More in-vitro bioactive, shorter-lived human chorionic gonadotrophin charge isoforms increase at the end of the first and during the third trimesters of gestation

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In the present study we analysed the dynamics of serum human chorionic gonadotrophin (HCG) charge isoform distribution throughout normal gestation and characterized some of the biological features of the several HCG glycoforms present in the circulation of pregnant women. Blood samples were obtained from normal pregnant women at 10-11, 12-15, 23-26 and 35-38 weeks of gestation. The sera were fractionated by preparative chromatofocusing and the separated HCG isoforms were identified and quantified by radioimmunoassay. The in-vitro biological activity and the plasma half-life of the several circulating HCG isoforms were determined by conventional methods. HCG isoforms became less acidic as pregnancy advanced. In samples taken at 10-11 weeks of gestation, the most acidic HCG molecules (pH <3.7) comprised >80% of total HCG recovered after chromatofocusing; this proportion decreased to 58, 60 and 47% in samples taken from weeks 12.1 to 38.4 of gestation. Meanwhile, the relative proportion of less acidic isoforms recovered within pH values 6.49-4.50 increased at the end of the first trimester (12-15 weeks), remained constant until weeks 23-26 and then increased further by the end of the third trimester. Less acidic isoforms had higher in-vitro biological potency per immunological unit than the more acidic analogues. Regardless of the trimester of pregnancy, the plasma half-life of the highly acidic (elution pH <3.7) isoforms varied from 84.4 to 150 min (116.3 ± 23.0; mean ± SD), whereas the corresponding half-life of mid-acidic (pH 4.25-5.31) and low-acidic (pH 5.74-6.50) HCG isoforms ranged from 31.0 to 115.3 (75.5 ± 20.6) and 15.3 to 58.3 (41.2 ± 14.3) min respectively (P <0.01, highly acidic versus mid- and low-acidic analogues and mid-acidic versus least acidic isoforms). The overall data indicate that the human trophoblast is able to regulate the exact intensity, biochemical composition and duration of the gonadotrophic stimulus secreted during the course of normal gestation. They also suggest that the decrease and maintenance of low serum HCG concentrations during the second and third trimesters of gestation may be partially caused by changes in the carbohydrate structure of the HCG molecule.

Key words: charge isoforms/human chorionic gonadotrophin/pregnancy

Introduction

Human chorionic gonadotrophin (HCG) is a sialic acid-enriched glycoprotein hormone synthesized and secreted by the placental syncytiotrophoblast (Simpson and MacDonald, 1981; Hoshina et al., 1982). The circulating concentrations of this hormone vary profoundly during pregnancy; in the first trimester, the concentration in the blood rapidly increases reaching a peak at ~8-12 weeks of gestation, thereafter declines, and then remains constant until term (Mishell et al., 1963; Reuter et al., 1980; Cole et al., 1984; Hay, 1985). Serum HCG concentrations also show significant amplitude-modulated pulsatile release throughout pregnancy; both the amplitude and the area of the HCG pulses are significantly higher during the first trimester than during mid- and late gestation (Díaz-Cueto et al., 1994). In addition to these quantitative variations, several studies have shown that the type of HCG molecules produced and secreted in early and late pregnancy are both structurally and functionally different (Fein et al., 1980; Wide and Hobson, 1987; Skarulis et al., 1992; Cole et al., 1993; Diaz-Cueto et al., 1994). HCG from women in late pregnancy bears a higher proportion of intrachain nicks (at positions β44-49) (Cole et al., 1993), is differently charged (Wide and Hobson, 1987; Sawitzke et al., 1994; Wide et al., 1994), exhibits reduced plasma half-life and in-vivo biological potency (Wide and Hobson, 1987) and shows lower biological to immunological relationships than HCG in early pregnancy (Díaz-Cueto et al., 1994).

