

Hyperglycosylated hCG

L.A. Cole*

*USA hCG Reference Service, Department of Obstetrics and Gynecology, University of New Mexico Health Sciences Center,
MSC10-5580, 1 University of New Mexico, 333 Cedar Street, Albuquerque NM 87131, USA*

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Abstract

Hyperglycosylated hCG (hCG-H) is a glycosylation variant of the hormone hCG. Here we review all that is known about this independently functioning molecule. As discussed, it is a very different molecule to the hormone hCG. First, hCG-H is produced by cytotrophoblast cells while regular hCG is made in syncytiotrophoblast cell. Second, it is an autocrine acting directly on the cells which produce it, while regular hCG is an endocrine acting on maternal corpus luteal cells. Third, hCG-H has minimal biological activity in promoting progesterone production compared to regular hCG. Fourth, hCG-H functions unlike regular hCG as an invasion promoter, whether invasion as in choriocarcinoma and testicular germ cell malignancies, or as in implantation of pregnancy. These functions seemingly occur through action on cytotrophoblast cell TGF β receptors. Fifth, hCG-H is an essential component for successful human implantation to prevent early pregnancy loss and spontaneous abortion. Sixth, hCG-H is critical for promoting the midtrimester hemochorial implantation, and for preventing preeclampsia. Seventh, measurements of hCG-H have advantages over measurements of regular hCG or total hCG, in detecting pregnancy, pregnancy outcome (failing or term pregnancy), predicting preeclampsia in pregnancy, or as a tumor marker for gestational trophoblastic diseases.

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1. Introduction

Hyperglycosylated human chorionic gonadotropin, acronym hCG-H, has been shown by multiple authors to have a very different oligosaccharide structure to regular hCG. It is also made by different cells and has very different biological function [1–12]. It is for these reasons that we call it an independent molecule to regular hCG. Likewise, tests for measuring hCG-H have independent uses to tests for regular hCG [13–41]. Its established functions are autocrine rather than endocrine, modulating trophoblast cell apoptosis, more resembling those of a cytokine than a hormone [2,4,42,43]. Here we review ongoing research regarding hCG-H. We review research on: (a) the structure of hCG-H; (b) immunoassays for hCG-H; (c) the source and invasive functions of hCG-H; (d) the biology of

hCG-H action; (e) hCG-H in pregnancy implantation; (f) hCG-H measurement of hCG-H in monitoring pregnancy outcome; (g) measurement of hCG-H in the prediction of preeclampsia; (h) measurement of hCG-H as a marker of invasive gestational trophoblastic disease; (i) measurement of hCG-H as a marker of Down syndrome pregnancy.

2. Structure of hCG-H

hCG is a heterogeneous molecule produced by trophoblastic cells in pregnancy and in gestational trophoblastic diseases including cancer of the trophoblastic cell or choriocarcinoma. It is also produced by other trophoblast sources including testicular germ cell malignancies [2,44].

The molecule contains an α -subunit comprising 92 amino acids and a β -subunit comprising 145 amino acids (Fig. 1). Between 25 and 40% of the molecular weight of hCG comes from the oligosaccharides [1,2,11], 2 N-linked oligosaccharides attached at residues 53 and 78 on the α -subunit

* Tel.: +1 505 272 6137; fax: +1 203 785 6367.

E-mail addresses: larry@hcglab.com, lac@unm.edu

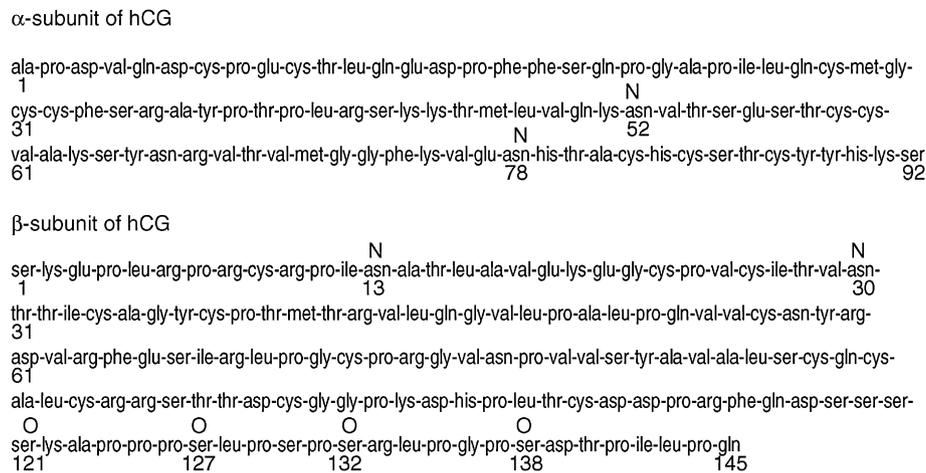


Fig. 1. Sites of attachment of oligosaccharides on hCG $\alpha\beta$ dimer.

