Early pregnancy human chorionic gonadotropin (hCG) isoforms measured by an immunometric assay for choriocarcinoma-like hCG

G Kovalevskaya¹, S Birken², T Kakuma³ and J F O’Connor¹,⁴

¹Irving Center for Clinical Research, Columbia University College of Physicians and Surgeons, 630 W 168th St., New York 10032, New York, USA
²Department of Medicine, Columbia University College of Physicians and Surgeons, 630 W 168th St., New York 10032, New York, USA
³New York Hospital-Cornell Medical Center, White Plains, New York 10605, New York, USA
⁴Department of Pathology, Columbia University College of Physicians and Surgeons, 630 W 168th St., New York 10032, New York, USA

Abstract

Human chorionic gonadotropin (hCG) exhibits molecular heterogeneity in both its protein and carbohydrate moieties. This communication describes changes in hCG isoforms detected directly in clinical samples. These isoforms, quantified in blood or urine specimens, show a progression of change throughout normal pregnancy. Early pregnancy produces a type of hCG that resembles, in terms of immunoreactivity, a major form of hCG excreted in choriocarcinoma. The isoforms predominate for the first 5–6 weeks of gestation and then diminish, being replaced with the hCG isoforms which predominate throughout the remainder of pregnancy. The alteration in hCG isoform content occurs in both blood and urine. The progression of isoforms is best delineated by calculating the change in the ratio of the two forms, as many hCG assays either do not detect or fail to discriminate among these isoforms. An analogous pattern of hCG isoforms was observed in patients with in vitro fertilization pregnancies. hCG isolated from the pituitary displayed binding characteristics similar to those of the hCG derived from normal pregnancy urine. The early pregnancy hCG isoforms appear to have a differential expression in normal pregnancy as opposed to pregnancies which will not carry to term, suggesting that a determination of the relative balance of hCG isoforms may have diagnostic application in predicting pregnancy outcome.


Introduction

Human chorionic gonadotropin (hCG) is produced by the placental trophoblast of normal pregnancy, and in gestational trophoblastic disease (hydatidiform mole and choriocarcinoma) (Hussa 1987). It is also produced in much smaller quantities by the pituitary (Birken et al. 1996) in both pre-and postmenopausal women and in men (Odell et al. 1990) and also in many non-gestational malignant tumors (Hussa 1987).

Structural variations in both the protein and carbohydrate portions of the hCG molecule have been well established. They may occur as a concomitant of its origin (Wide & Hobson 1987, Birken et al. 1996), or metabolic transformation (Nisula et al. 1989). The most prominent differences documented to date are between the hCG produced by choriocarcinoma and that of mid-first trimester normal pregnancy, with choriocarcinoma-derived hCG having a generally higher content of more highly branched oligosaccharides (Cole 1987, Elliott et al. 1997).

Even in normal pregnancy, multiple isoforms of hCG exist and their relative amounts vary as pregnancy progresses (Wide & Hobson 1987, Skarulis et al. 1992, Diaz-Cueto et al. 1994, 1996, Wide et al. 1994). Many of these determinations are based on isoelectric focusing (IEF) separation of the isoforms. This technique separates molecules based on their net charge, which in the case of glycoprotein hormones only reflects the degree of terminal carbohydrate sialylation (or less commonly, sulfation), and not major structural differences (Ulloa-Aguirre et al. 1990, Berger et al. 1993, Diaz-Cueto et al. 1994). However, these studies of microheterogeneity (by microheterogeneity, we refer to the permutations displayed by the carbohydrate groups) have not proven to have clinical utility, since they are both too expensive and labor intensive to perform routinely. Other studies have demonstrated, by analysis of carbohydrate structure and sugar content, differences in the carbohydrate moieties of normal pregnancy hCG as it progresses to term (Skarulis et al. 1992, Nemansky et al. 1998).