In the present study, we further explored some of the mechanisms by which the placenta modulates the intensity and duration of the HCG signal released during the course of
normal pregnancy. Our specific goals were: (i) to analyse the direction of the changes in charge isoform distribution of serum HCG throughout gestation; and (ii) to study and compare the relative in-vitro biological activity as well as the circulatory survival of the various charge HCG isoforms present in the circulation of women in different stages of pregnancy.

Materials and methods

Subjects and sample preparation

Serum samples were obtained from seven pregnant women, aged 22–32 years, at 20 min intervals for 24 h at the end of the first (12.1–14.8 weeks), second (23.1–26.4 weeks) and third (35.5–38.4 weeks) trimesters of pregnancy; details of the sampling conditions and procedures employed in the study of this subset of women have been reported elsewhere (Diaz-Cueto et al., 1994). The set of samples from each subject (73 samples/subject/trimester) were separately pooled, mixed at 8°C for 2 h, concentrated by dialysis at 4°C for 24 h against deionized water and thereafter against 0.01 M ammonium carbonate (pH 7.5) and freeze-dried. After lyophilization, each sample was stored frozen at −70°C until further analysis by chromatofocusing separation. Additional sera (20 ml each) from five pregnant women (aged 22–30 years) from whom a single blood sample was obtained at 10.0–10.8 weeks of gestation were also included in the study; samples from these women were also processed as described above. All the women were in good health and had conceived spontaneously with the exception of one woman who had received clomiphene citrate for ovulation induction. The length of gestation for each pregnancy was determined in accordance with the date of the last menstrual period. Approval from the ethical committee of the Institute and informed written consent from the volunteers were obtained.

Chromatofocusing of serum specimens

The isoforms of HCG present in each serum specimen were separated on the basis of charge by a previously described chromatofocusing procedure (Zambrano et al., 1995). Briefly, each lyophilized sample was re-dissolved in phosphate buffer (0.05 M, pH 7.4) to a volume equivalent to 1/10 (4/10 for samples from women in 10.0–10.8 weeks of pregnancy) of the original and the resultant concentrates were subjected to albumin removal by Affi-gel blue (Bio-Rad, Hercules, CA, USA) extraction. Each albumin-free sample (6 ml each) was transferred to a dialysis membrane tubing (molecular weight cut-off, 6000–8000; Spectrum Medical Industries, Los Angeles, CA, USA), dialysed at 4°C for 24 h against starting buffer (0.025 M imidazole–HCl, pH 7.4) and then applied to the top of a 30×1 cm chromatofocusing column of polybuffer exchange resin (PBE-94; Pharmacia Fine Chemicals, Piscataway, NJ, USA) which had been previously equilibrated with 15 volumes of starting buffer. Chromatofocusing was performed at 4°C and the column fractions (2 ml each) were collected at a flow rate of 1 ml/min. The pH of each fraction was measured, and when the column eluent reached pH 4.0, the eluent buffer [1:8 dilution of Polybuffer-74 (Pharmacia)] was changed to a solution of 1.0 M NaCl to displace those proteins bound at the lower limiting pH (salt peak). Each fraction was measured for HCG content by radioimmunoassay and then stored frozen at −20°C. After measuring the HCG content in each column eluate, fractions corresponding to pH values >7.0–6.50 (area I), 6.49–5.50 (area II), 5.49–4.50 (area III), 4.49–3.70 (area IV) and <3.7 (area V; salt peak) were separately pooled (one pool/pH area, per period of gestation) and concentrated as described above; the HCG content of each HCG pool was then measured by both radioimmunoassay and an in-vitro bioassay.

