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DEBATE—continued

Embryo implantation and GnRH antagonists

The search for the human placental GnRH receptor

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Hypothalamic gonadotrophin-releasing hormone (GnRH) plays a major role in the endocrine control of reproduction. Acting through its high affinity receptors on pituitary gonadotrophs, it regulates the secretion of gonadotrophins. In addition, GnRH also functions as a local regulator in a number of other cell lines and tissues, including the placenta. In a manner analogous to hypothalamic GnRH stimulation of LH and FSH from the anterior pituitary, GnRH was found to cause a dose-dependent release of human chorionic gonadotrophin (HCG) from the placenta. So began the search for a putative GnRH receptor in the human placenta. Although early radio-receptor studies reported specific binding, the properties of these 'putative' GnRH binding sites were found to differ significantly from those of their pituitary counterparts in several important respects. This was followed by a series of contradictory reports that led to more questions and opened up avenues for further investigations. Even after nearly two decades of research, the human placental GnRH receptor has not been characterized beyond all reasonable doubt. This review recalls the discovery, the controversies and unanswered questions concerning the human placental GnRH receptor.

Key words: GnRH receptor/ human placenta

Introduction

Gonadotrophin-releasing hormone (GnRH) is the key neural regulator of the reproductive process. GnRH released from the hypothalamus controls the synthesis and release of LH and FSH from the pituitary, which, in turn, affects gonadal function. In the pituitary, the primary site of action of GnRH is the gonadotroph, the cell that expresses high affinity GnRH receptors and secretes gonadotrophic hormones. The interaction between GnRH and its receptor is a critical event in the endocrine regulation of reproduction and therefore, understanding the regulation of the GnRH receptor is important for the study of normal reproductive physiology.

GnRH functions not only as a releasing hormone in the pituitary, but is distributed widely throughout the central and peripheral nervous system as well as in several extra-neural, neoplastic and non-reproductive tissues (Hsueh and Jones, 1981; Stojilkovic *et al*., 1994; Kakar and Jennes, 1995; Kottler *et al*., 1999), where it is believed to be involved in the regulation of cell growth and proliferation.

A role for GnRH in the human placenta

A plethora of in-vitro studies have demonstrated a functional role for GnRH in placental endocrinology, based on the observation that incubation of human placental minces with synthetic GnRH significantly increased the release of immunoactive human chorionic gonadotrophin (HCG) (for reviews, see Siler-Khodr, 1983; Belisle *et al*., 1984; Mathialagan and Rao, 1986a; Lin *et al*., 1995). In turn, HCG stimulates the synthesis and secretion of progesterone from the corpus luteum, which is required for early establishment and maintenance of pregnancy. HCG also up-regulates low density lipoprotein (LDL) receptor expression in the placenta, thereby enhancing the rate-limiting step in the biosynthesis of progesterone, which is indispensable for the maintenance of pregnancy (Gopi Shanker *et al*., 1998). The endocrine response to exogenous GnRH was dose-dependent, maximal at mid-gestation, and partly inhibited by an antagonist of GnRH (Siler-Khodr *et al.*, 1983), indicating the involvement of GnRH receptor-mediated downstream signalling. The release of HCG following GnRH stimulation (which in turn induces progesterone production) is reminiscent of the cascade that operates in the hypothalamo–pituitary–gonadal axis (Santra *et al*., 1999).

Placenta produces a decapeptide that is similar to hypothalamic GnRH

Since circulating concentrations of hypothalamic GnRH are far too low to sustain placental production of HCG, it was proposed that the placenta itself could produce GnRH or

GnRH-like peptides and also respond to it. This hypothesis was confirmed by several in-vitro and in-vivo experiments, some of which are described below.

