometrium expresses the proGnRH gene. Both the upstream and downstream transcriptional start sites of the GnRH gene are used in endometrial tissues.

Quantification of different transcripts derived from the human proGnRH gene in endometrial tissue during the endometrial cycle

Due to the low level of proGnRH mRNAs in endometrial cells, RT–PCR quantification was used to measure the relative abundance of the four different species of proGnRH mRNA in these cells. Two sets of DNA oligomer primers were used to quantitate the mRNA transcribed from the two different transcriptional start sites. To minimize the difference in efficiency of amplification of the first strand cDNA from that of mRNA from the two start sites, the two sense primers were synthesized only a few bp apart (Figure 2C). Thus, in the first
Figure 1. Analysis of the different transcripts derived from the GnRH gene in human endometrial tissue. (A) Reverse transcription–polymerase chain reaction (RT–PCR) amplification of RNA samples from human endometrial tissue with two sets of primers. (B) Autoradiography of RT–PCR product after transfer to nitrocellulose and hybridization with human GnRH cDNA probe. (C) Summary of RT–PCR assay of the different transcripts derived from the human GnRH gene in endometrial tissue. The filled boxes represent the hypothalamic-type exon; the open segment 5' to exon 2 corresponds with GnRH mRNA transcribed from the upstream start site with unspliced intron 1. The transcriptional start sites are shown as a solid bent arrow. Dashed bent arrows indicate the positions of the primers.

Our data show that the total number of GnRH transcripts were not significantly different throughout the endometrial cycle (Figure 3). However, the utilization of the two promoters was different between stages. The largest difference came from the mRNA transcribed from the downstream promoter and without intron 1. This mRNA was expressed at a very low level in the proliferative phase, increased dramatically by almost 10-fold (P < 0.01) in the early secretory phase, and decreased 5-fold in the late secretory stage.

Discussion

Initially, expression of the GnRH gene was reported only in the hypothalamus and placenta (Seeburg et al., 1984; Adelman et al., 1986); however, our previous studies have demonstrated...
Figure 2. Quantification of the transcripts derived from the human $proGnRH$ gene in MDA and JEG cells by reverse transcription–polymerase chain reaction (RT–PCR). (A) Autoradiography of a polyacrylamide gel of $[^{35}S]$-labelled PCR amplification of reference DNA by primers B/D. (B) Autoradiography of an amplification gel of $[^{35}S]$-labelled RT–PCR products amplified from the first strand cDNA of four species of mRNA with the primers H/D and B/D (dashed bent arrows) from human endometrial tissue. (C) Diagram of the of the human $GnRH$ gene with locations of the PCR primers (dashed bent arrows), and the four species of human $GnRH$ mRNA.

that human reproductive tissues, such as the ovaries, testes and mammary glands also express the $GnRH$ gene (Dong et al., 1993, 1997). Although $GnRH$ mRNA has been found in the rat endometrial stromal cells (Ikeda et al., 1996), and immunoreactive GnRH has been identified in porcine endometrial tissue (Li et al., 1993), expression of the $GnRH$ gene in human endometrial tissue has not been reported. The present results demonstrate for the first time the presence of $GnRH$ mRNA in human uterine endometrial tissue. Due to the low level of $GnRH$ mRNA in this tissue, demonstration of expression is based upon the combination of RT–PCR and Southern blot analyses. Amplification products were not detected in muscle tissue, hence the possibility of our observation resulting from contamination seems highly unlikely. Since we used a device known as an endometrial suction curette (Pipelle) to collect the endometrial tissue, the biopsy tissues were free from myometrium which has been shown to express the $GnRH$ gene. Furthermore, seven out of the 23 samples were checked for contamination by other tissues using a standard histological stain. No myometrial tissues were found in our collected samples. Therefore, contamination by $GnRH$ mRNA from myometrium was eliminated.

Our previous studies revealed that the human $GnRH$ gene uses two transcription start sites (one upstream and the other downstream) to produce different $GnRH$ mRNAs. The downstream transcription start site is widely used in all tissues which express the $GnRH$ gene. However, the upstream transcription start site is primarily utilized in the non-hypothalamic reproductive tissues (Dong et al., 1993, 1997). The use in reproductive tissues of a promoter other than the hypothalamic promoter reveals that there is a tissue-specific difference in $GnRH$ expression between the hypothalamus and reproductive tissues such as the placenta, ovaries, mammary glands and endometrial tissue.