This report describes the observation of the progressive changes of hCG isoforms throughout pregnancy by direct assay of clinical samples of urine or serum, using a combination of two IRMAs. One of these, a new IRMA,
is unique in that it is sensitive to changes in carbohydrate structure and can measure very early isoforms of hCG (O’Connor et al. 1998), whereas the second assay, a commonly used format, detects isoforms of hCG which predominate later in pregnancy. This observation takes on an added significance because of reports that certain pregnancy disorders (e.g. Down syndrome, pre-eclampsia, early pregnancy loss) are characterized by abnormal hCG levels (Bogart et al. 1987, Heinonen et al. 1996, Hurley et al. 1996, O’Leary et al. 1996, Onderoglu & Kabukcu 1997, Haddow et al. 1998). We have evidence that pregnancies which will not carry to term have an easily recognized difference in production of hCG isoforms (O’Connor et al. 1998). Thus, hCG isofrom production and distribution in pregnancy may prove to have a diagnostic role in the evaluation of pregnancy status. The method of direct IRMA analysis of early pregnancy isoforms described in this report is suitable for large scale studies using either serum or urine.

Materials and Methods

Hormones

The non-nicked hCG isolated from the CR127 preparation of hCG was used as a standard in both assays (Birken et al. 1993). The pituitary hCG was isolated as described (Birken et al. 1996). C5, a 100% nicked hCG having extra sugars on both N- and O-linked carbohydrate moieties, purified from the urine of a choriorcancinoma patient (Elliott et al. 1997), was supplied by Dr L Cole (Yale University School of Medicine). Although the C5 immunogen used in the development of B152 antibody was 100% nicked hCG isoform (i.e. had cleavages in the peptide backbone of loop 2 of the β subunit) the antibody did not discriminate nicked from non-nicked hCG (O’Connor et al. 1998).

The same serial dilutions of non-nicked hCG, pituitary hCG and C5 were used for binding characterization in hCG assays. Hormone concentrations of initial stock standard solutions were determined by amino acid analysis.

IRMAs

The methodology used in the construction and validation of the B109–B108* assay has been fully described elsewhere (O’Connor et al. 1988); the first antibody is the capture antibody, the second, with an asterisk, is a radioiodinated detection antibody. The B152–B207* assay has also been characterized (O’Connor et al. 1998). Both assays were performed with a slight modification of the published procedure: the capture antibody was adsorbed onto the wells of microtiter plates (Immulon IV, Dynatech, Chantilly, VA, USA) by incubating a 5 µg/ml solution (B109–B108* assay) or 25 µg/ml solution (B152–B207* assay) in coating buffer (0·2 M bicarbonate, pH 9·5) overnight at 4 °C. The coating antibody solution was aspirated, the plates washed (wash solution: 0·9% NaCl, 0·05% Tween 20) and blocked with a 1% solution of BSA in PBS with 0·1% sodium azide. Following incubation with the BSA solution (minimum 3 h at room temperature), the blocking solution was removed, the wells again washed with wash solution and 200 µl/well of the appropriate hCG standards were added in phosphate buffer B (PBS with 0·1% bovine gamma globulin and 0·1% sodium azide). After overnight incubation at 4 °C, the plates were again aspirated and wash. The 200 µl of 125I-labeled antibody (50 000–100 000 c.p.m.) were added to the wells, which were again incubated for 24 h at 4 °C. The tracer was aspirated, the plates washed with wash solution, the individual wells placed in glass tubes and the radioactivity determined in a Packard Cobra gamma counter (Downers Grove, IL, USA). Doses were determined by interpolation from a smoothed spline transformation of the data points.

All samples were stored frozen at −20 °C prior to assay. Because extreme values of sample pH may interfere with antibody binding, the urine pH was adjusted with 1·0 M Tris (pH 9·0), 50 µl/ml urine, prior to assay, so that the final pH was in the range 7·0–7·4 (O’Connor et al. 1988). Intra-assay variation was 6% for both assays, inter-assay variation was 12% for B109–B108* and 13% for B152–B207* assays. Sensitivity (least detectable dose) defined as +2s.d. from the zero calibrator, was 1·0 fmol/ml for the B109–B108* assay and 2·2 fmol/ml for the B152–B207* assay.

