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ABSTRACT

Total human chorionic gonadotropin (hCG) is high in maternal serum at 14-18 weeks of trisomy 21 (T21)-affected pregnancy, despite low placental hCG synthesis. We sought an explanation for this paradox. We first observed that in T21-affected pregnancies, maternal serum hCG levels peaked at around 10 weeks and then followed the same pattern throughout pregnancy as in controls, albeit at a higher (2.2-fold) level. After delivery hCG clearance was not significantly different from that in controls. We isolated cytotrophoblasts from 29 T21-affected placentas (12-25 weeks) and 13 gestational age- matched control placentas and cultured them for 3 days. In this large series we confirmed that in the culture medium of trophoblasts isolated from T21 placentas, hCG secretion was significantly lower (p <0.003) than in controls, in contrast to the high hCG in maternal serum of the same patients. In T21 cultured trophoblasts, transcripts of sialyltransferase-1 and fucosyltransferase-1 were abnormally high. In corresponding culture medium, hCG was abnormally glycosylated, highly acidic (pHi = 4.5) as shown by isoelectric focusing, immunoblotting and lectin binding and weakly bioactive (46% of control) as determined using the Leydig cell model. In conclusion, T21 trophoblast cells produced hCG that was weakly bioactive and abnormally glycosylated, but whose maternal clearance was unaltered.

INTRODUCTION

Trisomy of chromosome 21 (T21), which causes the phenotype known as Down syndrome, is the major known genetic cause of mental retardation and is found in around 1:800 live births. Screening strategies to identify women at increased risk of bearing a T21-fetus are based on maternal age, ultrasound signs (1) and maternal serum marker (2-5). Some of these markers, such as hCG, are of placental origin. When the fetus is trisomy 21-affected, it is now well established that total human chorionic gonadotropin (hCG) or its free ß subunit are raised (2 to 2.2 MoM) in maternal serum at 14-18 weeks of pregnancy. However, despite its widespread clinical use, the pathophysiology of this increase remains largely unknown.

hCG is composed of two subunits, one alpha common to all glycoprotein hormones (FSH, LH and TSH), and one specific beta subunit. The alpha subunit is a 92-amino-acid polypeptide with two N-linked oligosaccharides. The specific beta subunit is a 145-amino-acid polypeptide with two N-linked oligosaccharides and four O-linked oligosaccharides (6).

hCG is synthesized by the trophoblast, mainly by the syncytiotrophoblast, the outer layer of the chorionic villus (7). The syncytiotrophoblast is a very active endocrine unit, which secretes the vast majority of its hormonal products into the maternal circulation. The syncytiotrophoblast arises *in vivo (*8) and *in vitro (*9) from differentiation of cytotrophoblasts.

In trisomy 21, we recently confirmed previous observations by Eldar-Geva (10) that cultured cytotrophoblasts, isolated from trisomy 21-affected placentas, aggregate but fuse poorly or tardily (11). This is in agreement with previous macroscopic and histological observations pointing to an increase in the cytotrophoblast layer in trisomy 21 placentas (12-14). In addition, we demonstrated that this *in vitro* defect or

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delay in syncytiotrophoblast formation and function is characterized by a dramatic decrease in the synthesis and secretion of syncytiotrophoblastic pregnancy-associated hormones such as hCG, human placental lactogen (hPL), and human placental growth hormone (hPGH) (11,15). Similarly, we observed a significant decrease in mRNA levels of these hormones in trisomy 21 total placenta extracts, indicating a decrease in functional syncytiotrophoblast mass in trisomy 21-affected placenta (11).

In summary, trisomy 21-affected placenta presents morphological and functional anomalies, which result in a decrease in pregnancy-associated hormone production by the syncytiotrophoblast. Therefore, there is a discrepancy between the abnormally high maternal serum hCG values and the low placental rate of hCG synthesis. The aim of this study was to find an explanation for this paradox.

