Human chorionic gonadotrophin-β gene sequences in women with disorders of HCG production*

Lawrence C.Layman1,10, John L.Edwards4,7, William E.Osborne4,8, Douglas B.Peak6, Donald G.Gallup3, Sandra P.T.Tho2, Richard H.Reindollar8, Dorothy J.Roach4,9, Paul G.McDonough2 and Kenneth D.Lanclos5

1Section of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, The University of Chicago, Chicago, MC 2050, 2Section of Reproductive Endocrinology, 3Section of Obstetrics and Gynecology, 4Department of Obstetrics and Gynecology, 5Department of Biochemistry and Molecular Biology, Medical College of Georgia Augusta, GA, and 6Division of Reproductive Endocrinology, Department of Obstetrics and Gynecology, Tufts University School of Medicine, Boston, MA, USA

7Current address: 2660 10th Avenue, Suite 301, Birmingham, AL 35205, 8Current address: Provident OB/GYN Associates, 4750 Waters Avenue, Suite 400, Savannah, GA 31404, 9Current address: Reproductive Endocrinology, 530 Wells Fargo, Suite 116, Houston, TX 77090, USA

10To whom correspondence should be addressed

Women with recurrent abortion, primary unexplained infertility, and gestational trophoblastic neoplasia (GTN) manifest disordered human chorionic gonadotrophin (HCG) secretion. Mutations in the HCGβ/luteinizing hormone (LH)β gene complex could cause aberrant HCG production in these disorders. The purpose of this study was to determine whether HCGβ gene deletions occur in women with recurrent abortion or primary unexplained infertility, and whether HCGβ gene duplications are present in women with GTN. DNA was extracted from 10 patients with unexplained recurrent abortion, 10 patients with unexplained primary infertility, 12 patients with GTN, three partners of women with GTN, and 30 controls. Southern blots were constructed and hybridized with DNA probes for HCGβ-5 and the LHβ gene. No gene deletions were identified in patients with recurrent abortion or primary unexplained infertility. Likewise, no gene duplications were identified in women with GTN. A previously described MboI restriction fragment length polymorphism (RFLP) was identified in both patients and controls. A new PstI RFLP was also characterized, but was present in patients and controls. Deletion/duplication mutations in the HCGβ/LHβ gene complex do not appear to be common causes of aberrant HCG production in humans with these disorders.

Key words: gestational trophoblastic disease/HCGβ genes/infertility/LHβ genes/recurrent abortion

Introduction

Human chorionic gonadotrophin (HCG) is a member of the pituitary glycoprotein hormone family, which also includes luteinizing hormone (LH), follicle stimulating hormone (FSH), and thyroid stimulating hormone (TSH) (Pierce and Parsons, 1981). Each protein is a heterodimer, composed of an α-subunit common to all four proteins and a β-subunit which confers specificity (Pierce and Parsons, 1981). A single gene on the long (q) arm of chromosome 6 encodes the α-subunit (Fiddes and Goodman, 1981; Naylor et al., 1983), while a cluster of genes encode the HCGβ/LHβ complex on chromosome 19q (Policastro et al., 1983, 1986; Talmadge et al., 1983; Brook et al., 1985; Graham et al., 1987; Jameson and Lindell, 1988). There are six HCGβ genes and a single LHβ gene, which share >90% homology (Policastro et al., 1983, 1986; Talmadge et al., 1983; Brook et al., 1985; Graham et al., 1987; Jameson and Lindell, 1988; Jameson and Lindell, 1988). HCGβ-5 is the most abundantly expressed gene, although all of the other five HCGβ genes are also expressed to lesser degrees (Boime et al., 1982; Talmadge et al., 1984; Jameson et al., 1986; Bo and Boime; 1992; Jameson and Hollenberg, 1993). HCGβ is principally expressed in the placenta, but low levels of expression have also been demonstrated in the pituitary (Jameson and Hollenberg, 1993).