Incubation of placental trophoblasts with radioactive precursors resulted in the synthesis of a physiologically active decapeptide (Gibbons *et al*., 1975; Tan and Rousseau, 1982). The concentration of this GnRH-like substance was found to be higher in the placenta than in the maternal or fetal circulation or amniotic fluid. This decapeptide was found to share structural, immunological, chemical and biological similarities with hypothalamic GnRH (Gibbons *et al*., 1975; Siler-Khodr *et al*., 1983; Mathialagan and Rao, 1986b). The cDNA sequences of the precursor forms of hypothalamic and placental GnRH were also shown to be identical (Seeburg and Adelman, 1984; Kelly *et al*., 1991; Duello *et al*., 1993). Early immunofluorescence studies (Khodr and Siler-Khodr, 1978; Miyake *et al*., 1982) suggested that cytotrophoblasts of placental villi were the site of production of this decapeptide in the placenta. However, recent reports (Wolfahrt *et al*., 1998) indicate its production by both cytotrophoblasts and syncytiotrophoblasts in first and third trimester placentae.

Discovery and characterization of human placental GnRH binding sites

In 1981, for the first time, the presence of the GnRH receptor in the human placenta using radio-receptor assays was reported (Currie *et al*., 1981). This was also the first demonstration of extra-pituitary GnRH receptors in the human.

Crude membrane preparations from term placentae, placentae at 10–12 weeks gestation and one hydatidiform mole were found to bind GnRH agonists with high affinity and specificity. Native GnRH competed for binding with the same affinity whereas unrelated peptide fragments (oxytocin, thyrotrophin-releasing hormone, somatostatin) gave no or minimal displacement. Native GnRH was found to have the same affinity for the placental GnRH receptor as it had for GnRH receptors in the pituitary cell membranes, rat luteal cells or rat Leydig cells. However, GnRH agonists bound these tissues with a 100-fold higher affinity than the placenta. Three possible explanations were offered for this observation.

Firstly, the placenta could possibly possess a protease that acts on sites within the agonists not protected by D-amino acid substitutions and, therefore, the agonists could be degraded to the same extent as placental GnRH. This view was initially supported by the observation that the amount of [^{125}I]-GnRH agonist bound per mg protein decreased as protein concentration was increased. The following possibility, however, had to be considered. Differences in the abilities of radiolabelled GnRH isoforms to bind to rat pituitary and human placental receptors may indeed reflect differential inactivation of tracer during the binding incubation. Therefore, if one tracer were degraded more rapidly during incubation with placental membranes than the second, then the former would appear to be less capable of binding. This question was convincingly addressed by a study of the inactivation of various GnRH tracers following incubation with either rat pituitary or human placental membranes (Bramley *et al*., 1992). It was shown that, although different isoforms were degraded at different rates in these tissues, differences in the ability of the isoforms to bind to placental rather than pituitary sites were not related to differences in degradation of the tracers, but rather to differences in their specificity for the binding site.

Secondly, it was also possible that placental GnRH was structurally different from hypothalamic GnRH, in which case the GnRH receptor would have a higher affinity for the correct ligand and a lower affinity for both GnRH and the agonist. However, all available evidence, as recalled earlier in this review, supported the view that placental GnRH was indistinguishable from hypothalamic GnRH and therefore this possibility seemed unlikely although differential processing of the prepro-GnRH to yield other isoforms of GnRH could not be ruled out.

Thirdly, it was also suggested (Currie *et al*., 1981) that GnRH or GnRH agonists bind to a placental site, which was in fact a GnRH-degrading enzyme. However, it was later demonstrated that this was not so (Menzies and Bramley, 1992) and, by subcellular fractionation of placental tissue following density gradient centrifugation, it was shown that while degradation of radiolabelled ligand is associated with placental cytosol fractions, GnRH binding activity was associated largely with placental plasma membranes.

A further possibility remained that besides the presence of high affinity receptors for GnRH, which the study reported, there are also other receptors for GnRH which are structurally different and whose concentration varies in different sample preparations through gestation.

This additional possibility was corroborated by other studies (Belisle *et al*., 1984), which reported multiple binding sites for GnRH in enriched mid-term and term chorionic membrane preparations on the strength of binding data. These authors reported high affinity and low affinity binding sites for GnRH. Their results indicated that GnRH agonists and antagonists do not compete with the same receptor sites to displace native GnRH and that a population of these binding sites exhibited low affinity for GnRH. This observation was unexpected and difficult to explain since, in rat and bovine pituitary radioreceptor assays, inhibitory analogues of GnRH compete with synthetic GnRH for the same receptor sites.