MATERIALS AND METHODS

Maternal sera and placental tissue collection

Since 1988, maternal serum samples have been collected at the Hôpital Ambroise Paré from women included in second-trimester trisomy 21 maternal serum screening. In addition, all pregnant women in France undergo first-trimester maternal serum screening for toxoplasmosis. During the third trimester blood samples were taken in the case of abnormal ultrasound findings. The resulting serum samples were stored frozen at -20°C. Moreover, since 1996 maternal serum samples have been collected from women prior to termination of trisomy 21-affected pregnancy. French law allows termination of pregnancy with no gestational age limit when severe fetal abnormalities are observed. Trisomy 21 was diagnosed by karyotyping. The

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indications for chorionic villus sampling, amniocentesis or fetal blood sampling were advanced maternal age, ultrasound abnormalities or abnormal maternal serum markers. Demographic and pregnancy-related information (maternal age, weight, and gestational age at sampling) were entered in a database.

Samples of placental tissues were collected at the time of termination of pregnancy at 12-25 weeks of gestation (in weeks of amenorrhea) in trisomy 21-affected pregnancies and gestational age-matched control cases. Gestational age was confirmed by ultrasound measurement of crown-rump length at 8-12 weeks of gestation. Fetal Down syndrome was diagnosed by karyotyping of amniotic fluid cells, chorionic villi or fetal blood cells. Termination of pregnancy was performed in control cases affected by severe bilateral or low obstructive uropathy or major cardiac abnormalities.The karyotype of placental cells was checked in all cases (free trisomy 21 or normal).

For maternal serum hCG clearance, maternal blood samples were taken at the indicated times after delivery (placental expulsion) in 9 T21 pregnancies (free T21) and in 8 gestational age-matched controls (second trimester of pregnancy, fetus with severe bilateral uropathy or major cardiac abnormalities and normal karyotype). Informed consent was obtained in all cases. The resulting serum samples were stored frozen at -20°C.

Trophoblast cell culture

Villous cytotrophoblast culture was undertaken as previously described (11). Cytotrophoblasts were isolated from gestational age-matched controls (second trimester) and trisomy 21-affected placentas and cultured for three days. For each culture, cells were plated on 3 dishes. The culture medium of each dish was changed

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every 24 hours, collected and assayed for hormonal concentrations. The mean value of triplicates was calculated and was representative of hCG secretion at 72 hours. This was done for the 29 T21 cultures and the 13 control cultures.

RNA isolation and analysis

Total RNA was extracted from placental tissues and cultured cells following the procedure of QIAGEN (Courtabeuf, France). The total RNA concentration was determined at 260 nm and its integrity was monitored by 1% agarose gel electrophoresis.

Beta hCG transcript levels were quantified in total placental homogenates as previously described (16). Sialyl-transferase mRNA and fucosyl-tranferase mRNA levels were measured in cultured cells. Three dishes were pooled for each determination by quantitative RT-PCR assay as previously described (16). The nucleotide sequences of the primers are the following:available upon request (mvidaud@teaser.fr). The levels of transcripts were normalized using the RPLP0 gene (also known as 36B4) encoding human acidic ribosomal phosphoprotein P0 as an endogenous RNA control. The 24 h conditioned culture medium was collected at day three and frozen.

Affinity chromatographies

Lectin-affinity chromatographies were used to analyze glycosylation of total hCG in culture medium: 1) Triticum vulgaris which recognizes the N-acetyl glucose and sialyl groups of glycoproteins; 2) Tetragonolobus purpureas which recognizes alpha-fucosyl groups; 3) Ricinus communis which recognizes ß-galactosyl groups; 4) Concanavalin A a lectin which recognizes biantennary structures but neither tri- or

tetra-antennary structures nor biantennary structures of glycoproteins. Samples were applied to 0.5 ml columns of lectin (10 ml/h, room temperature) as described by Baenziger and Fiete (17). The first fraction, not bound by the lectin, was eluted in the starting buffer and the second fraction (lectin-bound) with starting buffer supplemented with 0.2 M of the lectin-specific ose. HCG was assayed in each fraction.