Placental HCG is produced by the early blastocyst and serves as the major biochemical marker of pregnancy (Shi et al., 1993). Although it is produced principally by the syncytiotrophoblasts, HCG is also secreted by the cytotrophoblasts (Lei et al., 1992; Shi et al., 1993). Recently, HCG has also been found to have functions within the placenta in addition to maintaining corpus luteum function (Shi et al., 1993). These functions of HCG include the differentiation of cytotrophoblasts into multinucleated syncytiotrophoblasts (Shi et al., 1993) and the regulation of its own synthesis through interaction with the LH/HCG receptor on placental cell membranes (Lei et al., 1992). The production of HCG appears to be important for luteal support (Soliman et al., 1994) and the prevention of fetal demise (Stevens, 1975).

© European Society for Human Reproduction and Embryology 315
We are not aware of studies investigating the presence of HCG\(\beta\) gene mutations in human endocrine disease. Patients with abnormalities in the production of HCG are potential candidates for the presence of mutations within the HCG\(\beta\) gene complex (Layman, 1991; Roach et al., 1992). Women with recurrent abortion demonstrate decreasing concentrations of serum HCG and subsequent miscarriage, whereas women with unexplained infertility either do not adequately produce HCG, or have only a transient (or even undetectable) rise of HCG (Speroff et al., 1994). In both instances, the mechanisms responsible for decreasing or low concentrations of HCG are unknown. Conversely, women with gestational trophoblastic disease overproduce HCG, which also suggests disordered HCG gene expression or production due to gene mutations (Speroff et al., 1994).

When a gene complex exists, such as LH\(\beta\)/HCG\(\beta\), there is an increased probability that meiotic errors could cause gene deletions and/or duplications. The purpose of the present study was to determine if HCG\(\beta\) gene mutations exist in three different groups of women with reproductive deficiency, gene deletions in recurrent abortion and primary unexplained infertility, and gene duplications in gestational trophoblastic neoplasia (GTN).

**Materials and methods**

**Patients**

Patients and controls were studied by Southern blot analysis to identify deletions or duplications in one or more of the six HCG\(\beta\) genes. Ten women had unexplained recurrent abortion, defined as two or more spontaneous abortions, with no identifiable aetiology. Spontaneous abortion was documented by the presence of a positive serum HCG and the presence shown by ultrasound of a fetal sac, or by pathological documentation of products of conception.

The definition of recurrent abortion is controversial, but, traditionally, it has been defined as three or more consecutive abortions (Hill et al., 1995; Stephenson, 1996), while other investigators use two or more miscarriages (McDonough, 1985; Warburton and Strobino, 1987; Plouffe et al., 1992). We chose to define recurrent abortion as two or more spontaneous abortions, with or without the presence of livebirths, because the prevalence of genetic, uterine, and autoimmune causes are similar to those with three or more abortions (McDonough, 1985; Warburton and Strobino, 1987; Plouffe et al., 1992). All recurrent abortion patients had a normal karyotype (as did their partner), hysterosalpingogram, endometrial biopsy, anticoagulant antibody, activated partial thromboplastin time, serum prolactin, TSH, and thyroxine. Ten women with unexplained infertility for 5 years were also studied. All patients had a normal hysterosalpingogram, diagnostic laparoscopy, postcoital test, endometrial biopsy, serum thyroid studies, prolactin, and normal ovulation documented by urinary LH testing or ultrasound follicular monitoring. All partners had a normal semen analysis by World Health Organization criteria. Twelve women with GTN had standard histological evidence of non-metastatic GTN, which included trophoblastic proliferation, hydropic avascular villi, and the absence of fetal tissue. Three men, who were partners of the women with GTN, and 30 normal fertile men without a prior history of recurrent abortion and GTN were also studied. The controls had normal puberty, demonstrated fertility, and no history of recurrent abortion. This study was approved by the Human Assurance Committee of the Medical College of Georgia, USA.

**Southern blot analysis**

DNA was extracted from peripheral white blood cells and Southern blots were constructed as described previously (Layman et al., 1993). Briefly, DNA was first extracted from peripheral leukocytes, and then digested separately with a variety of restriction enzymes: *PstI*, *XbaI*, *EcoRV*, *BamHI*, *HindIII*, *MboI*, *HindII*, and *KpnI*. DNA samples were electrophoresed in 1% agarose gels, denatured using alkali, transferred to nylon membranes, and then baked in a vacuum oven (Layman et al., 1993). Membranes were first prehybridized using Church’s solution and then hybridized at 65°C separately with HCG\(\beta\) and LH\(\beta\) probes (Gray, 1992).