However, despite their low affinity for GnRH, these binding sites were found to be saturable, of low capacity–high specificity for the ligand. The functional relevance of these sites in the production of bioactive HCG was also demonstrated (Belisle *et al*., 1984, 1987). Studies with placental cells from anencephalic fetuses showed a decreased binding capacity for GnRH and its agonists, when compared with normal trophoblastic cells, as well as a reduced capacity to produce HCG. These results also suggested that mechanisms dependent upon GnRH binding to its receptor are required for placental HCG production in normal pregnancies. These findings gained additional support from other studies (Iwashita *et al*., 1986), which confirmed the existence of low affinity (yet specific) binding sites for GnRH in human term placentae, again using binding studies. However, further approaches had to be taken to determine the biochemical nature of this receptor.

The presence of a 58 kDa binding site for GnRH in human

Table I. Locations in the gonadotrophin-releasing hormone (GnRH) receptor sequence to which the various primers used in reversetranscription–polymerase chain reaction (RT–PCR)/Southern blotting experiments were designed

Report	Study	Nucleotide positions of amplicons (according to Kakar *et al.,* 1992)	Comments
Wolfahrt *et al*. (1998)	Nested-RT–PCR	459–826 (primary amplicon, 368 bp)	The primers are exon-spanning. The primary amplicon is derived
		510–756 (secondary amplicon, 247 bp)	from all three exons of the GnRH receptor gene
Boyle *et al.* (1998)	RT–PCR	1–1049 (1049 bp)	The primers amplify the full-length GnRH receptor cDNA
Kakar and Jennes (1995)	RT–PCR and	706–987 (282 bp)	The primers amplify a sequence that spans portions of exons 2 and 3.
	Southern blot analysis		Probe for Southern analysis: human pituitary GnRH receptor cDNA representing its open reading frame.

term placental membranes was demonstrated (Escher *et al*., 1988) using photolabelling experiments followed by sodium dodecyl sulphate (SDS) gel electrophoresis. But again, their results suggested the association of this 58 kDa binding site with other proteins to form the functional GnRH receptor.

All studies described so far pointed to the presence of low affinity GnRH receptors in the human placenta that are distinct from the typical high affinity receptors for GnRH present in the pituitary. These data, however, did not provide a mechanistic explanation for the regulatory role of GnRH in placental HCG secretion. Characterization of GnRH receptor gene expression was required to define the functional role of GnRH in the regulation of HCG secretion by the placenta and the associated changes with gestational age.

Cloning and characterization of the human pituitary GnRH receptor (Kakar *et al*., 1992; Chi *et al*., 1993) allowed the availability of cDNAs and, therefore, the use of sensitive assays involving hybridization to radioactive probes.

Human placental *GnRH receptor* mRNA expression

In an attempt to investigate the distribution of the *GnRH receptor* gene transcript in the human placenta, in-situ hybridization assays were performed with samples at various gestational ages (Lin *et al*., 1995). GnRH receptor expression could be detected in both cytotrophoblasts and in syncytiotrophoblasts. The signals were abundant at 6 weeks gestation, peaked at 9 weeks, began to decline in the third trimester and, by term, the signals were undetectable by this assay. Also, the responsiveness of placental explants to GnRH stimulation in HCG was found to parallel the changing intensity of signals at different stages of gestation. This observation was in agreement with the previous binding studies (Bramley *et al*., 1994), which reported that specific GnRH binding activity was significantly reduced in membranes from placentae recovered at 10–20 weeks gestation, compared with 6–9 weeks. However, to quote Boyle *et al*., 'it is difficult to assess these results (Lin *et al.,* 1995), since it is not clear whether the sampling was sufficient and also, non-specific labelling does not appear to have been subtracted from total labelling' (Boyle *et al*., 1998).