The only mapping of the epitope sites thus far has established that the B108 and B207 tracer binding site corresponds to site II, the same site recognized by the SB6 antiserum (Vaitukaitis et al. 1972). The B152 capture site involves the carboxy-terminal portion of the hCG β subunit, since B152 will not bind simultaneously with a carboxyterminal peptide-specific monoclonal antibody. In the B109–B108* assay, B109 requires that the α and β subunits both be present in the hCG molecule, whereas B152 will bind either the intact, heterodimeric hCG molecule or its free β subunit (O’Connor et al. 1994, Birken et al., unpublished observations).

Patient samples

Urine samples from in vitro fertilization (IVF) patients were a gift from Dr L Cole. They included spontaneous abortion (n=14, range of gestational age 1·8–4·1 weeks from embryo transfer (ET)), ectopic pregnancies (n=7, gestational age 2·3–4·0 weeks) and normal pregnancy controls (n=65, encompassing the range 0·6–5·4 weeks from ET). Some of the normal pregnancy urine samples throughout the pregnancies were also obtained from Dr Cole. Others were obtained from the clinical practice of collaborating physicians at Columbia Presbyterian Medical Center
Matched serum/urine samples from the first \((n=12)\) and the third \((n=11)\) trimesters were provided by Dr A Kelly at CPMC. Trophoblast disease serum \((n=17)\) and urine \((n=28)\) samples were obtained from Dr Cole, but had been collected by Dr E Newlands (Charing Cross Hospital, London, UK). All specimen collection protocols were approved by the appropriate Institutional Review Board.

**Statistical analysis**

Polynomial regression models of log-transformed hormone ratios were used to describe the relationship between the change in ratio as a function of gestational age in normal pregnancy. A paired \(t\)-test was used to evaluate the relationship between matched serum and urine hormone ratios. Analysis of covariance (ANCOVA) was used to describe the time-adjusted relationship of hormone values in ectopic pregnancy and spontaneous abortion to those of normal pregnancy.

**Results**

**Immunoreactivity of different forms of hCG in the two IRMAs**

The relative binding of three different forms of hCG (urinary hCG, pituitary hCG and choriocarcinoma hCG C5) has been characterized in the two hCG assays (Fig. 1). Urinary non-nicked hCG and pituitary hCG are recognized nearly equally well by the two IRMAs, while C5 recognition is quite different. The B152–B207* assay is more sensitive to C5, which is to be expected because B152 antibody was developed and selected on the basis of higher affinity to C5. Urinary non-nicked hCG is purified from the CR127 preparation of pooled normal pregnancy hCG. Conversely, C5 is recognized with lower affinity by the B109–B108* assay, which has primary specificity for the hCG isoforms of later pregnancy.

The B152/B109 ratio measured in urine samples throughout the pregnancy

The relative concentrations of hCG isoforms in 103 normal pregnancy urine samples (5–39 weeks post last menstrual period (LMP)) were determined by the two IRMAs (B152–B207* and B109–B108*). Both because of the wide range of hCG concentrations in different samples, even at the same gestational age, and because neither of the assays is totally specific for the two (or more) families of hCG isoforms present, we find that presenting the data as a ratio of the observed two isoform groups more clearly delineates the change in isoform content as pregnancy progresses. This calculated ratio is shown in Fig. 2. In weeks 5–8 of pregnancy, the ratio of B152/B109 isoforms ranged from 6·2 to 1·3, indicating a predominance of the B152 isoform(s) in early pregnancy. During the 10–12 week period, the ratio ranged from 1·0 to 0·2, indicating that an inversion in hCG isoform content is occurring as pregnancy progresses. This decline in the ratio continues, ranging from 0·54 to 0·08 in the 15–18 week period and reaching an inflection point at 29 weeks.