(Fig. 1), and 2 N-linked on the β -subunit at 13 and 30 together with 4 O-linked oligosaccharides at residues 121, 127, 132 and 138 on the C-terminal extension (Fig. 1).

Variation occurs in the N-linked and O-linked oligosaccharide structures on hCG (Fig. 2), from differences in cellular metabolism and different expression of glycosyltransferase activities [7–12,45]. It has long been recognized that the hCG produced in choriocarcinoma or testicular germ cell malignancies is a larger molecule (>40,000 vs. 36,700 Da) than that produced through most of normal pregnancy [44,46,47]. In 1983 a significant difference was shown between the N-linked oligosaccharides on pregnancy hCG and those on choriocarcinoma hCG [7]. In the years that followed, 1.5–2-fold larger oligosaccharides were identified on hCG from choriocarcinoma, explaining the molecular size difference [7–12]. The term hyperglycosylated hCG (hCG-H) was coined for choriocarcinoma hCG, or the molecules with larger sugar structures, and regular hCG was defined as that from the bulk of pregnancy with normal sugar structures.

In 1997 it was demonstrated that the 4 O-linked oligosaccharides is the principal difference between choriocarcinoma or testicular germ cell malignancy hCG and pregnancy hCG [11]. As shown in Fig. 2, there are 2 principal types of O-linked oligosaccharides, monoantennary (OM) and biantennary (OB). While first trimester normal pregnancy urine hCG contained 12.3–19% of the larger OB sugar structure (regular hCG, $n = 6$ individuals, mean = 15.6%), choriocarcinoma urine hCG contained 48 to 100% OB chains (hCG-H, $n = 6$ individuals, mean 74.2%) (Fig. 2) [11]. Lesser differences have been shown in the 4 N-linked oligosaccharide structures on pregnancy and choriocarcinoma molecules [8,11]. While in first trimester normal pregnancy biantennary (NB) structures predominate at the two sites on the β -subunit of hCG, the larger triantennary (NT) structure predominates in choriocarcinoma cases (Fig. 2) [8,11]. While in normal pregnancy monoantennary (NM) and NB structures predominate at the two sites on the α -subunit of hCG, biantennary, alternative (NBa) and NT structures predominate in choriocarcinoma cases (Fig. 2) [8,11]. Significant variation has also been observed in the sialic

acid and fucose contents of hCG from pregnancy and choriocarcinoma [7–12].

In 2006, Valmu et al. evaluated the oligosaccharides for the first time on a site by site basis using mass spectrometry methods and multiple regular hCG and hCG-H preparations from pregnancy, choriocarcinoma and testicular germ cell malignancies [12]. They found a greater fucose content of N-linked oligosaccharides in cancer cases [12]. They demonstrated for the first time site-specific difference in O-linked oligosaccharides. They showed the constant presence of predominantly OB structure at Serine residue 121 (68–89%), with variable structures on Ser 127, 132 and 138, primarily OM on regular hCG in molecules isolated from regular pregnancy, and OB on hCG-H in molecules from cancer cases. In conclusion, it appears that difference in glycosylation at Ser 127, 132 and 138 are the greatest discriminator of regular hCG and hCG-H.

3. Immunoassays for hCG-H

A specific monoclonal antibody to hCG-H (antibody B152) was generated against an hCG preparation with 100% OB structure O-linked oligosaccharides [13] produced by a single patient with choriocarcinoma (patient C5) [11]. This antibody detects the presence of structure OB at Ser residue 132 in the middle of the variable 3 O-linked oligosaccharides (Fig. 1) [13]. Using this monoclonal antibody, a two-antibody (B152 plus anti- β tracer) microtiter plate assay was established for detecting hCG-H with 4 h incubation [6,13,15]. This test detected hCG-H with 0% cross-reactivity with pure regular hCG [1,16,17]. It has 60% cross reactivity with the hCG-H free β -subunit [1,13]. Nichols Institute Diagnostics (San Clemente, CA) marketed an automated chemiluminescence hCG-H assay using antibody B152 and an anti- β tracer on their Nichols Advantage immunoassay platform [14]. This assay has been FDA approved for pregnancy-related applications. The automated Nichols Advantage assay, however, with its short 20 min incubation time, more poorly detected the hCG-H free β -subunit ($\sim 20\%$) [16,17]. New tests are now available

O-LINKED OLIGOSACCHARIDESMonoantennary (OM) SA α 2,3 Gal β 1,3 GalNAc-O-

Biantennary (OB)

SA α 2,3 Gal β 1,4 GlcNAc
 β 1,6
 SA α 2,3 Gal β 1,3 GalNAc-O-

N-LINKED OLIGOSACCHARIDES

Monoantennary (NM)

SA α 2,3 Gal β 1,4 GlcNAc β 1,2 Man
 α 1,3
 Man β 1,4 GlcNAc β 1,4 GlcNAc-N-
 α 1,6
 Fuc