Isoelectrofocusing (IEF) was performed as previously described (18) using a monoclonal antibody against free ß-hCG (Ab FBT10, generous gift of Pr. J-M Bidart, Institut Gustave Roussy, Villejuif, France) (19).

Hormone assay

Total hCG was measured in culture media and maternal serum using the ACS180SE instrument (Bayer, Fernwald, Germany).

hCG biological activity assay

Biological activity of secreted hCG was tested on Leydig cells (MA.10 cells, generous gift of Pr. M. Ascoli, University of Iowa, Iowa City, USA) as previously described (20). hCG levels were first assayed in the culture medium of trophoblasts. Different amounts of culture medium were added for 4 h to 2.10^6 MA-10 cells cultured on gelatin-coated 30 mm dishes. Progesterone was then assayed in the MA-10 cell incubation medium. Results were expressed as progesterone concentration per number of cells for each tested concentration of hCG present in the control and T21 trophoblast culture medium. Progesterone was assayed using the ACS180SE instrument (Bayer, Fernwald, Germany).

Statistical analysis

Statistical analysis was performed using the Statview F4.5 software package (Abacus Concept, Inc ; Berkleley, CA, USA). Values are presented as the mean± SEM. Comparisons were performed using Student's t-test. $p < 0.05$ was considered significant.

RESULTS

Maternal serum hCG levels

Maternal serum hCG levels were analyzed throughout pregnancy in a large cohort of control and T21-affected pregnancies (figure 1). We confirmed that, in trisomy 21 cases, hCG levels were increased during the periods 7-13 weeks and 14-18 weeks. In addition, we observed that the hCG levels peaked around 10 weeks in both trisomy 21 and control cases, with no gap between the two peaks. Moreover, we noted that this increase continued during the third trimester. The discrepancies between MoMs in normal (1 MoM) and trisomy 21-affected cases (2.16 MoM) were approximately constant throughout gestation.

Trophoblast culture

We confirmed in a large series of primary cultures the significant ($p = 0.003$) decrease in hCG secretion in the culture medium of trophoblast cells isolated from T21 placentas (n = 29, mean ± SEM: 337 ± 62 IU/L) as compared to gestational age-matched control placentas (n = 13, mean ± SEM: 930 ± 246). Likewise, as previously described (11), we confirmed in this series that in the total homogenates of the

corresponding placentas the transcript levels of beta hCG measured by real-time quantitative RT-PCR were significantly decreased (p<0.01) in T21-affected placentas (mean ± SEM: 4139 ± 1554) as compared to controls (mean ± SEM: 19494 ± 5812). In contrast, hCG levels in maternal serum corresponding to these placentas were significantly higher (p <0.0001) in trisomy 21-affected pregnancies (median of MoM = 2.3) than in control pregnancies (median MoM = 1.0).

Maternal serum hCG clearance

We studied the disappearance of hCG in the maternal serum after delivery and therefore after expulsion of the placenta. The percentage of hCG remaining in maternal circulation at different times after delivery (table 1) was not significantly different between normal and T21-affected pregnancies.

hCG bioactivity

We tested the ability of culture medium from normal and trisomy 21 trophoblasts to stimulate steroid production in Leydig cells possessing LH receptors, which bind hCG. At equal concentration of hCG in the culture medium (10^{-10} M), the ability of culture media of trisomy 21 trophoblasts to stimulate Leydig cell progesterone secretion was significantly (p < 0.0008) decreased (figure 2).

Trophoblast glycosylation activity

mRNA levels of two enzymes involved in the glycosylation pathway, sialyl-transferase-1 (which adds a sialyl group to antennary structures) and fucosyl-transferase-1 (which adds a fucose to the first N-acetyl-glucosamine of glycoproteins), were significantly higher (p <0.05) in cultured trophoblasts isolated

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from trisomy 21 placenta (n = 5) than in control cases (n = 5) (mean ± SEM: 27 ±15 vs 2.5 ± 1 and 9 ± 3 vs 3 ± 1, respectively). Lectin binding differed between hCG secreted by normal and trisomy 21 trophoblast cultures (table 2). IEF and immunobloting (figure 3) of hCG present in culture medium from normal trophoblasts revealed a band with a pHi at 7.3, whereas in culture medium from T21 trophoblast a more acid band (pHi = 4.5) was present.