In the same year, other authors (Kakar and Jennes, 1995) reported the direct demonstration of the human placental *GnRH receptor* mRNA. Using primers based on the sequence of the human pituitary *GnRH receptor* cDNA, they amplified the placental GnRH receptor message by reverse transcription–polymerase chain reaction (RT–PCR) and performed Southern blotting under high stringency conditions to determine the authenticity of the product obtained. However, in the absence of sequence information, it is difficult to conclusively comment on the extent of identity to the pituitary counterpart. Also, the RT–PCR product that hybridized to the human pituitary cDNA sequence represents only a small fragment (see Table I). It is highly likely that corresponding sequences differ outside this region.

This was soon followed by studies (Wolfahrt *et al.,* 1998), which demonstrated the presence of the *GnRH receptor* mRNA in human trophoblasts throughout gestation, using in-situ RT–PCR and exon-spanning primers (see Table I). These results were contradictory to previous studies (Lin *et al*., 1995), which suggested that *GnRH receptor* mRNA expression decreases with gestation and disappears at term. This is perhaps due to the fact that the technique of in-situ RT–PCR used by Wolfahrt *et al.* was more sensitive than in-situ hybridization and could help demonstrate the GnRH receptor message, even in cells from term placentae, which earlier studies could not. However, this study could not determine whether the GnRH receptor message detected was actually translated into a functional protein product.

In 1998, the sequence of a human placental cDNA (Boyle *et al*., 1988) was shown to be identical to that of the pituitary *GnRH receptor* cDNA, as previously reported (Kakar *et al*., 1992; Chi *et al*., 1993). Using specific primers (designed based on the human pituitary *GnRH receptor* cDNA sequence, see Table I), they amplified the human placental *GnRH receptor* cDNA by RT–PCR and sequenced the clones. This study, however, leaves room for doubt. The source of the RT–PCR products is questionable because the authors have not ruled out endometrial contamination in the first trimester placental samples collected. A myriad of studies, reported over the last decade, have demonstrated the presence of the GnRH receptor in the endometrium (Imai *et al*., 1994a,b; Chatzaki *et al*., 1996; Raga *et al*., 1998; Takeuchi *et al*., 1998). Since the endometrium and the placenta are intimately associated with each other during pregnancy, it is important to ensure the absence of endometrial contamination, more so, since even traces of it in placental samples would give a grossly erroneous picture of the genes expressed by the placenta, given the sensitivity of the RT–PCR technique. Studies from our labora-

tory (Rao *et al*., 2000) demonstrate that specific endometrial messages can be amplified by RT–PCR from as little as 0.01% contribution from endometrial cDNA. Also, this study (Boyle *et al*., 1988) reported the amplification of the receptor cDNAs from 6 and 8 week placental samples but not from 5 and 7 week samples. This is difficult to explain, given that maternal HCG concentrations rise exponentially for the first 6 weeks of gestation, peak at weeks 8–10 and only decrease thereafter.

However, discounting the above-mentioned inconsistencies, the results based on RT–PCR and in-situ hybridization (Kakar and Jennes, 1995; Lin *et al*., 1995; Boyle *et al*., 1998; Wolfahrt *et al.,* 1998), indicated that the action of GnRH in the placenta could indeed be mediated through its typical high affinity receptors. These were in sharp contrast to the earlier binding studies that suggested that the receptors might differ. An obvious question that had to be addressed was the copy number of the GnRH receptor gene in the human genome. The answer to this question made explanations to the above observations more difficult. Genomic Southern blot analysis indicated its presence in a single copy (Fan *et al*., 1994). However, since the human GnRH receptor gene has multiple transcription sites and polyadenylation signals (Fan *et al*., 1995), it is possible that there may be a differential usage of promoter and/or polyadenylation signals. In a preliminary study (Rodway *et al*., 1995) the detection of GnRH receptor transcript in the human placenta was described (for review, see Leung and Peng, 1996). Unlike in the pituitary where a major transcript of 4.7–5 kb was found (Kakar *et al*., 1992; Chi *et al*., 1993), Northern blot analysis of human placental tissue, as well as two placental choriocarcinoma cell lines, JEG-3 and JAR, revealed a major transcript of 1.2 kb.