Biantennary (NB)

SA α 2,3 Gal β 1,4 GlcNAc β 1,2 Man
 α 1,6
 Man β 1,4 GlcNAc β 1,4 GlcNAc-N-
 α 1,3
 SA α 2,3 Gal β 1,4 GlcNAc β 1,2 Man
 α 1,6
 Fuc

Biantennary Alternative (NBa)

SA α 2,3 Gal β 1,4 GlcNAc β 1,4 Man
 α 1,3
 Man β 1,4 GlcNAc β 1,4 GlcNAc-N-
 α 1,6
 Fuc
 SA α 2,3 Gal β 1,4 GlcNAc β 1,2 Man

Triantennary (NT)

SA α 2,3 Gal β 1,4 GlcNAc β 1,2 Man
 α 1,6
 Man β 1,4 GlcNAc β 1,4 GlcNAc-N-
 α 1,3
 SA α 2,3 Gal β 1,4 GlcNAc β 1,4 Man
 α 1,6
 Fuc
 SA α 2,3 Gal β 1,4 GlcNAc β 1,2 Man

Fig. 2. Principal structures of *O*- and *N*-oligosaccharides attached to regular hCG and hCG-H. GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; Man, mannose; Gal, galactose; Fuc, fucose.

or in development. An ultra sensitive chemiluminescence microtiter plate test is now available from Meso Scale Discovery (Gaithersburg MD, USA) and automated tests are being planned by other major immunoassay manufacturers.

As published, the majority of total hCG immunoreactivity in serum and urine samples in early pregnancy samples is due to hCG-H [6,17–22]. As shown in Table 1, hCG-H accounts for 92% (median) of total hCG in the 3rd complete week of gestation or the week that immediately follows implantation, 73% in the 4th week or the week following missing menses when most pregnancy testing is done, and then declines rapidly thereafter [17,21,22]. It accounts for 14% in the 7th week of gestation and <2% in the second and third trimesters of pregnancy [17,21,22]. Clearly a pregnancy test needs to appropriately detect hCG-H.

As shown in Table 2, only 3 of 14 professional laboratory serum pregnancy tests equally or near-equally (sensitivities for hCG-H <5% different from sensitivity for regular hCG) detect hCG and hCG-H [16,17]. The majority, 9 of 14 tests,

Table 1

The proportion of total hCG immunoreactivity in urine samples due to hCG-H in early pregnancy [17,19]

Gestational age	n	Median percentage hCG-H	Range of hCG-H (%)
3rd complete week	75	92	55–100
4th complete week	63	73	15–100
5th complete week	45	50	10–100
6th complete week	23	26	7.9–53
7th complete week	22	14	2.3–21
2nd trimester	154	1.9	0–31
3rd trimester	50	2.0	0–14

Total hCG was measured in the DPC Immulite total hCG test, which detects hCG and hCG-H equally [17]. hCG-H was measured in the Nichols Advantage hCG-H test, and hCG-H ng/ml was expressed as a percentage of total hCG. Gestational age is presented as complete weeks, where the 3rd complete weeks is 3 weeks 0 days to 3 weeks 6 days since last menstrual period, or in most cases, the week following implantation.

Table 2
Sensitivity of commercial hCG tests

Test or device	hCG only	hCG-H only	hCG-H sensitivity (%)
A. Professional laboratory tests			
Roche Elecsys	76%	167%	220
Tosoh A1A600	95%	97%	102
DPC Immulite	102%	99%	97
Dade Stratus	89%	85%	96
Ortho Vitros ECI	89%	80%	91
Bayer Centaur	121%	107%	89
Wako hCG-CTP	86%	75%	87
Bayer ACS180	119%	102%	86
London radioimmunoassay	112%	95%	85
New Haven radioimmunoassay	107%	91%	85
Abbott AxSym	110%	86%	79
Dade Opus	90%	69%	68
Dade Dimension	90%	50%	54
Serono MAIAclone	94%	4%	4
B. Point of care tests			
Beckman Icon 25	25 IU/l	50 IU/l	50
Quidel Quick Vue hCG Combo	25 IU/l	50 IU/l	50
C. Over the counter tests			
Answer	12.5 IU/l	12.6 IU/l	100
Equate	12.5 IU/l	12.5 IU/l	100
First Response, Early Result	9.4 IU/l	9.4 IU/l	100
K-Mart, American Fare, Easy to Read	25 IU/l	25 IU/l	100
Clear Blue Easy, One Minute	12.5 IU/l	25 IU/l	50
Clear Plan Easy	25 IU/l	50 IU/l	50
EPT	12.5 IU/l	25 IU/l	50
Eckerd, One-Step	50 IU/l	100 IU/l	50
Inverness Medical, Early	25 IU/l	50 IU/l	50
Inverness Medical, Early, Cassette	12.5 IU/l	50 IU/l	50
Save-on, Osco, One-Step	25 IU/l	50 IU/l	50
Confirm	6.3 IU/l	25 IU/l	25
Rite Aid, One-Step	25 IU/l	100 IU/l	25
Target Brand, One-Step	25 IU/l	100 IU/l	25
Walgreens, One Step	12.5 IU/l	100 IU/l	13

