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The biological function of hyperglycosylated hCG

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ABSTRACT

Objective: Hyperglycosylated human chorionic gonadotropin (hCG) is a variant of hCG made by cytotrophoblast cell. Here we examine the role of hyperglycosylated hCG in placenta growth and invasion. **Methods:** JEG−3 choriocarcinoma cells and term cytotrophoblast monolayer culture were prepared. The effect of supplemental hyperglycosylated hCG and hCG was investigated. Growth of these cells was examined by increase in cell number. Invasion was investigated using Matrigel basement membrane cells. The proportion of cell invading Matrigel was determined. **Results:** Term cytotrophoblast cell and JEG−3 choriocarcinoma cells grew to 5 427±834 cells (109%) and 7 114±553 cells (142%). With the supplementation of hyperglycosylated hCG, they grew significantly wider to 7 633±177 cells (142%) and 10 315±1 477 cells (206%). With the supplementation of hCG they diminished to 4 227±769 cells (78%) and 5 620±657 cells (79%). Term cytotrophoblast cell and JEG−3 choriocarcinoma cells penetrated Matigel membranes to (40.0± 10.0)% and (46.0±9.8)%. Hyperglycosylated hCG significantly enhanced penetration to (76.0± 13.0)% and (84.0±6.6)%. hCG diminished penetration to (32.0±9.1)% and (32.0±4.5)%. **Conclusions:** Hyperglycosylated hCG enhances both cytotrophoblast growth and cytotrophoblast cell invasion. hCG minimally suppresses growth and invasion.

1. Introduction

Hyperglycosylated human chorionic gonadotropin (hCG) is a variant form of hCG produced by normal and cancerous placental cells. While placental syncytiotrophoblast cells make the hCG hormone, placental cytotrophoblast cells produce hyperglycosylated hCG, the hCG variant[1,2]. With the first discovery of hyperglycosylated hCG, an hCG dimer with much larger oligosaccharide side chains[3,4], studies indicated that it controlled trophoblast implantation and uterine invasion[5,6]. With these alternative functions to the hormone hCG and its alternative carbohydrate structure, it was renamed "invasive trophoblast antigen"[7,8]. A letter from the World Health Organization in 2003 stated that it shared amino acid sequence with hCG, and that it therefore had to have "hCG" as part of its name; the term hyperglycosylated hCG was chosen. As shown in 2006, hyperglycosylated hCG independently promotes trophoblast invasion and choriocarcinoma growth *in vivo* and *in*

vitro[2,6]. As shown, blockage of hyperglycosylated hCG with specific hyperglycosylated hCG antibody blocked human choriocarcinoma growth *in vitro* in cell cultures and *in vivo* in nude mice[2,6].

Studies in 2011 showed that hyperglycosylated hCG, like hCG free beta−subunit or a hyperglycosylated hCG free beta−subunit[9], seemingly acts biologically as an autocrine growth factor, antagonizing transforming growth factor−beta (TGF beta) action on cancer cells, like hCG free beta−subunit[9]. This antagonistic action of hyperglycosylated hCG (also called choriocarcinoma hCG) has been independently shown to blocks cell apoptosis[10], like hCG free beta−subunit. Multiple studies show that antagonism of TGF beta will block cellular apoptosis, promote cell growth, and promote cell metalloproteinase and collagenase production, leading to cell invasion[11−18]. Here, we tie this research together, examining the biological activity of hCG and hyperglycosylated hCG and showing that hyperglycosylated hCG acts biologically by specifically promoting both cell growth and cell invasion by the placenta in pregnancy and cancer. Two cell models are examined, JEG−3 choriocarcinoma cells and isolated term placenta cytotrophoblast cells.

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2. Materials and methods

2.1. Total hCG assay

Total hCG in serum and culture fluid was measured using the Siemens Immulite 1000 assay. This assay measures hCG, nicked hCG, hyperglycosylated hCG, nicked hCG missing beta–subunit C–terminal peptide, free beta–subunit, nicked free beta–subunit, and nicked free beta–subunit missing beta–subunit C–terminal peptide equally[19].