**Figure 1** Binding curves for three hCG types in the B152–B207* assay (upper panel) and the B109–B108* assay (lower panel).
At that time, the ratio reached a value of around 0.06, after which it displayed a rise to a range from 0.20 to 0.07 in the 37–39.5 weeks of gestation time period.

Statistical analysis involved fitting the log-transformed ratio data to second and third order polynomial regression models. Since the third order term was not significant (likelihood ratio $\chi^2(1)=1.32$, $P=0.25$), the second order model was used ($r^2=0.793$). The log B152/B109 ratio reached an inflection point at LMP=29 weeks, based on this model.

The B152–B207* values reflect a measurement of the B152 isoform in terms of later pregnancy hCG equivalents, not in absolute quantities. It must be emphasized that the 'absolute' concentrations measured in the B152 assay cannot be compared with the results of the B109 assay on an equimolar basis, since the potency of the hyperglycosylated isoform is much higher in the B152 assay vis-à-vis the standard, i.e. normal later first trimester pregnancy hCG. The actual molar values of this isoform are of the order of tenfold less than those recorded in the assay. For this reason, we have chosen not to analyze absolute molar quantities of the two analytes, but only the ratio of the two measurements.

The B152/B109 ratio in matched serum/urine samples in the first and third trimesters of pregnancy compared with hCG from JAR cells

The B152/B109 ratio in serum is analogous to that found in matched urine samples and undergoes a similar change as pregnancy progresses (Fig. 3). The B152/B109 ratio in the cell supernatant from JAR cells (a choriocarcinoma-derived cell line) was similar to that of early pregnancy.

The B152/B109 ratios of both serum and urine hCG concentrations are significantly higher in the first trimester as compared with the third trimester of normal pregnancies (Table 1). Significant differences between serum and urine hCG concentration ratios as well as log-transformed ratios in early (5–6 weeks) and late (36–39 weeks) gestation were evaluated by paired $t$-tests (Table 2). In both the first and third trimesters, urinary B152/B109 ratios were significantly higher than serum ratios, indicating that there was a preferential clearance of the B152-recognized isoform into urine, regardless of the relative concentrations of the two isoforms.

The B152/B109 ratio in urine samples from IVF patients

In urine samples from IVF patients (1–4 weeks post ET) the B152/B109 ratio was again between 2 and 8 and decreased as pregnancy progressed (Fig. 4), similar to that observed in natural conceptions. The effect of pregnancy duration with respect to outcome variables could best be represented by a linear or quadratic function. ANCOVA models including the second order week were fitted to the general equation: Outcome=($t$-effect of time post ET)+($e$-effect of diagnosis). After an appropriate ANCOVA model was determined, the least square means (adjusted for week post ET effect) were compared among the
normal pregnancy, ectopic pregnancy and spontaneous abortion populations (Table 3). The log-transformed values of both B109–B108*- and B152–B207*-measured hCG forms discriminated both ectopic pregnancy and spontaneous abortions from normal pregnancy ($P = 0.0001$). The ratio of the log-transformed values discriminated abortion from normal pregnancy ($P = 0.016$). However, neither the ratio of B152/B109 nor the log of that ratio discriminated either of the pregnancy disorders from normal pregnancy.

### hCG analysis of trophoblastic disease samples

Trophoblast disease serum (17 samples) and urine (28 samples) were obtained from patients post therapy and hence contained low hCG levels. Due to limited amounts of sample, all of these specimens were run at a 1:10 initial dilution. hCG levels in serum were low. The highest hCG concentration in serum was 202 fmol/ml in the B152–B207* assay, with a corresponding value of 148 fmol/ml in the B109–B108* determination. Six of seventeen samples in serum had detectable levels, with four out of six having a higher value in the B152–B207* assay. Of the 15 out of 28 positive urine samples, however, 14 out of 15 had higher levels in the B152–B207* assay than in the B109–B108* assay, with the highest hCG value being 20 000 fmol/ml in the B152–B207* assay and 18 715 fmol/ml in the corresponding B109–B108* assay. Due to the small sample size, no statistical treatment was performed on these data, but even in these post-treatment patients the B152/B109 ratio was $>1$, which corresponds to the early pregnancy hCG isoform ratio.