Pure regular hCG (CHO cell recombinant hCG, 0% hCG-H) and pure hCG-H (Preparation C5, 0% regular hCG [5]) were calibrated by absorbance at A278 [11]. The concentrations were then assessed blindly in 14 professional laboratory tests [16,17]. Percentages are the assay value divided by the absolute concentration. hCG-H sensitivity was the relative sensitivity for detecting hCG-H vs. hCG. Point of care and over the counter pregnancy test devices were tested for sensitivity by evaluating using multiple concentrations of absolute calibrated hCG and hCG-H in at least sextuplicate [18,19]. Sensitivity results are presented. These are the lowest concentration at which all devices were positive. hCG-H sensitivity was the relative sensitivity for detecting hCG-H vs. hCG.

poorly detect or fail to detect this critical pregnancy marker [16,17]. Similarly, only 4 of 15 home pregnancy tests or over the counter tests equally detected regular hCG and hCG-H [18,19]. The vast majority of tests (11 of 15 over the counter and 2 of 2 point of care) poorly detected hCG-H [18,19]. Over the counter or point of care devices make claims like “99% accurate” or “100% accurate,” and “Use as Early as the First Day of Missed Period” or “Use Test Before You Expect Your Period.” Claims like these are concerning, especially when the device does not appropriately detect the principal hCG analyte present in early pregnancy samples.

It is now 2006, 23 years since the literature first described hCG-H or choriocarcinoma hCG [7], and 8 years since publications started to show that this is the principal hCG immunoreactivity produced in early pregnancy [20]. Still, most manufacturers continue to ignore hCG-H and do not optimize assays for detecting hCG-H. This is partly due to the non-availability of a formal standard or WHO standard or lack of interest by WHO in making such a standard. Until a standard is made, most pregnancy tests will remain sub-optimal with poor recognition of hCG-H and early pregnancy.

4. The source and invasive functions of hCG-H

hCG-H accounts for the major proportion of total hCG forms produced in times of trophoblast invasion, whether at the time of trophoblast invasion at implantation extremely early in pregnancy, or the trophoblast invasion that marks choriocarcinoma cases and testicular germ cell malignancy cases [1,2,5,6,12,13,17–21,29,48]. Only small proportions of hCG-H are made in the absence of trophoblast invasion (<2% of total hCG), as in the bulk of gestation (second and third trimesters) and in non-invasive trophoblast tumors. As such, the presence of significant hCG-H is a virtual absolute marker of ongoing invasion or malignancy in these cancers and in early pregnancy [2,29,31,32,49].

hCG-H is unique to cytotrophoblast cells or stem trophoblast cells the principal cells of choriocarcinoma and most testicular germ cell malignancies and of blastocysts at the time of implantation or pregnancy [1,2,5,6]. hCG-H is not, however, significantly produced by mature or differentiated syncytiotrophoblast cells, the predominant cells in trophoblast tissue through the length of pregnancy and in non-malignant gestational trophoblastic diseases [5,11,17,29].

Four model systems have been used for studying the invasive functions of cytotrophoblast cells. The first two are JAR and JEG-3 lines of choriocarcinoma cells, clones of the same patient's human choriocarcinoma cytotrophoblast cells [50]. The third is fresh cytotrophoblast cells isolated, purified and cultured from human placentas at 6 and 7 weeks of gestation, and prepared by the Kliman method [51]. The fourth is NTERA testicular embryonal carcinoma (testicular germ cell malignancy) cell line. All four cytotrophoblast models produce exclusively hCG-H and small amounts of hCG free β -subunit [2,12,17,52] and no detectable regular hCG [17].

As published by 3 independent groups using these 4 models, hCG-H (or choriocarcinoma hCG), but not regular hCG, directly modulates the cell growth, tumor formation and invasion by early pregnancy cytotrophoblasts, choriocarcinoma cytotrophoblasts and testicular germ cell malignancy cells, *in vitro* and *in vivo* (nude mouse human xenograph models) [1–4]. This was shown by cell culture growth studies, cell culture Matrigel invasion chamber model studies *in vitro*, and by athymic nude mouse studies with the human cytotrophoblast cell transplants (xenographs) *in vivo* [1–4]. As shown in my laboratory, monoclonal antibodies to hCG-H completely blocked the hCG-H promoted invasion in nude mice xenographs *in vivo*, and culture models *in vitro*,