2.2. Hyperglycosylated hCG assay

Hyperglycosylated hCG was measured in the immunometric assay using B152 ant–hyperglycosylated hCG[2,6]. This assay detects hyperglycosylated hCG and it free beta–subunit only, with no cross–reactivity (<0.1% reactivity) with hCG[2,6].

2.3. Biological activity

Biological activity at the hCG/luteinizing hormone (LH) receptor was measured in the assay using isolated Sprague–Dawley rat ovaries, as described previously[20]. Briefly, ovaries were collected 6 d post hCG injection, and luteal cells were prepared by the method of Behrman *et al*[21]. Briefly, luteinized cells were finely minced with a razor and dispersed in Minimal Essential Medium (Gibco, Grand Island NY) containg 0.1% (v/v) bovine serum albumin. Minced luteal cells were separated by Percoll density gradient (Pharmacia, Uppsala, Sweden). In sextuplicate, hCG preparations were added to 12 mm×75 mm glass tubes containing luteal cells, $2.5×10^6$ cells per tube. After 90 min incubation at 37 °C, progesterone concentration was determined by radioimmunoassay. Assay results were determined as µg/mL progesterone or per µg/mL hCG, and concentrations were determined as a percentage relative to concentration of control, P8 pregnancy hCG, which has been shown not be nicked and normally glycosylated[3].

2.4. JEG–3 cell line experiments

To examine cell growth, JEG–3 choriocarcinoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) with 10% (v/v) fetal bovine serum (Gibco) to approximately 70% confluency in 5% (v/v) carbon dioxide incubator. Then 5 000 of the trypsin–dispersed cells were cultured for 24 h in defined medium without fetal bovine serum, in sextuplicate, with no hCG form added, with added hyperglycosylated hCG (C5) or added regular hCG (CR127), 10 times the concentration normally produced by 5 000 cells over a 24 h period (163 ng total), in T–25 culture flasks. After 24 h, cell were dispersed with trypsin and counted using a Millipore (Billerica, MA, USA) automated cell counter.

To investigate cell invasion using Matrigel medium, 70% confluent JEG–3 choriocarcinoma cells were trypsin dispersed, and then 5 000 cells were placed on Matrigel basement membranes and control inserts in quadruplicate. Concentrations of hCG CR127 and hyperglycosylated hCG C5 used to promote invasion were 10 times that normally produced by the cells (163 ng total).

2.5. Term cytotrophoblast monolayer culture experiments

Cytotrophoblast cell were purified by Percoll density centrifugation from trypsin dispersed normal term pregnancy placenta villous trophoblast tissue, using the methods published by Kliman *et al*[22,23]. To investigate cell growth, a total of 5 000 cells were cultured for 24 h in a 60 mm dish in 1 mL defined DMEM with no fatal bovine serum, with no additive, with hCG batch CR127 or hyperglycosylated hCG C5, 10 times that concentration normally produced by 5 000 cells over a 24 h period (19.8 ng total). After 24 h, cells were dispersed and counted using a Millipore (Billerica, MA, USA) automated cell counter.

To investigate cell invasion using Matrigel medium, 5 000 cells were placed on Matrigel basement membranes and control inserts in quadruplicate. Concentrations of hCG CR127 and hyperglycosylated hCG C5 used to promote invasion were 10 times that normally produced by these cells (19.8 ng total).

2.6. Matrigel invasion assay

Matrigel (BD Biosciences, San Jose, CA, USA) matrix suspended basement membranes were used to assess cell invasion. These basement membranes comprise neurons, Sertoli cells, vascular endothelial cells and heptocytes. Five thousand JEG–3 or term cytotrophoblast cells were plated onto Matrigel membranes and control inserts, 5 000 cells per membrane and per control insert (Biocoat Matrigel invasion membranes; BD Biosciences, Bedford, MA, USA). Cells were cultured at 37 °C for 24 h in DMEM contining 10% (v/v) fetal bovine serum (controls), and with 19.8 ng (term cytotrophoblast cells) and 81 ng (JEG–3) hCG (hCG CR127 or hyperglycosylated hCG C5, each condition on quadruplicate membranes. Matrigel membranes were processed and percentage invasion was calculated as suggested by the manufacturer in package inserts. Briefly, membranes were rehydrated in the incubator for 2 h before use. Membranes and control inserts are then plated (25 000 cells in 0.5 medium per plate). Plates were cultured for 24 h, and membranes removed from the inserts using a scalpel. Membranes are transferred to a slide using Cytoseal mounting medium (Stephens Scientific Inc., Riverdale, NJ, USA), exposing the under surface or the invaded cells. Cells were stained with DIF–Quick Stain (IMEB Inc., Chicago, IL, USA) to mark nuclei. Invaded cells were counted at five marked places, and the count averaged. Cell penetration or invasion of membranes was directly compared to that of correspondingly cultured control inserts and the percentage invasion was calculated using the formula provided by the manufacturer.