### Discussion

We have developed a method to directly profile changes of hCG isoforms in serum or urine throughout pregnancy. Two IRMAs for hCG are employed, each based on monoclonal antibodies to different hCG epitopes. The B109–B108* assay is a commonly used intact hCG assay to the heterodimeric-dependent epitope. A new assay, B152–B207*, is most likely sensitive to the carbohydrate portion of hCG carboxy-terminal peptide. The same standard non-nicked hCG was used in both assays. Non-nicked hCG was employed, since the B109 assay reacts poorly with nicked forms of hCG, while the B152 assay does not discriminate between nicked and non-nicked forms of the hormone. The B152 assay detected with greatly enhanced sensitivity hCG isoforms which appear earlier in pregnancy than isoforms measured by the B109 assay (O’Connor et al. 1998). Prior to development of the new IRMA system described in this report, it was not possible to readily discern the changes in hCG isoforms from very early pregnancy to mid pregnancy. The only available procedure for examining these changes was IEF of every patient specimen followed by immunoassay of every focused fraction (Ulloa-Aguirre et al. 1990, Berger et al. 1993). The IEF pattern reflects the heterogeneity of the charged sugar, sialic acid, which varies with the multi-antennary structures of the carbohydrate moieties in which sialic acid is the terminal sugar. Although we do not yet know the precise nature of the isoform epitopes being
measured, the evidence for carbohydrate discrimination is based upon the hyperglycosylated structure of the immunogen, C5, used to develop the B152 monoclonal antibody and the antibody’s reactivity with the hCG isoforms found in the JAR choriocarcinoma cell line. C5 hCG was isolated from a choriocarcinoma patient and has been thoroughly characterized as to its protein and carbohydrate content and structure (Elliott et al. 1997). It has been shown that C5, and hCG from other choriocarcinoma subjects, differ in the protein moiety mainly by the presence of an increased number of nicked sites and by increased glycosylation relative to the hCG of normal pregnancy. In comparison with the hCG of normal pregnancy, choriocarcinoma-derived hCG has increased fucosylation of the N-linked biantennary oligosaccharides in the β subunit. In addition, the O-linked oligosaccharides in preparation C5 (a form of hCG produced from a single patient with choriocarcinoma) has a 100% tetrasaccharide core on the carboxy-terminal region of the β subunit. Normal mid pregnancy hCG has only 10–20% of this structure (Elliott et al. 1997). These observations, plus our own determination that the hCG synthesized by the JAR choriocarcinoma cell line provides a B152/B109 isoform ratio similar to that observed in early pregnancy, lead us to the conclusion that, in very early pregnancy, the developing trophoblast secretes an isoform of hCG which resembles that produced in choriocarcinoma.

We have also tested recognition of pituitary hCG, since its N-Asn carbohydrates differ somewhat from those of placental hCG, bearing a closer resemblance to those of human luteinizing hormone, which have both sialic acid and sulfate groups (Birken et al. 1996). The carbohydrate structure of the β carboxy-terminal portion of pituitary hCG is not yet known. Since B152 did not recognize any substantial differences between pituitary and placental hCG (Fig. 1), differences in N-Asn recognition are unlikely. In terms of the carboxy-terminal carbohydrates, it appears that pituitary and placental hCG (mid pregnancy isoforms) may be similar, assuming the O-linked carbohydrate on the C5 antigen is part of the epitope of B152.