preventing cytotrophoblast cell invasion and cell growth and blocking any ongoing tumorigenesis [1,2]. Addition of pure hCG-H, but not pure regular hCG, promoted growth and invasion in cell culture models [1,2]. Two independent studies solidly confirm these observations. As shown by Lei et al. [3], antisense DNA to hCG α -subunit blocked all choriocarcinoma hCG production in JAr choriocarcinoma cells (hCG-H is the only form of hCG made in these cells [17]). The use of antisense DNA to hCG α -subunit blocked invasion, cell growth and tumorigenesis by choriocarcinoma in nude mice xenographs *in vivo* [3]). This confirms our studies showing that hCG-H is essential for cell invasion to occur. Similarly, further independent studies by Hamada et al. [4] using antisense DNA to hCG β -subunit also confirmed our finding regarding the critical requirement of hCG-H for invasion to occur. Considering our studies [1,2] and these independent reports [3,4], it is concluded that hCG-H is secreted by cytotrophoblast cells and acts on the same cells, or through an autocrine mechanisms acts on the same cells, to promote invasion. Considering these findings, it is also concluded that hCG-H is critical for early pregnancy cytotrophoblast cell invasion, i.e. for implantation, and essential for choriocarcinoma and testicular germ cell malignancy cytotrophoblast cells to grow or metastasize.

While regular hCG functions by promoting progesterone production at the corpus luteal LH/hCG receptor, hCG-H functions poorly in this hormonal capacity, with 1/25th the biological activity of regular hCG [53]. hCG-H is in multiple ways a distinct molecule from regular hCG, it is significantly larger (>40,000 vs. 36,700), it is produced by separate cells (cytotrophoblast vs. syncytiotrophoblast), it has an autocrine rather than an endocrine function, and it has a separate function promoting growth and invasion and tumor formation. This is a unique situation where identical gene sequences code for the α - and β -subunits of both hCG and hCG-H polypeptides [11], yet two independent molecules are produced. Identical polypeptides form the common backbone of two very separate molecules—regular hCG as a steroid promoting hormone and hCG-H as an invasion promoter—distinguished only by variation in the extent of glycosylation.

5. The biology of hCG-H action

As described above, hCG-H has a completely separate biological function to regular hCG. We consider here whether hCG-H acts on a separate receptor to regular hCG (hCG/LH receptor) to promote growth and invasion. Noting again that hCG-H is poor at binding the LH/hCG receptor and mimicking regular hCG's steroidogenic action, with 1/25th the biological activity of regular hCG [53], we consider other modes of action.

X-Ray crystallography studies demonstrate that the hCG β -subunit is unusual in having a unique cystine knot configuration within its structure [54,55]. This rare structure comprises a specific arrangement of two contiguous disulfide bonds, and the peptide chains linking them, penetrated and knotted by a third disulfide bond [54–57]. This cystine knot

structure has only been found elsewhere on transforming growth factor β (TGF β) and on 3 other cytokines [56,57]. Several authors, including Lei et al. [3], investigating choriocarcinoma hCG (hCG-H) and trophoblast invasion mechanisms have suggested that the cystine knot structure may make the molecule like a cytokine and explain its autocrine involvement in trophoblast invasion [2,3,54,55].

Multiple studies have shown that TGF β 1, -2 and -3 and their common receptors are all key elements in trophoblast invasion [42,56–71]. Multiple studies have also shown and repeatedly confirmed that trophoblast cell invasion, whether in implantation, choriocarcinoma or testicular germ cell malignancies, involves a mechanism involving blockage of TGF β -controlled apoptosis, which enhances cell proliferation [59,61,62,68–71]. Interestingly, Hamada et al., in addition to showing choriocarcinoma hCG (hCG-H) as the promoter of invasion, demonstrated a mechanism involving blockage of apoptosis, which enhances cytotrophoblast cell proliferation, leading to cell growth and invasion [4]. This links hCG-H promotion of cell growth and invasion to blocking apoptosis. It also indicates a mechanism involving modulation of a TGF β controlled process.

The relationship between hCG-H, cell invasion, cystine knot cytokine and TGF β -controlled mechanisms such as apoptosis and trophoblast invasion seems more than a coincidence for the following reasons: first, hCG-H is critical to trophoblast invasion [1–4]; second, hCG-H directly blocks apoptosis [4]; third, hCG-H has the cystine knot structure unique only to TGF β and 3 other cytokines [4,54,55]; fourth, the vast abundance of research showing that TGF β -receptor functions are key in trophoblast invasion [31,32,42,49,52,56–71], through modulation of apoptosis [59,61,62,68–71]. It all ties together neatly. However, the link is not yet proven. Once this link is proven we can consider new concepts in cancer cure or therapy.