3. Results

Regular hCG/LH hormonal biological activity of hyperglycosylated hCG was measured examining progesterone activity in isolated rat corpora lutea cells. As shown in Table 1, three pregnancy hCG urine preparations were not nicked *at all*[3], and yielded the highest progesterone production per μg/mL hCG. Preparation P8 yielded 7.4 μg/mL, and preparation P1 yielded 7.4 μg/mL. Preparation P7 yield a slightly higher value, 8.2 μg/mL. The concentration of O– and N–oligosaccharides (average) indicates that these three pregnancy hCG preparations were minimally hyperglycosylated 17.1% and 18.4%, respectively. Three other pregnancy hCG preparations were invariably nicked at beta 47–48, 12%, 58% and 59%, respectively (Table 1). Nicking diminished the LH/hCG biological activity of hCG. These three preparations had diminishing biological activities relative to extent of nicking, 6.6 (89% of P8), 3.7 (50%) and 3.6 μg/mL (50%), respectively.

One hCG preparation, M4, was 100% nicked at beta 47–47, this preparation has a biological activity of 1.8 μg/mL or 24% of P8 activity. Considering all nine pregnancy and hydatidiform mole preparations and nicking proportion and biological activities in Table 1, a linear regression relationship was observed between biological activity and proportion nicking (*r*=0.950). By linear regression analysis, 100% nicking corresponded to a biological activity of 1.12 μg/mL or 15.1% of control P8 pregnancy hCG. It is concluded that nicking reduces biological activity by 15.1%.

Considering choriocarcinoma hCG molecules, JEG–3 cell line hCG, C7, C3 and C5 hCG, preparations were invariably nicked, 0%, 0%, 24% and 100%. The biological activity

of the two 0% nicked preparations, JEG–3 and C7 were 3.7 μg/mL (50% of P8) and 3.4 μg/mL (46%), respectively. Indicating that hyperglycosylation decreases biological activity by an average of 48%. Two of these hyperglycosylated choriocarcinoma hCG preparations were considerably nicked, C3 hCG and C5 hCG, with biological activities of 3.0 (40%) and 1.5 μg/mL (20%). It is inferred that these reduced biological activities are due to a combination of proportional nicking and hyperglycosylation. In preparation C3, 40% activity, this is close to the combined estimated action of nicking and hyperglycosylated hCG (24% nicking=80% activity, hyperglycosylation=38% activity). In preparation C5, 20% activity, an exceptionally high activity was noted (100% nicking=15.1% activity, hyperglycosylation=7.2% activity). Both hyperglycosylation and nicking clearly diminish biological activity at the hCG/LH receptor.

Antagonistic TGF beta biological activity of hyperglycosylated hCG molecules was assessed[9]. Action of 1 ng/mL hCG on promoting growth of JEG–3 choriocarcinoma cells was assessed using 6×10^5 to 8×10^5 cultured cells in an approximately 70% confluent flask. An antagonistic value of 1.0% represents growth enhancement by 1.0%. As found, C5 hyperglycosylated hCG was the best promoter, 2.4% (73.9% hyperglycosylated N– and O–linked oligosaccharides), C3 hyperglycosylated hCG was the second best, 2.2% (68.1% hyperglycosylated N– and O–linked oligosaccharides), and JEG–3 hyperglycosylated hCG was the poorest promoter, 1.7% (51% hyperglycosylated N– and O–linked oligosaccharides). A linear regression correlation was found between proportion hyperglycosylated oligosaccharides and calculated TGF beta antagonistic activity (*r*=0.999). Three pregnancy preparations, P7, P3 and P5, were found to have

Table 1
The biological and antagonistic activity of purified hCG urines, standards and cell line medium.