A further possible explanation for this discrepancy could involve the presence of multiple transcripts for the human placental GnRH receptor gene that may arise due to alternative splicing. This is possible because the open reading frame in this gene is distributed among three exons. Alternative splicing is known to occur in numerous members of the G-protein-coupled receptor family, and is correlated with differences in affinity, potency, coupling efficiency, specificity, subcellular localization or sensitivity to desensitization of the receptor. Interestingly, such variant transcripts, differing in their sequence, have been described for the mouse and human pituitary GnRH receptor gene (Zhou and Sealfon, 1994; Grosse *et al*., 1997). Variant transcripts of the GnRH receptor gene have also been shown to exhibit a tissue-specific pattern of expression (Kottler *et al*., 1999). The functional relevance of these variant transcripts is, however, a matter of speculation. Even if the sequence of the transcripts turns out to be identical, the encoded proteins could perhaps differ in their post-translational processing, yielding proteins that differ in their phosphorylation or glycosylation states. Multiple receptor isoforms, if present, could modulate the function of GnRH by exhibiting variable affinities and could therefore explain earlier binding data in the light of recent observations made on the strength of RT–PCR and in-situ hybridization assays. Studies from our laboratory also support this possibility. Using primers based on the sequence of the human pituitary *GnRH receptor* cDNA, we have amplified and cloned the bonnet monkey pituitary GnRH receptor (Santra *et al*., 2000). However, we were unable to amplify the human placental sequence using these primers under all conditions tried (unpublished observations). Using another set of exon-spanning primers, we have amplified and cloned an internal fragment of the human placental GnRH receptor cDNA (unpublished observations). However, we have not yet been able to obtain a full-length fragment by RT–PCR. It is possible that the human pituitary and placental sequences differ while being conserved at the exon–intron junctions so that, amplification occurs only with exon-spanning primers. Furthermore, the failure to amplify the placental *GnRH receptor* mRNA consistently at all stages of gestation, using the same set of primers, also suggests that there might be different receptors expressed at different stages of gestation.

In the midst of a sea of unanswered questions and a host of possibilities, it is slowly beginning to dawn why the placental receptor sequence has eluded detection for so long. The technical modifications (e.g. nested PCR or reamplification PCR) described in the previous reports (Boyle *et al*., 1998; Wolfahrt *et al*., 1998), to amplify the sequence, suggest that either the *GnRH receptor* mRNA is a rare transcript or it may have a very short half-life. It is also possible that *GnRH receptor* mRNA may be translated as rapidly as it is transcribed, with the result that the net accumulation of mRNA within the cell, at any given point in time, is below the limits of detection. Again, our own observations (unpublished data) are also in concord with these and contrary to those of Kakar and Jennes (1995), who reported detection of this mRNA species without the need for any special technical modifications to enrich *GnRH receptor* mRNA in the total RNA pool. Perhaps more extensive sampling and screening of placental libraries might reveal additional forms present in small amounts. This is highly probable, considering a previous report (Troskie *et al*., 1998). Their study using PCR amplification of genomic DNA with degenerate primers (designed to amplify the extracellular loop 3), supports the concept of existence of multiple GnRH receptor sub-types in vertebrates. Again, whether there does truly exist more than one physiologically significant form of the receptor, only time will tell.

Conclusions

The characterization of the human placental GnRH receptor is of utmost importance because it contributes directly to the study of HCG regulation and hence, to our understanding of placental endocrinology. Ever since the discovery of GnRH binding sites in the human placenta, a myriad of studies have attempted to characterize the 'putative' receptor. Early studies based on radioreceptor assays revealed that GnRH agonists bind the placental site with a much lower affinity than they bind the corresponding sites on pituitary cells. These results suggested that the receptors might differ. In sharp contrast to the binding data, recent reports based on RT–PCR and in-situ hybridization assays suggest that the human pituitary and placental GnRH receptors may indeed be identical. This is difficult to explain, considering the fact that the GnRH receptor gene exists as a single copy gene in the human genome. However, a careful analysis of the gene structure points to several possibilities for this observation. The utilization of multiple transcription sites, polyadenylation signals and splice

junctions could result in the formation of several isoforms of the placental receptor. Generation of multiple isoforms could have a physiological significance and could be another level of control of GnRH receptor gene expression. The story is, however, far from complete. There are more questions than answers as of now. The search for the human placental GnRH receptor continues.

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