The hCG-H–TGF β –apoptosis relationship hypothesis is in many ways confirmed by the parallel studies by Butler et al. with hCG free β -subunit rather than hCG-H [43,72,73]. For many years, hCG free β -subunit expression by numerous epithelial carcinomas was considered nothing more than an epiphenomenon [74,75]. Studies by Butler et al. established that the production of hCG free β -subunit directly correlated with the rate of cell growth [43]. Initial studies showed that the presence of hCG free β -subunit in the cell culture system could increase cell populations by up to 50% and this was brought about by a proportional reduction in the number of cells undergoing apoptosis. They confirmed that hCG free β was acting as an anti apoptotic factor and could competitively reverse apoptosis induced by TGF β [43]. A key autocrine role was demonstrated for the free β -subunit of hCG, even though the free β -subunit has been shown to have zero ability to bind the LH/hCG receptor or to promote cAMP or progesterone production at the LH/hCG receptor [76,77]. When cancer cells were treated with antibodies to free β -subunit, apoptosis blocked by free β -subunit resumed and cell growth declined [73]. They later showed that the free β -subunit of hCG binds to and antagonizes a TGF β -RII sized

(70 kDa) membrane protein (Butler et al., manuscript submitted).

A TGF β receptor and an apoptosis mechanism have been clearly shown for free β -subunit [43,72,73]. We now consider the parallels between the mechanisms seen with free β -subunit and the hCG-H applications shown in choriocarcinoma and testicular germ cell malignancies. A study from Khoo et al. shows that in choriocarcinoma a molecule with the exact molecular size of hCG-H binds the TGF β -RII receptor on cytotrophoblast cells [42]. This is the same receptor as was shown binding the free β -subunit on bladder cancer cells. Taken together, data strongly indicates that both hCG-H and free β -subunit inhibit apoptosis in cancer cells, and both seemingly function through the TGF β -RII receptor. While the link between hCG-H—cystine bridge—apoptosis and the TGF β RII receptor seems quite solid, it still needs to be confirmed. We await this confirmation.

Just as the hCG produced in testicular germ cell malignancies and choriocarcinoma is hyperglycosylated (hCG-H) [2,11,12], the free β -subunit is also hyperglycosylated [12]. Commonness is noted firstly between forms of β -subunit made by different cancer cells, and secondly between hCG-H and free β -subunit and their function in cancer promotion. It is hypothesized that trophoblast cells have the concentration dynamics (law of mass action) to make dimers like hCG-H while non-trophoblastic cancers, like the bladder and endometrial cancer cells investigated by Butler et al., lack the combination dynamics to make hCG-H dimers and instead make only the separate subunits. Both hCG-H and free β -subunit seemingly have the same essential exposed β -subunit element that promotes invasion and malignancy.

The cystine knot structure is present in both regular hCG and hCG-H. We ask why would only hCG-H and not regular hCG interfere with apoptosis and promote growth and invasion. As published [11,12,53,78], hCG-H, but not regular hCG, in urine and serum is almost always extensively cleaved between β -subunit Val 44 and Leu 45 and/or Gly 47 and Val 48 (5 of 5 purified molecules from choriocarcinoma cases [11]). This is not observed in normal pregnancy regular hCG [11]. These cleavage sites are folded on top of an α -subunit N-linked oligosaccharide in regular hCG [11]. This site is rapidly cleaved by leukocyte elastase on hCG-H or free β -subunit, with only minimal cleavage occurring on regular hCG [53]. This site is close to the cystine knot structure on the β -subunit [11,55,62]. We infer that the large oligosaccharides on hCG-H limit the tightness of regular hCG folding, uniquely exposing the molecule to proteases and exposing the cystine knot. Consistent with this finding is the demonstration that the subunits of choriocarcinoma hCG dissociate much more rapidly than regular pregnancy hCG [78,79]. We infer that exposure of the cystine knot in the β -subunit of hCG-H differentiates the action of hCG-H from regular hCG.

6. hCG-H in pregnancy implantation

As published, the majority or all of total hCG immunoreactivity in serum and urine samples in early pregnancy samples

is due to hCG-H [6,17–22]. As shown in Table 1, hCG-H accounts for a 92% (median) of total hCG in the 3rd complete week of gestation or the week that immediately follows implantation. This is no surprise when considering the early stage of differentiation of hatched blastocyst cells. The proportion of hCG-H rapidly declines thereafter as cytotrophoblast cells fuse to intermediate cells and to syncytiotrophoblast cells [5,6,17]. By the second and third trimesters of pregnancy, hCG-H accounts for <2% of total hCG [17,21,22].

As noted by O'Connor et al. in 1998, an unduly low proportion or the absence of hCG-H occurs in pregnancy marking early pregnancy losses prior to the sixth week of gestation [20]. In further studies by Kovalevskaya et al. [21] and our group [22], all failing pregnancies, whether early pregnancy losses or miscarriages after 6 weeks of gestation, were associated with unduly low proportions or absence of hCG-H. As noted, a simple cut-off of 13 ng/ml hCG-H in serum could be used to differentiate a pregnancy with failure outcome (<13 ng/ml) from that with term outcome (>13 ng/ml) between 4 and 7 weeks of pregnancy [22]. As discussed earlier, hCG-H has a clear role in promoting the invasive process in implantation of pregnancy and cancer, seemingly through a TGF β modulated apoptosis mechanism. If we consider the findings of O'Connor et al. [20], Kovalevskaya et al. [21] and our own findings [22], indicating extremely low production of hCG-H or absence of hCG-H in failing pregnancies, and the research showing ineffective implantation as the root of pregnancy failures [80–88], then a link is likely between hCG-H and failing pregnancies.