Hormonal assays

Radioimmunoassay of HCG

The radioimmunoassay for HCG was performed using reagents provided by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK, Bethesda, MD, USA) as previously described (Diaz-Cueto et al., 1994). The HCG–CR121 standard (10–100 ng/nickel; Birken et al., 1991) was radiolabelled by the chloramine-T method, and this same preparation was employed to construct the standard curve. Upon fractionation by chromatofocusing, this HCG standard exhibits a predominantly acidic pH distribution profile with two peaks identified at pH values 4.80–4.10 and 4.0–3.20 as well as an additional component bound at the lower limiting pH (pH <3.2); this is very similar to that of its analogue HCG–CR127 whose extent of nicking is ~30% (Birken et al., 1991). Anti-HCG–H80 (batch 2) at a final dilution of 1:100 000 was used as antiserum. Cross-reactivities of this antiserum with HCG α- and β-subunits are 1.2 and 3.2%, respectively, and the antiserum recognizes intact and nicked molecules equivalently (Cole and Kardana, 1992). To avoid interassay variations, samples from a single chromatofocusing column or period of pregnancy were analysed in the same assay run. Results are expressed as ng of the HCG–CR121 standard or as IU according to the WHO Third International Standard of HCG for immunosassay (coded 75/537) (1 mg of HCG–CR121 = 8000 IU) as appropriate. The sensitivity of the assay was 0.125 ng (1.0 IU) HCG–CR121/tube and the intra-assay coefficient of variation was <7.5%.

In-vitro bioassay of HCG

The in-vitro bioactivity of HCG was assessed employing the mouse Leydig cell testosterone production assay described by Van Damme et al. (1974) and Diaz-Cueto et al. (1994). The standard curve was constructed using the HCG–CR121 preparation. In samples from women at 10–11 weeks of gestation, the in-vitro biological activity of HCG was measured in a single serum sample, whereas in those women studied at the end of the first, second and third trimesters, the activity was determined in serum pools made from aliquots of each individual series of samples (one pool per subject/trimester) as described previously (Diaz-Cueto et al., 1994). To avoid interassay variations, all samples from a single subject or HCG isoform concentrates (pH areas I–V) from a single gestational period were tested in the same assay at 8–10 different concentrations in triplicate incubations; the mean intra-assay coefficient of variation was <10%. The quantity of testosterone produced in vitro was determined by radioimmunoassay, as previously described (Ulla-Aguirre et al., 1985). All samples from a single bioassay were analysed for testosterone content in the same batch; the mean intra-assay coefficient of variation was <5%. Tests for parallelism among the slopes generated by the different HCG specimens (standard and unknowns) assayed by the in-vitro bioassay were performed following the method of De Lean et al. (1978).

Disappearance of HCG from rat circulation

HCG isoforms identified after chromatofocusing of samples from one subject at 10.1 weeks and two women at 12.2, 23.1–24.2 and 36.2 weeks of gestation were concentrated as described above and used to assess the half-life of the HCG isoforms in circulation. For this purpose, a jugular vein from adult Long Evans male rats weighing 380–400 g was prepared and cannulated under ether anaesthesia. The animals were slowly injected i.v. with ~50 ng of immunogenic HCG. Blood samples (150 μl) were taken at 5 and 10 min after the injection and subsequently at regular time-intervals (10–60 min) during the ensuing 7–8 h. After each bleeding, the circulating volume was maintained with 0.9% NaCl. Samples were left to clot at 4°C and then centrifuged at 1000 g for 10 min. Serum was removed and stored frozen at −20°C until the HCG radioimmunoassay. Each HCG
isoform was assayed in a single animal. To determine the plasma half-life of each injected HCG specimen, an interactive computer program for statistical and pharmacokinetic analysis of data (PKC-ALC) (Shumaker, 1986) was used as described elsewhere (Ulloa-Aguirre et al., 1992).

Statistical analysis

One-way analysis of variance was employed to determine differences between groups. When differences existed, t-tests were used to determine which means were significantly different. Linear regression analysis was performed to determine the degree and significance of the association between the median elution pH value of the HCG isoforms and their corresponding plasma half-life. \( P < 0.05 \) was considered to be statistically significant.