DISCUSSION

The major endocrine component of human placenta is the villous trophoblast and mainly the syncytiotrophoblast, which arises *in vivo* and *in vitro* from fusion of mononucleated cytotrophoblasts. We previously demonstrated *in vitro* (11, 15), and confirmed in the present study in a large number of primary trophoblast cultures (n = 29), that in trisomy 21-affected placenta formation of syncytiotrophoblast is defective or delayed. The genetic overexpression of copper-zinc superoxide dismutase is involved in this abnormal trophoblast fusion and differentiation (16). These results are in agreement with previous macroscopic and histological observations of T21-affected placentas which reveal delayed maturation of chorionic villi and syncytiotrophoblastic hypoplasia with a persistent cytotrophoblastic layer in the third trimester (12,14). They point to a decrease in the syncytiotrophoblast mass in T21-affected placentas and therefore to a decrease in hCG synthesis. We confirmed in this study our previous observation of a significant decrease in hCG transcript levels in total placental homogenats from T21-affected placentas as compared to gestational age-matched controls. These results do not agree with previous observations of different authors describing increased or no change in hCG transcript levels or protein levels. Eldar-Geva (10) reported an increase in hCGα and hCGβ

mRNA in trophoblast cells of T21-affected placenta. This was based on a northern analysis performed with cells cultured for 24hours. In this study the secretion of hCG at 24 hours is extremely variable from one T21 cell culture to another one. In addition the secretion of hCG at 24 hours in normal cells or in T21 cells is higher than the one observed at 72h and 96h. This suggests a contamination of cytotrophoblast cell cultures by fragments of syncytiotrophoblast, which explains the highest transcript levels of hCG. Indeed it is now well established that hCG transcript levels and hCG secretion are higher in the syncytiotrophoblast as compared to the cytotrophoblasts (9, 21-24). Jauniaux *et al* (25) and Newby *et al* (26) have compared the levels of hCG and/or of its free subunits, detected by immunoassay in placental total homogenate extracts. This is a different methodological approach, focused on the protein levels and thereby depending upon the specificity of the antibodies. In addition total placental extracts are contaminated by maternal blood which may lead to artefactual increase in hCG levels and therefore do not directly reflect the trophoblast synthesis of hCG. In addition, Newby *et al*, did not find an increase in hCG by immunohistochemistry.

The defect in trophoblast differentiation pointing to a delayed placental maturation in T21 prompted us first to check that the increased hCG levels observed during first- and second-trimester maternal screening are not related to a delay in the physiological peak of hCG in the maternal circulation. In normal pregnancies, hCG concentrations rise rapidly in maternal serum, peaking at 9-10 weeks of gestation, followed by a fall during the second trimester. The factors involved in the peak of hCG during pregnancy are widely debated and remain to be elucidated (see 27 for review). Maternal serum hCG peaked in trisomy 21-affected pregnancies at exactly

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the same time (10 weeks) as in normal pregnancies and these high levels of hCG persisted throughout pregnancy in the case of trisomy 21.