Recently, we investigated this hCG-H—failing pregnancy relationship [23]. We monitored hCG and hCG-H and the proportion of hCG due to hCG-H daily in 110 women through multiple menstrual cycles until they achieved pregnancy [23]. As indicated by Wilcox et al. [86,87], the day of first detection of hCG (hCG >1 mIU/ml) was assumed as the day of implantation. Forty-three women achieved normal term pregnancies. On the day of implantation the average proportion of hCG-H in these cases was $87 \pm 17\%$ (mean \pm standard deviation). In all 43 cases >50% hCG-H was detected. Twenty women achieved only miscarriages of pregnancy. On the day of implantation the average proportion of hCG-H in these cases was $44 \pm 39\%$. A significant difference was observed between the term outcome and failing pregnancies by *t*-test, $p < 0.00001$. In 13 of these 20 miscarriage cases <50% hCG-H was detected. All pregnancies going to term produced >50% hCG-H at the time of implantation. It is therefore inferred that a minimal proportion of hCG-H is critical for successful pregnancy outcome, and that insufficient production of hCG-H is at the root of ineffective implantation leading to pregnancy failures.

Given the role shown for hCG-H in invasion, it is inferred that hCG-H is critical for effective implantation to occur. It was also concluded (see previous section) that cytotrophoblast cells produce hCG-H which acts as an autocrine antagonist on the TGF β receptor to inhibit apoptosis. Through this mechanism, cytotrophoblast cell propagation and implantation occur.

7. hCG-H measurement of hCG-H in monitoring pregnancy outcome

In 2002, Kovalevskaya et al. discovered unusually low proportions of hCG-H in pregnancies that spontaneously abort in the first trimester of pregnancy [21]. The low proportions started when hCG was first detected and continued until the time of spontaneous abortion. While this was an important discovery, no clinical application was indicated. Two years later these findings were confirmed by Sutton-Riley et al. [22].

Sutton-Riley et al. showed that a simple single point cut-off of 13 ng/ml hCG-H in serum could be used between 4 and 7 weeks of pregnancy to differentiate a failure outcome (<13 ng/ml) from term outcome (>13 ng/ml) [22]. This 13 ng/ml cut-off was pivotal in detecting 73% of failures (spontaneous abortion and ectopic pregnancy) at 5% error rate, compared to 42% detection at this same error rate for a comparable cut-off for hCG. A significant difference was observed between the area under the ROC curve results, or test accuracies using hCG and hCG-H ($p < 0.00005$).

8. Measurement of hCG-H in the prediction of preeclampsia

Preeclampsia is the leading cause of prenatal infant mortality and maternal mortality in pregnancy today [89–91]. Preeclampsia occurs as a consequence of abnormal invasion by the trophoblast and the uterine spiral arteries in human pregnancy [91–95]. Whereas all mammalian embryos undergo implantation shortly after conception, humans are the only species known to undergo a deep trophoblastic implantation in the second trimester [91–95]. This provides for modification of spiral arteries that results in an increase in the blood flow to the placenta [92]. Preeclampsia is believed to be the result of failure to achieve or to complete this second implantation leading to hypoxia and nutritional deficiencies, compensated for by raised maternal blood pressure [91–95].

As discussed earlier in the first half of this article, hCG-H is the signal for cytotrophoblast cell invasion during pregnancy. We consider the relationship between hCG-H production and preeclampsia. Mid-trimester hCG-H was measured in 568 women with normal singleton pregnancies and no history of preeclampsia, 14–21 weeks of gestation, undergoing amniocentesis because of maternal age concerns [96]. Preeclampsia developed in 26 of these women (4.6%). A significant correlation was observed between low hCG-H and subsequent development of preeclampsia ($p = 0.001$). The mean hCG-H level was significantly greater in normals than in those destined to develop preeclampsia (43 ng/mg vs. 20 ng/mg), $p = 0.002$ [96]. There was a progressive increase in risk for developing preeclampsia as hCG-H levels decreased [96].

Low maternal mid-trimester hCG-H levels predict risk for developing subsequent preeclampsia. Considering the key role of hCG-H in trophoblast invasion, this supports the concept that preeclampsia results from poor or failure of mid-trimester second implantation [91,93–95]. Thus, hCG-H is not only critical to invasion at implantation, but critical to

the second implantation and the development of preeclampsia. There probably cannot be a better marker of preeclampsia than the measurement of the molecule that is responsible for the second implantation, which is at the root of the disease.