Preparation	Concentration by amino acid analysis (μg/mL)	hCG/LH bioactivity μg/mL (% of P8 result)	Nicking at beta 47–48 from amino acid sequence (%)	Hyperglycosylated from O– + N–linked sequence[3,4] (%)	Antagonist activity (%)
From pregnancy					
P7 (3)	1 130	8.2 (>100%)	0	18.4	<0.1
P8 (3)	970	7.4 (100%)	0	17.1	
P1 (3)	620	7.4 (100%)	0		
CR127 (3)	850	6.6 (89%)	12	12.0	<0.1
P3 (3)	530	3.6 (48%)	59	10.9	<0.1
P5 (3)	1 460	3.7 (50%)	58		
From hydatidiform mole					
M2 (3)	1 490	8.7 (>100%)	0	35.1	
M1 (3)	1 100	6.4 (86%)	15	14.7	
M4 (3)	3 400	1.8 (24%)	100	24.4	
From choriocarcinoma					
JEG–3 (2)	340	3.7 (50%)	0	51.0	1.7
C7 (3)	741	3.4 (46%)	0	58.0	
C3 (3)	320	3.0 (40%)	24	68.1	2.2
C5 (3)	2 130	1.5 (20%)	100	73.9	2.4

The bioactivity at the hCG/LH receptor was determined in isolated rat ovaries, results are progesterone production (μg/mL) in response to hCG (μg/mL). The % nicking was determined from amino acid sequence analysis[3], and the % hyperglycosylated hCG is an average of the % tetrasaccharide core O–linked oligosaccharides, and the % triantennary (% GGG and GGGF) N–linked oligosaccharides on hCG beta–subunit[3]. The activity of TGF beta antagonism was expressed as % enhancement of cell growth of JEG–3 choriocarcinoma cells.

no measurable TGF beta antagonism biological activity, even though the proportions hyperglycosylated oligosaccharides were measurable, 18.4%, 12.0% and 10.9%. It is inferred that a minimal or cut–off hyperglycosylation may be required to achieve measurable activity.

Trophoblast cells, JEG–3 choriocarcinoma cell line and term cytotrophoblast monolayer cells, 5 000 cells, were cultured 24 h. As shown in Table 2, in both cell line media, total hCG was equal to hyperglycosylated hCG concentration, indicating that only hyperglycosylated hCG was being produced. In JEG–3 cell line, 16.3 ng hCG was being produced by 5 000 cells. In term cytotrophoblast monolayer cells, 1.98 ng hCG were being produced by 5 000 cells (Table 2).

The ability of hCG to promote normal trophoblast and choriocarcinoma cell growth was investigated. Cells, 5 000, were cultured in the presence of no additive, a 10 fold excess concentration of hCG CR127 over endogenously produced hCG (10 times that produced by 5 000 cells or 163 ng, Table 2, for JEG–3 cells and 19.8 ng, Table 2, for term cytotrophoblast monolayer cells), or a 10 fold excess concentration of hyperglycosylated hCG C5 (10 times that produced by 5 000 cells or 163 ng, Table 2, for JEG–3 cells and 19.8 ng, Table 2, for term cytotrophoblast monolayer cells). As found (Table 3), those cell without supplement grew minimally due to endogenous hyperglycosylated hCG, 5 427±834 cells (109%) (term cytotrophoblast monolayer) and 7 114±553 cells (142%) (JEG–3 cells). hCG CR127 slightly inhibited cell growth 4 227±769 cells (78%) (term cytotrophoblast monolayer) and 7 114±553 cells (79%) (JEG–3 cells). Hyperglycosylated hCG C5, in contrast, significantly promoted cell growth, 7 633±777 cells

(152%) (term cytotrophoblast monolayer) and 10 315±1 477 cells (206%) (JEG–3 cells) (*t* test, *P*<0.000 05 and *P*=0.002 1). It was inferred that hyperglycosylated hCG promotes cell growth.