The HCG\(\beta\)-5 probe is a 900 bp fragment containing part of intron 1, and all of the second and third exons (Talmadge et al., 1983). The LH\(\beta\) probe is a 2.2 kb insert complementary to the entire LH\(\beta\) gene (Talmadge et al., 1983). Both probes were purchased from the American Tissue Culture Collection, Rockville, MD, USA. The HCG\(\beta\)-5 probe was radio-labelled by the random primer method, denatured by boiling, and hybridized with the membranes (Layman et al., 1993). The blots were then washed at high stringency using 0.1X sodium chloride/sodium citrate (SSC)/0.1% sodium dodecyl sulphate (SDS) at 65°C, followed by autoradiography at –70°C; the film was then developed. After hybridization with the HCG\(\beta\) gene probe, blots were stripped of radiation signals by boiling in deionized water for 30 s and reprobed with radiolabelled LH\(\beta\).

**Results**

Southern blots of DNA digested with the enzymes *BamHI*, *EcoRV*, *HindIII*, *HindII*, and *XbaI* revealed similar sized restriction fragments in all three groups of study patients and in controls. Multiple fragments are produced (Figures 1–4) due to cross-hybridization with all of the genes in the HCG\(\beta\) gene complex. Identical fragment sizes were produced using either the LH\(\beta\) or HCG\(\beta\)-5 DNA probes. The enzyme *BamHI* cuts genomic DNA to produce a 36 kb fragment containing all six HCG\(\beta\) genes and a 6.6 kb fragment containing the single LH\(\beta\) gene (Policastro et al., 1986). Upon digestion of genomic DNA from all individuals with *BamHI*, the expected fragments were obtained in patients and controls (Figure 1) demonstrating the absence of deletions or duplications. Digestion of genomic DNA with the enzyme *KpnI* yields seven different fragments containing each separate HCG\(\beta\) and LH\(\beta\) gene (Figure 2) (Policastro et al., 1986). No deletions of any of the six HCG\(\beta\) genes or the LH\(\beta\) gene were identified in any patient or control (Figure 2). This was true for patients with recurrent abortion and unexplained primary infertility. Similarly, no gene duplications indicated by an increased number or intensity of bands on the radiograph, were identified in women with GTN or their partners.

An *MboI* restriction fragment length polymorphism (RFLP) that had been previously described (Rouch et al., 1992) was identified in both patients and controls (Figure 3). The *MboI* polymorphism, probably due to the loss of an *MboI* restriction recognition site (and loss of a 2 kb band), was seen in three of 10 women with recurrent abortion, four out of 10 women with primary unexplained infertility, and three out of 12 GTN women, but in none of their partners (Figure 2). Similarly, eight out of 30 controls lacked the 2 kb *MboI* fragment. A new polymorphism detected with the enzyme *PstI* was also identified using the HCG\(\beta\)-5 and LH\(\beta\) probes (Figure 4). This
HCGβ gene mutations in human disease

**Figure 1.** A radiograph of DNA digested with *Bam*H1 and hybridized with the human chorionic gonadotrophin (HCG)β-5 probe. This enzyme cuts between HCGβ and the luteinizing hormone (LH)β gene such that all the HCGβ genes reside on a large 36 kb fragment, and the LHβ gene on the 6.6 kb fragment. Lanes 1–5 are DNA samples from representative recurrent abortion patients and lane 6 is a control.

**Figure 2.** A radiograph of Kpnl-digested DNA from representative patients with recurrent abortion (lanes 1–3), primary infertility (lanes 4,5), and controls (lanes 6,7).

**Figure 3.** A radiograph of DNA digested with *Mbo*I and hybridized with luteinizing hormone (LH)β shows the polymorphic fragment (presence or absence of the 2 kb fragment) described previously (Roach et al., 1992). Lanes 1–3 are DNA samples from representative women with recurrent abortion, lanes 4–6 from gestational trophoblastic neoplasia (GTN) patients, and lanes 7 and 8 are from controls. The 2 kb fragment is absent in lanes 3, 4, 8.