Results

Serum HCG concentrations at 10-11 and 12-15 weeks of gestation were significantly higher than those at 23-26 and 35-38 weeks (mean ± SEM; 10-11 weeks, 88 ± 9 IU/l; 12-15 weeks, 77 ± 18; 23-26 weeks, 8 ± 2 and 35-38 weeks, 10 ± 2 IU/l). \( P < 0.05 \) 10-11 and 12-15 versus 23-26 and 35-38 weeks; not significant 10-11 versus 12-15 weeks and 23-26 versus 35-38 weeks. The in-vitro biological activity/immunoactivity (B/I) HCG ratio also decreased as the gestational age progressed (mean B/I ratio at 10-11 weeks, 1.5 ± 0.12; 12-15 weeks, 1.14 ± 0.14; 23-26 weeks, 0.87 ± 0.22 and 35-38 weeks, 0.79 ± 0.12). \( P < 0.05 \) 10-11 versus 12-15, 23-26 and 35-38 weeks, and 12-15 versus 23-26 and 35-38 weeks; not significant 23-26 versus 35-38 weeks.

Separation by chromatofocusing of serum concentrates obtained during different gestational ages disclosed the presence of multiple (5-8) immunoreactive charge HCG isoforms (Figure 1). Refocusing of concentrates prepared from each HCG peak identified after chromatofocusing of a serum specimen from a woman at 12.2 weeks of gestation yielded single peaks of HCG activity for components with elution pH values 6.68-6.63 (Figure 2). Re-chromatofocusing of a minor component recovered at pH 4.30-4.39 resulted in the identification of significant quantities of HCG activity in both the original elution pH value and the salt peak; conversely, refocusing of HCG present in the latter component disclosed the presence of some HCG immunoreactivity in fractions corresponding to pH 4.30-4.39.

HCG isoforms became less acidic as pregnancy advanced. At 10-11 weeks of gestation, the most acidic HCG molecules (present in area V, pH <3.7) comprised >80% of total HCG recovered after chromatofocusing; this proportion progressively decreased to 58, 60 and 47% throughout weeks 12.1-38.4 of gestation (Figure 3). Meanwhile, the relative proportion of less acidic isoforms recovered in pH areas II and III (pH 6.49-4.50) increased at the end of the first trimester (12-15 weeks), remained constant until weeks 23-26 and then increased further by the end of the third trimester. The relative proportions of HCG recovered in pH areas I and IV slightly increased as pregnancy advanced; these changes, however, were not statistically significant.

The in-vitro biological activity of HCG recovered within the different pH segments of chromatofocusing separations from each gestational is shown in Figure 4. The amount

Figure 1. Distribution of human chorionic gonadotrophin (HCG) isoforms after chromatofocusing of serum specimens collected from a woman sampled at (a) 10.8 weeks and from a subject studied at (b) 12.2, (c) 24.8 and (d) 38.5 weeks of gestation. Arrows indicate the addition of 1 M NaCl to the chromatofocusing columns. Vertical broken lines separate isoforms with pH values >5.0 and <5.0.
of HCG tested for in-vitro bioactivity was determined by radioimmunoassay of each HCG specimen at several dose levels; all concentrates displaced $[^{125}]$-labelled HCG from the antibody in a parallel fashion (not shown). HCG material present in concentrates I and II from 10–11 weeks and in area I from 36–39 weeks was insufficient to assess the bioactivity of their corresponding HCG isoforms. As shown in Figure 4, all HCG concentrates induced significant and parallel dose-dependent testosterone production curves when analysed in the mouse Leydig cell bioassay. The dose-response curves of the most acidic HCG isoforms identified in fractions with pH values $<4.49$ (areas IV and V) were consistently shifted to the right of the dose-response curves for the isoforms with pH values $\geq 4.50$ (pH areas I to III). Thus, the less acidic isoforms had higher in-vitro biological potency per immunological unit (as assessed by radioimmunoassay) than the more acidic analogues.