The ability of cells to invade a membrane was assed using Matrigel matrix suspended basement membrane cells. The proportion of cell penetrating the basement membrane was assessed as a measure of invasion. Term cytotrophoblast monolayer cells, 5 000, were cultured on a Matrigel basement membranes in the presence of no additive, a 10 fold excess concentration of hCG CR127 (10 times that produced by 5 000 cells or 19.8 ng for term cytotrophoblast monolayer cells, Table 2), or a 10 fold excess concentration of hyperglycosylated hCG C5 (10 times that produced by 5 000 cells or 19.8 ng for term cytotrophoblast monolayer cells, Table 2). As found, with term cytotrophoblast monolayer cells, 40% of cells (control cultures) naturally invaded the membrane (Table 4). By comparison, hCG CR127 inhibited invasion to (32±9.1)%. Hyperglycosylated hCG C5, in contrast, significantly enhanced membrane penetration to (76±13)% (t test, *P*=0.005 4).

JEG–3 choriocarcinoma cells, 5 000, were cultured on a Matrigel basement membranes in the presence of no additive, a 10 fold excess concentration of hCG CR127 (10 times that produced by 5 000 cells or 163 ng, Table 2), or a 10 fold excess concentration of hyperglycosylated hCG C5 (10 times that produced by 5 000 cells or 163 ng, Table 2). As found, 46% of cells (control cultures) naturally invaded the membrane (Table 4). By comparison, hCG CR127 inhibited invasion to (39±4.5)%. Hyperglycosylated hCG C5, in contrast, significantly enhanced membrane penetration to (84±6.2)% (t test, *P*=0.001 2).

Table 2
Total hCG concentration in culture fluids.

Cells	Count	Total hCG in medium (ng/mL)	Total hCG per 5 000 cells (ng)	Hyperglycosylated hCG in medium (ng/mL) (%)
JEG–3 cell line	7 979±954	13.00±1.30 in 2 mL	16.30	13.00±1.40 (100%)
Term cytotrophoblast, fresh	5 310±1,134	2.10±0.33 in 1 mL	1.98	2.20±0.17 (>100%)

Cells were cultured for 24 h in sextuplicate in defined medium (JEG–3 cells from 70% confluency flasks, sub–cultured, 5 000 cells per culture) and term cytotrophoblast cells, fresh, 5 000 cells per harvest), then cells counted and concentration of hCG determined. JEG–3 cells were cultured in T25 flasks with 2 mL medium and term cytotrophoblast cells in 60 mm dishes with 1 mL culture medium.

Table 3
Effect of hCG and hyperglycosylated hCG on cytotrophoblast cell growth.

Cell	Cell line and additive	Cell count after 24 h culture (% 5 000 cells)
Term pregnancy cytotrophoblast cells	No supplement	5 427±834 (109%)
	Hyperglycosylated hCG C5 added, 19.8 ng (10×)	7 633±777[a] (152%)
	With regular hCG CR127 added, 19.8 ng (10×)	4 227±769[b] (78%)
JEG–3 choriocarcinoma cell line	No supplement	7 114±553 (142%)
	Hyperglycosylated hCG C5 added, 163 ng (10×)	10 315±1 477[c] (206%)
	With regular hCG CR127 added, 163 ng (10×)	5 620±657[d] (79%)

Trophoblast cells, 5 000 total (term cytotrophoblasts were fresh cultures, JEG–3 cells were 70% confluent cultured cells subcultured), were cultured 24 h in sextuplicate in defined medium with added hyperglycosylated hCG C5 or regular hCG CR127, 10 times that concentration normally produced by 5 000 cells over a 24 h period, 81.0 and 19.8 ng, cells were then counted. JEG–3 cell were cultured in T25 flasks with 2 mL medium and term cytotrophoblast cells in 60 mm dishes with 1 mL culture fluids. [a]A significant difference observed compared to control, *P*=0.005 4; [b]No difference observed compared to control, *P*=0.28; [c]A significant difference observed compared to control, *P*=0.001 2; [d]No significant difference observed compared to control, *P*=0.26.

Table 4

Action of hyperglycosylated hCG and regular hCG on term cytotrophoblast cell invasion and choriocarcinoma cell invasion in Matrigel membrane chambers.