Polymorphism, which most likely results in the loss of a *Pst*I site (and a 3.8 kb fragment), was detected in one out of 10 recurrent abortion patients, two out of 10 infertility patients, and two out of 12 GTN patients (Figure 4). The 3.8 kb *Mbo*I fragment was also absent in five of 30 controls. The *Mbo*I and *Pst*I polymorphic fragments were identified in similar
frequencies in all three patient groups and controls. This finding provides strong evidence that these fragments are indeed benign DNA polymorphisms in the HCGβ/LHβ complex rather than causative gene mutations.

Discussion

HCG is a member of the pituitary/placenta glycoprotein hormone family, as are LH, FSH, and TSH. Each is composed of a common α-subunit and a specific β-subunit (Pierce and Parsons, 1981). The HCGβ genes are expressed in the placenta, and the LHβ gene is expressed in the pituitary due to the utilization of a different promoter (Jameson and Hollenberg, 1993). Only minimal expression of HCGβ has been demonstrated in the pituitary gland (Jameson and Hollenberg, 1993). Although all six HCGβ genes are expressed to some degree in humans, HCGβ-5 is the principal gene expressed in the placenta (Boime et al., 1982; Talmadge et al., 1984; Jameson et al., 1986; Bo and Boime, 1992; Jameson and Hollenberg, 1993). For this reason, the HCGβ-5 gene was selected as the hybridization probe for this study, although it is likely that other HCGβ probes would have produced similar, if not identical, band patterns (as did the LHβ probe).

This complex HCGβ/LHβ gene cluster appears to have arisen only recently in evolution by duplication from an ancestral LHβ gene (Talmadge et al., 1984). Evidence in support for this comes from the observation that only primates and horses possess chorionic gonadotrophin (CG). Lower animals such as bovine and murine species lack CG (Talmadge et al., 1984). Comparison of the sequence homology of LHβ and HCGβ supports the hypothesis that HCGβ arose by duplication of an ancestral LHβ gene, producing a protein subunit which acquired a new function (Talmadge et al., 1984a). HCG now assumes a major role in the successful completion of pregnancy in these species.

Mutations in pituitary glycoprotein hormone genes cause human disease (for review, see Fauser and Hsueh, 1995). Gene mutations have already been described for LHβ (Weiss et al., 1992), FSHβ (Matthews et al., 1993; Layman et al., 1995), and TSHβ (Hayashizaki et al., 1989; Dacou-Voutetakis et al., 1990). Homozygosity for a missense mutation in the LHβ gene was identified in a male with hypergonadotropic hypogonadism and oligozoospermia (Weiss et al., 1992). In this instance, immunoactive LH was elevated, but bioactive LH was decreased since receptor binding was diminished (Weiss et al., 1992). In women with delayed puberty, elevated serum concentrations of LH, but undetectable amounts of FSH (both basally and after stimulation with gonadotrophin-releasing hormone), point mutations and deletions have been described in the FSHβ gene in at least two different families (Matthews et al., 1993; Layman et al., 1995). Both of these FSHβ gene mutations appear to affect α-β dimer association and binding to the FSH receptor (Matthews et al., 1993; Layman et al., 1995). Likewise mutations in the TSHβ have been identified in some rare autosomal recessive forms of hypothyroidism with decreased concentrations of TSH (Hayashizaki et al., 1989; Dacou-Voutetakis et al., 1990).

We are not aware of any studies of HCGβ gene structure in human disease. The HCGβ gene complex represents a reasonable candidate for gene mutations in women with reproductive failure; for example, in unexplained primary infertility, recurrent abortion, and GTN (Layman, 1991; Roach et al., 1992). When highly homologous sequences reside near each other, there is a predisposition to deletion and duplication events during meiosis. Such is the case in gene deletions of the α-globin gene in α-thalassaemia (Higgs et al., 1989), the 21-hydroxylase B gene in congenital adrenal hyperplasia (White et al., 1987), the growth hormone gene in autosomal recessive forms of growth hormone deficiency (Phillips et al., 1981), and repeated elements close to the steroid sulphatase gene in X-linked ichthyosis (Yen et al., 1990). Similarly, gene duplication can occur and produce disease, as has been described in the overproduction of the thyroid binding globulin (Bartalena and Robbins, 1992). We did not identify HCGβ gene deletions or duplications in the present study. However, an MboI polymorphism previously characterized (Roach et al., 1992),
and a newly described PstI polymorphism, were seen in both patients and controls. The frequency of HCGβ gene polymorphisms was not different in any of the patient groups or controls, suggesting that they are not causative in women with unexplained recurrent abortion, unexplained primary infertility, or GTN. Point mutations that do not alter a restriction cut site or small deletions and insertions would escape detection by Southern blot analysis. However, the purpose of the present study was to determine if meiotic errors resulting in gene deletions or duplications of the HCGβ genes exist in these patients. Large gene deletions or duplications should have been detected if they were present.