Injection of individual HCG isoforms into male rats revealed that the plasma half-life of circulating HCG increased as the pH value of the corresponding isoforms declined. Regardless of the trimester of pregnancy, the plasma half-life of the highly acidic (elution pH $<3.7$) isoforms varied from 84.4 to 150 min (mean ± SD; 116.3 ± 23.0), whereas the corresponding half-life of mid-acidic (pH 4.25–5.31) and low-acidic (pH 5.74–6.50) HCG isoforms ranged from 31.0 to 115.3 (75.5 ± 20.6) and 15.3 to 58.3 (41.2 ± 14.3) min respectively ($P < 0.01,$
the spectrum of cytoplasmic and functional events (Ulloa-Aguirre et al., 1990). Employing this system, which reproduces blast secretes different molecular forms of HCG during its morphological and functional differentiation in culture (Ulloa-Aguirre et al., 1986) may be actually produced by the placenta and then released to the periphery.

In the present study, we have shown that the pattern of pH distribution of circulating HCG changes throughout pregnancy. The earliest change detected in the specimens analysed was a significant decrease in the relative proportion of the most acidic isoforms (pH <3.7) with a concomitant increase in isoforms with pH values 6.49–4.50. This initial shift towards secretion of less acidic isoforms occurred between 11–15 weeks of gestation, a finding which corroborates a previous study employing a different charge-based separation technique (Wide et al., 1994). Using chromatofocusing to fractionate the different HCG glycoforms, we were able to detect the occurrence of a second major shift towards the production of less acidic/sialylated HCG isoforms during the third trimester. This observation indicates that in addition to alterations in its peptide backbone (i.e. nicking and cleavage) (Cole et al., 1993), the structural modifications of HCG secreted during the later stages of gestation also involve changes in the degree of sialylation of its oligosaccharide structures. Thus, the decrease and maintenance of low serum HCG concentrations during the second and third trimester of gestation may be partially caused by these changes in both the peptide and carbohydrate structures of the HCG molecule.

All serum HCG isoforms exhibited lower in-vitro bioactivity than the highly purified urinary HCG standard, suggesting that the isoform-containing specimens lost some biological activity, but not immunoactivity, during the various experimental procedures employed in their isolation. Nonetheless, as previously observed with the HCG isoforms released in vitro (Ulloa-Aguirre et al., 1990), less acidic serum isoforms exhibited higher bioactivity per immunological unit than the more acidic variants. This finding is also in agreement with a recent study demonstrating that less acidic and basic isoelectric forms of molar HCG show higher thyroid-stimulating activity in vitro than their more acidic counterparts (Yoshimura et al., 1994). The difference in bioactivity between the less and more acidic isoforms of HCG may be due to the presence of more negatively charged carbohydrate residues (i.e. sialic acid) in the acidic analogues (Ulloa-Aguirre et al., 1990; Yoshimura et al., 1994), which in turn may decrease the interaction between the hormone and its receptor at the target cell (Ulloa-Aguirre et al., 1984). In fact, it has been demonstrated that some less sialylated/glycosylated HCG species may be more potent than intact HCG in stimulating cAMP production in Chinese hamster ovary cells expressing functional human thyroid stimulating hormone (TSH) receptors (Yoshimura et al., 1993). Less likely, the reduced relative potency of the more acidic isoforms may be due to more extensive or selective intrachain nicking of their corresponding β-subunit (Cole et al., 1991). There is no evidence, however, that the highly acidic HCG isoforms are more prone to deactivation by nicking than

Discussion

We have previously demonstrated that the human cytotrophoblast secretes different molecular forms of HCG during its morphological and functional differentiation in culture (Ulloa-Aguirre et al., 1990). Employing this system, which reproduces in vitro the spectrum of cytoplasmic and functional events normally occurring during early gestation (Kliman et al., 1987), we found that the secretion of the most acidic/sialylated HCG molecules progressively increased as the morphofunctional differentiation of the cytotrophoblasts into syncytiotrophoblasts progressed. These observations indicated that some of the isoforms of HCG previously identified in serum and urine (Lichtenberg et al., 1986) may be actually produced by the placenta and then released to the periphery.
Figure 4. The ability of increasing doses of human chorionic gonadotrophin (HCG) present in five different pH segments (I–V; see Figure 3 legend) from chromatofocusing separations of serum samples obtained at (a) 10–11, (b) 12–15, (c) 23–26 and (d) 35–38 weeks of gestation to induce testosterone production by mouse interstitial cells. The dose is expressed in terms of HCG-CR121 as measured by radioimmunoassay.