Cell	Cell line and additive	Mean penetration±standard deviation (%)
Term pregnancy cytotrophoblast cells	Control cultures	40±10
	Hyperglycosylated hCG C5, 19.8 ng (10×)	76±13[a]
	Regular hCG CR127, 19.8 ng/mL (10×)	32±9.1[b]
JEG–3 choriocarcinoma cells	Control cultures	46±9.8
	Hyperglycosylated hCG C5, 81 ng (10×)	84±6.2[c]
	Regular hCG CR127, 81 ng (10×)	39±4.5[d]

Isolated cytotrophoblast cells were prepared from fresh placenta. Isolated cytotrophoblasts and 70% confluent JEG–3 choriocarcinoma cells, were separately cultured 24 h (5 000 cells) on Matrigel basement membranes and control inserts in quadruplicate. Concentrations of hCG CR127 and hyperglycosylated hCG C5 used to promote invasion were 10 times that normally produced by the cells, 19.8 ng total for term pregnancy cytotrophoblast and 81.0 ng total for JEG–3 choriocarcinoma cells. The underside of Matrigel basement membranes, containing penetrated or invaded cells, was stained and counted. Cell penetration was compared with that of control inserts. The percentage penetration or invasion was calculated using the formula described by the manufacturer. [a]A significant difference observed compared to control, P=0.005 4; [b]No difference observed compared to control, P=0.28; [c]A significant difference observed compared to control, P=0.001 2; [d]No significant difference observed compared to control, P=0.26.

4. Discussion

As established, syncytiotrophoblast cell produce the hormone hCG which acts on an LH/hCG receptor[1–6,24–26]. Pregnancy cytotrophoblast cells, produce a hyperglycosylated variant of hCG, which controls growth and promotion of cytotrophoblast cells, and invasion by cytotrophoblast cells[2,6,24,25]. Hyperglycosylated hCG is seemingly an autocrine, which apparently antagonizes TGF beta activity[2,6,9]. As found, hyperglycosylated hCG binds a hCG/LH hormonal receptor. Hyperglycosylated hCG is commonly nicked at beta 47–48, so the effect of nicking on biological activity was first assessed. As found by regression analysis, nicked hCG reduces biological activity of hCG to 15.1% of un–nicked P8 pregnancy hCG. Hyperglycosylated hCG has an average of 48% of the biological activity of minimally hyperglycosylated hCG or P8 pregnancy hCG. Most hyperglycosylated hCG molecules are nicked and hyperglycosylated. Therefore, the proportion of nicking must be assessed along with hyperglycosylation, to estimate the hCG/LH receptor biological activity.

TGF beta antagonistic activity was assessed for hCG. Three hyperglycosylated hCG choriocarcinoma preparations were assessed along with three pregnancy preparations. The antagonistic activity was assessed by examining promotion of choriocarcinoma cell growth. This activity was directly related to the proportion of hyperglycosylation for carbohydrate structure studies[3]. Of the three choriocarcinoma preparations, C5 (the most hyperglycosylated) had the greatest antagonistic activity. None of the three pregnancy preparations had any antagonistic activity.

The action of C5 hyperglycosylated hCG on promoting cellular growth was investigated. JEG–3 cultured cells comprise only cytotrophoblast cells[2]. JEG–3 cultured cells like term cytotrophoblast monolayer cultured cells were significantly promoted to grow by hyperglycosylated hCG (P=0.002 1 and P<0.000 05). It is inferred that normal pregnancy term and choriocarcinoma cytotrophoblast cells are similar, and the growth of both cells are promoted by the autocrine activity of hyperglycosylated hCG. In contrast, hCG CR127 did not promote cell growth but seemingly inhibited or limited cell growth to 78% (term cytotrophoblast monolayer cells) and 79% (JEG–3) of no supplement values (P=0.027 and P=0.001 8).