The discrepancy between decreased placental hCG production and high maternal serum levels in T21-affected pregnancies points to an abnormal clearance of this hormone in the maternal-placental compartment. Maternal serum hCG clearance depends on maternal hepatic catabolism, renal excretion, ovarian uptake and placental uptake. In this study we observed that after placental expulsion, the disappearance of hCG in the maternal serum was similar in normal and T21-affected pregnancies. This suggested that in the absence of placenta, the clearance of hCG in the maternal compartment was the same. Therefore this pointed to an abnormal placental hCG clearance in T21-affected pregnancies. In addition, we observed for the first time that hCG produced by T21-affected trophoblasts had a low bioactivity, as shown by the well- established Leydig cell assay (20). Therefore, hCG produced by T21-affected trophoblasts will have little ability to stimulate the LH/hCG receptors in its main target organs, and therefore in the placenta. Indeed, the placenta is an organ which possesses a large number of hCG receptors (28) and hCG plays a major role via an autocrine process in trophoblastic differentiation (29-31). Furthermore, the placenta presents a large surface of exchange due to the presence of microvilli at the surface of the syncytiotrophoblast bathing in the maternal blood. This favors the capture of circulating maternal hCG by trophoblast receptors (13). Therefore, the increased maternal hCG levels observed in T21-affected pregnancies might be related to different abnormalities: 1/ a decreased number of hCG receptors in T21-affected placentas or an abnormality of these receptors. hCG receptors in T21-affected placentas are poorly known. However, an increase in receptor gene

expression has been described in these placentas (25). 2/ a decreased binding and/or internalization of this low bioactive hCG secreted by T21-affected trophoblast.

HCG is a complex glycoprotein hormone whose molecular size and carbohydrate content change in physiological processes (gestation) or in pathological conditions such as choriocarcinoma (32-35). Based on the use of a monoclonal antibody (B152 hCG) raised against the hyperglycosylated choriocarcinoma hCG, a hyperglycosylated isoform produced by the first-trimester cytotrophoblast cells (but not syncytiotrophoblast) can be detected in the maternal blood and urine only during the first 6-7 weeks of pregnancy (36-38). Cole *et al*. investigated the composition of the N-linked and O-linked oligosaccharide side chains in hCG. In Down syndrome pregnancies, hyperglycosylated hCG (H-hCG), also called invasive trophoblast antigen (ITA), was found in maternal serum and urine in higher proportion than in controls, and its measurement was proposed as a potential alternative to hCG in Down syndrome screening during the first or second trimester (39-46).

In the present work, for the first time, hCG glycosylation was studied comparatively at the source of hormone production, ie cultured trophoblast cells from age-matched second-trimester control and T21-affected placentas. The demonstration of an abnormally glycosylated hCG synthesized by trisomy 21 placental cells was based on: 1) fucosyl-transferase-1 and sialyl-tranferase-1 transcript increases in T21 trophoblast cells; 2) different binding of the secreted hCG to different lectins, 3) detection by IEF of a highly acidic form of hCG in the T21 cell culture medium. The IEF pattern reflects the heterogeneity of the charged sugar (sialic acid), which varies with the multi-antennarity and/or moiety of the N- or O-oligosaccharide chains in which sialic acid is the terminal sugar. Glycosylation

modification of polypeptide hormones is known to modulate their activity on target cells. Interestingly, this T21-hCG had a low bioactivity as compared to hCG secreted by gestational age control trophoblasts.

In conclusion due to the pleiotropic role of this hormone in human pregnancy, maintenance of corpus luteum, trophoblast differentiation, endometrium vascularization, etc. (7,47) these results suggest that this abnormal highly glycosylated weakly bioactive hCG might be implicated in the highly frequent spontaneous abortion observed in this aneuploidy.

Acknowledgements

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Legends of figures

Figure 1. Transversal study of maternal serum levels as a function of gestational age of total hCG (control cases: n = 894; trisomy 21: n = 499)**.**

Empty circle = controls; black triangle = trisomy 21-affected pregnancy. Each point represents a single measurement for one patient. Inset shows median values.

Figure 2. Bioactivity of hCG secreted by normal and T21 trophoblast cells. hCG produced by trophoblast cells was measured by immunoassay in conditioned media from cultures of control (n = 5) and T21 trophoblasts (n = 5). Different volumes of these media corresponding to the indicated concentrations of hCG were added to MA-10 Leydig cell culture. Progesterone was assayed in MA-10 culture media 3 hours later.