Figure 5. The relationship between the charge, expressed as the median elution pH value, and the plasma half-life of individual human chorionic gonadotrophin (HCG) isoforms obtained after chromatofocusing of serum specimens from a subject sampled at 10.1 weeks of pregnancy (∅) and from two women studied at 12.2, 23.1 and 24.2, and 36.2 weeks of gestation (open and filled symbols respectively). A median elution pH value of 3.0 was arbitrarily assigned to the salt peak (pH <3.7).
in which the serum specimens were obtained, less acidic HCG isoforms disappeared faster from the circulation of rats than their more acidic counterparts. Although previous studies aimed to determine the pharmacokinetics of the various HCG isoforms have been performed exclusively on rodents (Graesslin et al., 1984; Wide and Hobson, 1987), the observation that the relative abundance of the less acidic serum HCG isoforms is considerably reduced as compared to that present in placental extracts (Lichtenberg et al., 1986), strongly suggests that a similar plasma behaviour may also occur in the human and that, after being secreted, these less acidic variants are cleared faster from the circulation than the more acidic/sialylated isoforms. Interestingly, we were unable to find within each particular group of charge isoforms (highly acidic, mid-acidic and low acidic) a clear relationship between the specific week of pregnancy and the plasma half-life of HCG. Although the extent of nicking was not analysed in the isoforms tested, the data suggest that variations in sialic acid content may be more critical than the relative extent of intrachain nicks in determining the circulatory survival of the hormone. Nevertheless, additional studies including a larger number of the different charge isoforms need to be performed in order to corroborate this hypothesis.

The factors involved in the regulation of HCG isoform production are unknown. Changes in the production and secretion of sex steroids and other non-steroidal factors such as gonadotrophin-releasing hormone (GnRH) and inhibin, have been associated with structural modifications in follicle stimulating hormone (FSH) and luteinizing hormone (LH) molecules (Galle et al., 1983; Padmanabhan et al., 1988; Phillips and Wide, 1994). Although modifications in glycosylation and sialylation of HCG throughout gestation could be subserved by changes in serum concentrations of oestradiol and progesterone, there is no firm evidence supporting such a causal relationship. On the other hand, it has been shown that several non-steroidal factors from placental and extraplacental origin are involved in HCG production and secretion in vitro (Belisle et al., 1984; Petraglia et al., 1989; Nishino et al., 1990; Ohashi et al., 1992; Barnea et al., 1993), and cAMP has proved to exert a regulatory role in the production of acidic isoforms in cultured human cytotrophoblasts (Ulloa-Aguirre et al., 1990). Whether these factors are involved in the control of the synthesis and secretion of the different charge isoforms remains to be determined.

Although the molecular heterogeneity of HCG is well established, both the functional role of the hormone during the second and third trimesters of pregnancy and the physiological significance of such a variety of isoforms for a single hormone still remain to be more clearly defined. Aside from its critical role in regulating corpus luteum function during early pregnancy, sever other endocrine, paracrine and autocrine regulatory effects at both the maternal and fetal level have been proposed (Huhtaniemi et al., 1977; Braunstein, 1988; Hoermann et al., 1991; Lei and Rao, 1992; Licht et al., 1993; Eta et al., 1994). Although speculative, the various isoforms may selectively participate in the control of a variety of responses at different target cells and during particular periods of gestation requiring a more precise control of the intensity and/or duration of the HCG stimulus. In addition to inducing thyroid activity (Yoshimura et al., 1994), some HCG isoforms may preferentially act on other target cells through distinct HCG receptors (Mellroy, 1992). From this perspective, HCG heterogeneity may represent an additional, fine-tuning mechanism through which the placenta regulates different maternal and fetal functions throughout gestation.

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