The action of C5 hyperglycosylated hCG was investigated for promoting invasion through a Matrigel basement membrane. JEG–3 cultured cells like term cytotrophoblast monolayer cultured cells were significantly promoted to invade their way through a basement membrane by hyperglycosylated hCG (P=0.001 2 and P=0.005 4). It is inferred that normal pregnancy term and choriocarcinoma cytotrophoblast cells are similar, the growth of both are promoted by the autocrine activity of hyperglycosylated hCG. In contrast, hCG CR127 did not promote significant invasion through the basement membrane, but rather, inhibited or limited invasion to 85% (term cytotrophoblast monolayer cells) and 85% (JEG–3) of no supplement values (P=0.28 and P=0.26).

It has been inferred that hyperglycosylated hCG antagonizes TGF beta through its type II receptor. This is consistent with the known functions of TGF beta antagonists[11–18]. Hyperglycosylated hCG significantly promotes normal term cytotrophoblast and choriocarcinoma cell growth, and normal term cytotrophoblast and choriocarcinoma invasion. Other studies show that hyperglycosylated hCG controls pregnancy implantation at the blastocyst during pregnancy[24,25]. It is inferred that this implantation is part of the invasive function of hyperglycosylated hCG. Hyperglycosylated hCG also controls cytotrophoblast cell growth during pregnancy. This apparently represents placental growth during the course of pregnancy. As indicated, hyperglycosylated hCG promotes placental growth and hemochorial placentation during the course of pregnancy[26].

Hyperglycosylated hCG action in choriocarcinoma also involves promotion of cell growth and invasion or malignancy. As found, when JEG–3 choriocarcinoma cells are transplanted into nude mice, cells grow rapidly forming metastases. An antibody to hyperglycosylated hCG

completely blocks all growth and invasion[2], consistent with hyperglycosylated hCG alone promoting growth and invasion.

It is concluded that while the hormone hCG during pregnancy promotes progesterone steroidogenesis, uterine spiral artery angiogenesis, uterine growth, blocks phagocytosis and immuno–rejection of invading cells, and promotes fetal growth[27], hyperglycosylated hCG is a completely independent molecule, controlling root cytotrophoblast cell growth and invasion by cytotrophoblast cells.

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] Kovalevskaya G, Kakuma T, Schlatterer J, O'connor JF. Hyperglycosylated HCG expression in pregnancy: cellular origin and clinical applications. *Mol Cell Endocrinol* 2007; 260–262: 237–243.

[2] Cole LA, Dai D, Butler SA, Leslie KK, Kohorn EI. Gestational trophoblastic diseases: 1. Pathophysiology of hyperglycosylated hCG–regulated neoplasia. *Gynecol Oncol* 2006; **102**(2): 145–150.

[3] Elliott MM, Kardana A, Lustbader JW, Cole LA. Carbohydrate and peptide structure of the α– and β–subunits of human chorionic gonadotropin from normal and aberrant pregnancy and choriocarcinoma. *Endocrine* 1997; **7**(1): 15–32.

[4] Cole LA. O–Glycosylation of proteins in the normal and neoplastic trophoblast. *Troph Res* 1987; **2**: 139–148.

[5] Cole LA. hCG, its free subunits and metabolites in pregnancy and trophoblastic disease. *J Reprod Med* 1998; **43**: 3–10.

[6] Cole LA, Khanlian SA, Riley JM, Butler SA. Hyperglycosylated hCG (hCG–H) in gestational implantation, and in choriocarcinoma and testicular germ cell malignancy tumorigenesis. *J Reprod Med* 2006; **51**(11): 919–929.

[7] Cole LA, Shahabi S, Oz UA, Bahado–Singh RO, Mahoney MJ. Hyperglycosylated human chorionic gonadotropin (invasive trophoblast antigen) immunoassay: A new basis for gestational Down syndrome screening. *Clin Chem* 1999; **45**(12): 2109–2119.

[8] Cole LA, Khanlian SA, Sutton JM, Davies S, Stephens ND. Hyperglycosylated hCG (invasive trophoblast antigen, ITA) a key antigen for early pregnancy detection. *Clin Biochem* 2003; **36**(8): 647–655.

[9] Cole LA, Butler SA. Hyperglycosylated hCG, hCGβ and hyperglycosylated hCGβ: interchangeable cancer promoters. *Molec Cellul Endocrinol* 2012; **349(2):** 232–238.