Studying women with GTN for HCGβ gene mutations appears reasonable since HCG concentrations are markedly increased in women with this disorder, and HCG regulates its own synthesis (Lei and Rao, 1992). Further evidence for HCG interaction on placental cells is provided by Lei et al. (1992), who demonstrated that the HCG/LH receptor is expressed in trophoblasts from a choriocarcinoma cell line. Since placenta from GTN has been reported to demonstrate two copies of paternal chromosomes (i.e. is imprinted) in a high percentage of cases (Kajii, 1977; Goshen et al., 1994), male partners of GTN patients were also studied for gene mutations. Unfortunately, only three males were able to be recruited for DNA analysis, which reduced the likelihood of drawing any meaningful conclusions. Imprinted genes appear necessary for normal placental development, i.e. contributions from maternal and paternal genomes are necessary for normal development (Cross et al., 1994). This is indicated by placental and fetal demise when only maternal or paternal genomes are present. When there are two copies of the paternal genome and an absent maternal genome, trophoblastic neoplasia with little fetal development occurs (Cross et al., 1994). Similarly, fetal and trophoblast development are abnormal when there are two copies of the maternal genome and no paternal genome (Cross et al., 1994). These data suggest that one copy of each of the maternal and paternal genomes is essential for normal fetal and placental development (Cross et al., 1994). In addition, several investigators have suggested that HCG itself may be an imprinted gene (Goshen et al., 1994).

Placental development and normal endocrine function during pregnancy are complex events involving the production of a myriad of hormones, growth factors, proto-oncogenes, and other peptides (Cross et al., 1994). During pregnancy, the trophoblast appears to regulate HCG isoforms, with a greater predominance of bioactive isoforms with shorter half-life at the end of the first and third trimesters (Diaz-Cueto et al., 1996). Normal placental development does not occur in each of the three disorders studied, although the exact mechanisms are not known. HCG is expressed as early as the 6–8 cell stage of fetal development and is detectable 9–11 days after ovulation in maternal serum (Bonduelle et al., 1988). It is well known that placental HCG is a biochemical marker for pregnancy, but there has also been recent evidence that HCG has additional functions to the stimulation of steroidogenesis in the corpus luteum. HCG appears to be involved in stimulating differentiation of cytotrophoblasts into syncytiotrophoblasts (Shi et al., 1993), and is produced by both cell types. In cytотrophoblast cell culture, HCG induces cell aggregation, and also results in increased expression of cadherin (a protein involved in cell adhesion) and HCG/LH receptor mRNA (Shi et al., 1993). In addition, there is increased production of biochemical markers for syncytiotrophoblasts, namely human placental lactogen (hPL), SP1, and cytokerin (Shi et al., 1993). Further evidence for HCG involvement in placental function is the observation that HCG appears to regulate its own synthesis in placental cells through interaction with the HCG/LH receptors (Lei and Rao, 1992). These data provide strong evidence for HCG function in pregnancy.

Clinical evidence supporting the need for HCG comes from the demonstration that early spontaneous abortion can be induced in primates by the administration of anti-HCG antibodies (Stevens, 1975). One group has even suggested that prolonged HCG administration to women may have some benefit in the prevention of miscarriage in women undergoing ovulation induction (Blumenfeld and Ruach, 1992). Derangements in HCG production could potentially produce reproductively failure manifested by recurrent pregnancy loss, unexplained infertility, or gestational trophoblastic neoplasia. Although the HCG/LHβ gene complex displays DNA polymorphisms, gene mutations were not identified in women with recurrent abortion, primary unexplained infertility or GTD.

References


Yen, P.H., Li, X.M., Tsai, S.P. et al. (1990) Frequent deletions of the human X chromosome short arm result from recombination between low copy number repetitive elements. Cell, 60, 603–610.

Received on July 29, 1996; accepted on January 23, 1997