Even in normal pregnancy, the hCG values obtained vary widely according to the characteristics of the immunological reagents employed (Cole & Kardana 1992, Cole et al. 1993). We hypothesize that the two assays described in this report primarily detect hCG isoforms at opposing ends of this spectrum, each primarily recognizing a subset of closely related molecules in the continuum of early to later pregnancy hCG molecular forms.

We have retained the use of normal pregnancy hCG as the standard in the B152–B207* assay, despite its decreased affinity in this antibody configuration. The reasons for this include the limited and unrenewable supply of C5 (which was isolated from the urine of a single patient) and the variability in data which would result from investigations using different standards. The consequences of this choice are that the early pregnancy hCG isoforms have markedly increased immunopotency over that of normal pregnancy and hence their molar quantities are overestimated in this assay. We use this difference in affinity to our advantage by employing a ratio of the molar results of two assays (B152 and B109). Either assay taken alone obscures this change due to the wide excursion of hCG values which occur in normal pregnancy.

Others have documented progressive changes in hCG isoforms throughout pregnancy. Skarulis et al. (1992) found that the fucose content of both intact hCG and also its free β subunit increased as pregnancy progressed. Diaz–Cueto et al. (1996), investigating the IEF pattern of

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Adjusted $r^c$</th>
<th>Diagnosis</th>
<th>Pairwise difference$^d$</th>
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<tbody>
<tr>
<td>Log(ratio B152/B109)$^a$</td>
<td>0.51</td>
<td>0.89</td>
<td>0.41</td>
</tr>
<tr>
<td>Log(B109–B108*)$^a$</td>
<td>0.56</td>
<td>21.33</td>
<td>0.0001</td>
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<tr>
<td>Log(B152)/log(B109)$^b$</td>
<td>0.45</td>
<td>4.34</td>
<td>0.016</td>
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<tr>
<td>Log(B152–B207*)$^b$</td>
<td>0.50</td>
<td>26.94</td>
<td>0.0001</td>
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$^a$ANCOVA model with 2nd order polynomial coefficient (or parameter).
$^b$ANCOVA model with only 1st order (linear) coefficient.
$^c$Adjusted $r^c$ is an $r^c$ adjusting number of coefficients on the ANCOVA model so that comparisons of two $r^c$ with different ANCOVA models with different numbers of coefficients are meaningful.
$^d$Pairwise difference is based on t-test comparing the least-square means of outcome variables (after adjusting effect of week of ET).

Degrees of freedom (df1, df2) for t-test are 2,82) for a model with only linear coefficient and (2,81) for a model with both linear and 2nd order coefficient.
circulating hCG throughout pregnancy, found that in early pregnancy more than 80% of the hCG isoforms were acidic. This fraction decreased to less than half (47%) late in the third trimester (Diaz-Cueto et al. 1996). In contrast, Wide & Hobson (1987) found that the hCG of early pregnancy was more ‘choriocarcinoma-like’ by virtue of its greater biological activity than the hCG of normal pregnancy. Fein et al. (1980), in a study which employed gel filtration, determined that first trimester hCG was a larger size than that of the third trimester. Treatment with exoglycosidases eliminated the size differential, indicating that the first trimester hCG was more highly glycosylated (Fein et al. 1980).

A significant number of spontaneous abortions and ectopic pregnancies occur in IVF pregnancies. We did not find a difference in the ratio of the isoforms between either of these two categories as compared with normal controls, possibly a consequence of low statistical power. However, a significant difference was found between the B152 hCG isoforms levels in normal pregnancy and spontaneous abortion. This supports our previous finding in early pregnancy loss, where diminished or absent levels of the B152 isoforms characterized an early pregnancy loss (O’Connor et al. 1998).

The specimen limitations discussed above preclude our reaching any definitive conclusion on the analysis of trophoblastic disease samples. However, it appears, as might be anticipated, that the B152 assay is more sensitive than the B109 assay in detecting hCG immunoreactivity in the blood and in the urine of trophoblastic disease patients, even after treatment.

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