[10] Hamade AL, Nakabayashi K, Sato A, Kiyoshi K, Takamatsu Y, Laoag–Fernandez JB, et al. Transfection of antisense chorionic gonadotropin β gene into choriocarcinoma cells suppresses the cell proliferation and induces apoptosis. *J Clin Endocrinol Metab* 2005; **90**(8): 4873–4879.

[11] Schuster N, Krieglstein K. Mechanisms of TGF–beta–mediated apoptosis. *Cell Tissue Res* 2002; **307**(1): 1–14.

[12] Liu YX, Gao F, Wei P, Chen XL, Gao HJ, Zou RZ, et al. Involvement of molecules related to angiogenesis, proteolysis and apoptosis in implantation in rhesus monkey and mouse. *Contraception* 2005; **71**(4): 249–262.

[13] Murphy G, Reynolds JJ, Whitham SE, Docherty AJ, Angel P, Heath JK. Transforming growth factor beta modulates the expression of collagenase and metalloproteinase inhibitor. *EMBO J* 1987; **6**(7): 1899–1904.

[14] Qureshi HY, Sylvester J, El Mabrouk M, Zafarullah M. TGF–beta–induced expression of tissue inhibitor of metalloproteinases–3 gene in chondrocytes is mediated by extracellular signal–regulated kinase pathway and Sp1 transcription factor. *J Cell Physiol* 2005; **203**(2): 345–352.

[15] Staun–Ram E, Shaleu E. Human trophoblast function during the implantation process. *Reprod Biol Endocrinol* 2005; **3**: 56–68.

[16] Fisher SJ, Cui TY, Li Z, Hartman L, Grahl K, Zhang GY, et al. Adhesive and degradative properties of human placental cytotrophoblast cell *in vitro*. *J Cell Biol* 1989; **109**: 891–902.

[17] Khoo NK, Bechberger JF, Shepherd T, Bond SL, McCrae KR, Hamilton GS, et al. SV40 Tag transformation of the normal invasive trophoblast results in a premalignant phenotype I Mechanisms responsible for hyperinvasiveness and resistance to anti–invasive action of TGFbeta. *Intl J Cancer* 1998; **77**(3): 429–439.

[18] Akhurst RJ. TGF–beta antagonists: Why suppress a tumor suppressor? *J Clin Invest* 2002; **109**(12): 1533–1536.

[19] Cole LA, Shahabi S, Butler SA, Mitchell H, Newlands ES, Behrman HR, et al. Utility of commonly used commercial human chorionic gonadotropin immunoassays in the diagnosis and management of trophoblastic disease. *Clin Chem* 2001; **47**(2): 308–315.

[20] Cole LA, Kardana A, Andrade–Gordon P, Gawinowicz MA, Morris JC, Bergert R, et al. The heterogeneity of human chorionic gonadotropin (hCG). III. The occurrence amd biological and immunological activities of nicked hCG, *Endocrinol* 1991; **129**(3): 1559–1567.

[21] Behrmann HR, Preston SL, Hall AK. Cellular mechanism of the antigonadotropic action of luteinizing hormone–releasing hormone in the corpus luteum. *Endocrinol* 1980; **107**(3): 656–664.

[22] Kliman HJ, Nestler JE, Sermasi E, Sanger JM, Strauss JF. Purification, characterization, and *in vivo* differentiation of cytotrophoblasts from human term placentae. *Endocrinol* 1986; **118**(4): 1567–1582.

[23] Kliman HJ, Feinman MA, Strauss JF. Differentiation of human cytotrophoblasts into synctiotrophoblasts in culture. *Trop Res* 1987; **2**: 407–421.

[24] Sasaki Y, Ladner DG, Cole LA. Hyperglycosylated hCG the source of pregnancy failures. *Fertil Steril* 2008; **89**: 1871–1786.

[25] Cole LA. Hyperglycosylated hCG and pregnancy failures. *J Reprod Immunol* 2012; **93**(2): 119–122.

[26] Cole LA. hCG and Hyperglycosylated hCG, Promoters of Villous Placenta and Hemochorial Placentation. Placenta: Functions, Development and Disease; New York: Nova Publishers; 2012 (in press).