9. Measurement of hCG-H as a marker of invasive gestational trophoblastic disease

hCG-H accounts for the major proportion of total hCG forms produced in times of trophoblast invasion, whether at the time of trophoblast invasion at implantation or the trophoblast invasion that marks choriocarcinoma cases and testicular germ cell malignancy cases [1,2,5,6,12,13,17–21,29,48]. Only small proportions of hCG-H are made in the absence of trophoblast invasion (<2% of total hCG). As such, the presence of significant hCG-H is a virtual absolute marker of ongoing invasion or malignancy [2,29,31,32,49]. This makes hCG-H invaluable as a tumor marker for identifying the presence of invasive disease and the need for chemotherapy.

A quiescent or inactive form of gestational trophoblastic disease has been identified. It is marked by continuous low serum hCG results, persisting for periods ranging from 3 months to 16 years in the absence of hCG-H [29–34]. It may be considered as a pre-malignant state since approximately 1 in 5 cases transforms into GTN or choriocarcinoma [29–34]. We have reviewed the histology slides from two cases undergoing surgery for this condition. In both cases, intermediate and highly differentiated syncytiotrophoblast cells were observed, with a clear absence of the cytotrophoblast cells that mark invasive disease [1,29]. The cells of quiescent gestational trophoblastic disease, the differentiated syncytiotrophoblast cells are commonly slow-growing cells that will not respond to chemotherapy.

Persistent low levels of hCG occur in the months after evacuation of a hydatidiform mole, after spontaneous abortion of pregnancy, or after chemotherapy for a persistent mole, choriocarcinoma or gestational trophoblastic neoplasm. The question arises whether to treat these cases with chemotherapy, as is common practice. If quiescent disease is present, chemotherapy would neither work nor be warranted. This need for chemotherapy is marked by hCG-H.

The USA hCG Reference Service is a clinical laboratory specializing in the detection of hCG and in the management of gestational trophoblastic diseases [29–32]. In their experience with 82 invasive gestational trophoblastic disease cases (choriocarcinoma, gestational trophoblastic neoplasm and invasive hydatidiform mole) hCG-H accounted for $50 \pm 39\%$ of the total hCG (range 27–100%) regardless of the magnitude of the total hCG result (5.2 to 597,000 mIU/ml). The USA hCG Reference Service also consulted on 69 cases with quiescent gestational trophoblastic disease. These cases were marked by the virtual absence of hCG-H. In these cases hCG-H accounted for $0.47 \pm 2.1\%$ (range 0–10%). Using a cut-off of 20% hCG-H, 100% of active disease cases could be identified at 0% false positive. As such, hCG-H can be used to absolutely discriminate active disease of cases requiring therapy [29–32]. The USA hCG Reference Service findings

with quiescent gestational trophoblastic disease have now been confirmed independently by 2 other groups, showing the importance to differentiate active and quiescent disease [97,98].

The USA hCG Reference Service knows now of a case that died due to over treatment of quiescent gestational trophoblastic disease. Quiescent gestational trophoblastic disease does not respond to chemotherapy. In this case, when the patient's hCG result was not suppressed by chemotherapy, the patient was assumed to be resistant to chemotherapy and further, and further chemotherapy administered. Patient died as a complication of chemotherapy. It is very important in cases with persistent low hCG results <212 mIU/ml to exclude quiescent disease before resorting to chemotherapy [29–32].

10. Measurement of hCG-H as a marker of Down syndrome pregnancy

In 1997 a preponderance of hCG-H was demonstrated in structure studies in mid-trimester Down syndrome pregnancies and then later confirmed by using the B152-based hCG-H immunoassay [25–27]. This was a surprising finding. This enigma has been explained in recent years by the finding of a defect in cytotrophoblast differentiation into syncytiotrophoblast cells resulting in an accumulation of cytotrophoblasts, the hCG-H producing cells, in Down syndrome cases [99,100].

Variable results have been reported when using hCG-H to predict Down syndrome pregnancy in the first and second trimesters of pregnancy. This is largely due to the use of the long-incubation manual assay compared with the short incubation automated Nichols Advantage test. In second trimester pregnancy Down syndrome prediction (14–24 weeks of gestation), using serum or urine samples and the manual test 80% and 81% detection has been reported [6,36]. Using the automated test, 55 and 60% detection has been recorded in large multi-center trial [39,101]. In first trimester screening (11–13 weeks gestation) 60% detection has been recorded with the automated test in USA and Europe multi-center trials [28,102]. In all cases, hCG-H has higher sensitivity than total hCG alone in the second trimester [6,39,101], and than hCG and its free β -subunit alone in the first trimester [28,102].

Sutton et al. [24] investigated the difference in results between the manual and automated screening tests. As found, the principal isoform of hCG-H elevated in Down syndrome cases, whether first trimester or second trimester, is hCG-H deficient in sialic acid [24]. This major isoform of hCG-H was poorly detected by the Nichols Advantage hCG-H test and is reflected in the decreased detection rate. We eagerly await a new automated hCG-H test that equally or solely recognizes sialic-acid deficient hCG-H.

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