Although the actual function of GnRH in the human uterus is not fully understood, the presence of $GnRH$ mRNA, as well as the GnRH receptor and its mRNA in endometrial tissue, strongly suggests paracrine and/or autocrine functions of human GnRH in this reproductive tissue. Endometrial tissue plays an important role in implantation and supporting embryo
GnRH gene expression in endometrial tissue

Figure 3. (A) Levels of GnRH mRNA during the endometrial cycle. Data are expressed relative to 10^{-5} fmol DNA standard. The DNA standard was prepared from the same size DNA template (see Materials and methods). Values refer to the percentage of total mRNA transcript species present in each tissue fraction. Results are expressed as mean ± SEM. The data were evaluated by analysis of variance. Asterisks indicate different proGnRH mRNA species, as shown in (B).

development during pregnancy. In order to achieve a successful implantation, communication between the embryo and uterus is essential. Recent studies from our laboratory and from others have demonstrated the presence of GnRH and GnRH receptors in both the embryo and uterus, which suggests that GnRH may play some role in implantation (Imai et al., 1994; Seshagiri et al., 1994; Ikeda et al., 1996).

Studies from in-vitro culture of peri-implantation monkey embryos in a well established culture system (Seshagiri et al., 1994), demonstrated that the secretion of GnRH (pg/ml) by embryos (n = 20) commenced from low levels (0.32 ± 0.05) during the prehatching blastocyst stage to 0.7 ± 0.08 at 6–12 days (hatched blastocyst attachment stage) and 1.30 ± 0.33 at ≥13 days (proliferation of trophoblast cells). It is interesting to note that although the GnRH mRNAs are not significantly different throughout the endometrial cycle, the GnRH mRNA transcribed from the downstream transcriptional start site differs with the stages. Specifically, the 497bp GnRH mRNA is at the lowest level during the proliferative phase, and dramatically increases (~10-fold) during the early secretory
phase and subsequently decreases during the late secretory phase. Our previous studies suggest that the 499 bp GnRH mRNA could be translated into proGnRH protein most efficiently (Dong et al., 1997). Thus this change during the early secretory phase may have some physiological relevance since, during the early secretory phase, the endometrium is preparing for the implantation of the embryo.

The autocrine and paracrine functions of GnRH have also been reported in other reproductive tissues such as ovary and placenta (Bramley and Menzies 1996; Furger et al., 1996). Although GnRH receptor mRNA has been found in the ovary (Peng et al., 1994), the existence of specific binding site for the GnRH in this tissue is controversial. This inability to determine the binding sites may be due to different expression of the GnRH receptor gene in ovarian tissue or to a lack of an efficient method of detection. Bramley and Menzies (1996) have demonstrated that the degradation of GnRH tracers may prevent the measurement of specific GnRH binding sites in some tissues. Despite this, the direct action of GnRH and its analogues have been demonstrated in ovarian cancer cell(s) and other reproductive organ cancer cells (Imai et al., 1994). Furthermore, Furger et al. (1996) have demonstrated that GnRH inhibited the follicle stimulating hormone-induced response in primary cultured human granulosa–lutein cells.

In summary, this study reports the presence of GnRH mRNA in human endometrial tissue for the first time. It is difficult to predict exactly how human uterine GnRH affects the development and implantation of the embryo; however, the presence of GnRH and GnRH receptors, both in the embryo (Seshagiri et al., 1994) and endometrial tissue (Imai et al., 1994), as well as the differential GnRH gene expression during the early secretory phase, provides physiological evidence that human GnRH may play a significant role in the local function of the uterus.

Acknowledgements

Kerlin Marcelin was supported by National Medical Fellowship from Bristol-Myers Squibb Inc, and the Commonwealth Foundation. This work was supported by a NIH Grant (IR29HD/CA30244 to K.W.D.). We wish to thank Barbara Atkinson for proofreading.

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Received on February 17, 1998; accepted on June 9, 1998