$*p < 0.05$; $***p < 0.001$

Figure 3. hCG isoforms secreted by normal (n = 3) and T21 trophoblast cells (n = 3) as revealed by isofocusing and immunoblot.
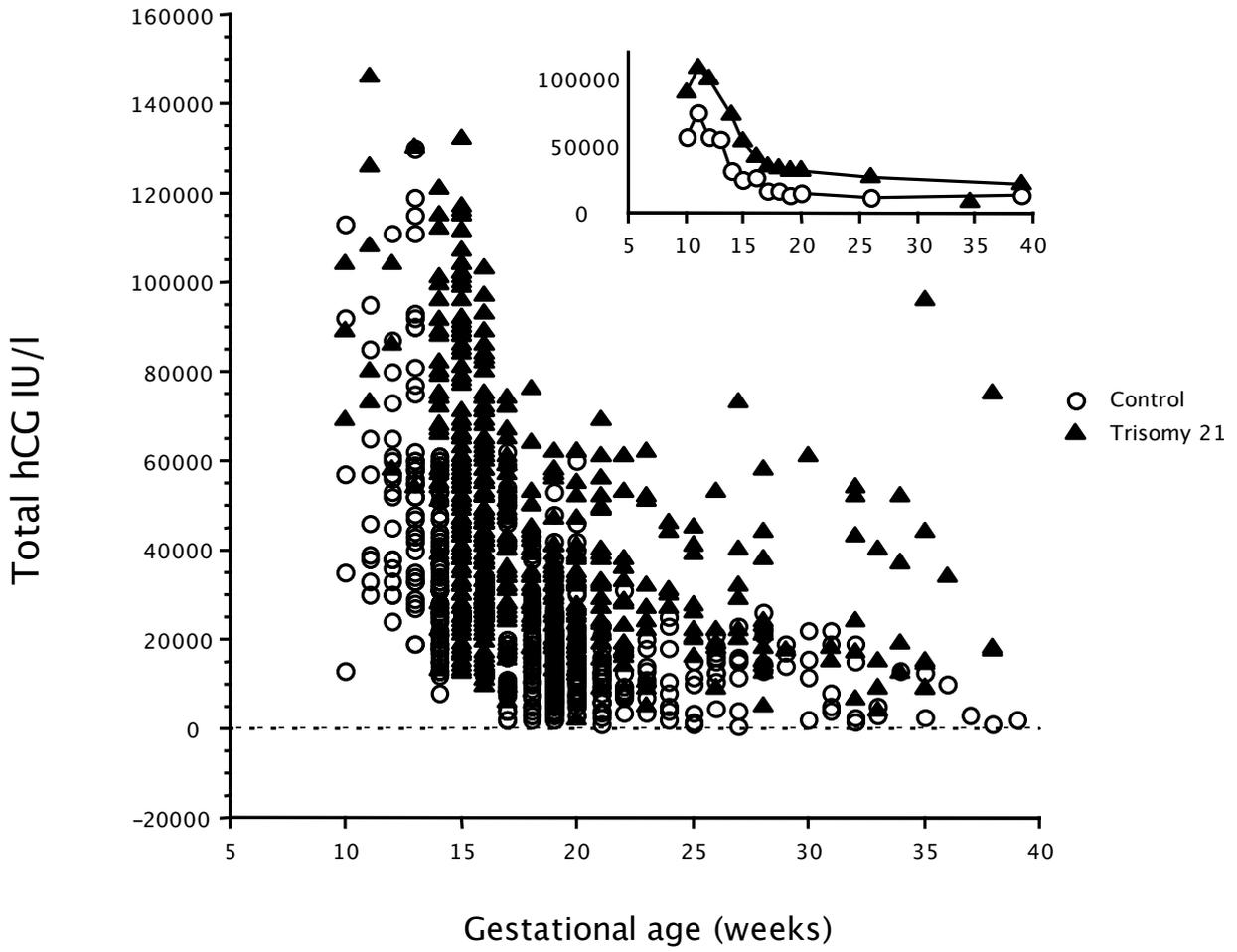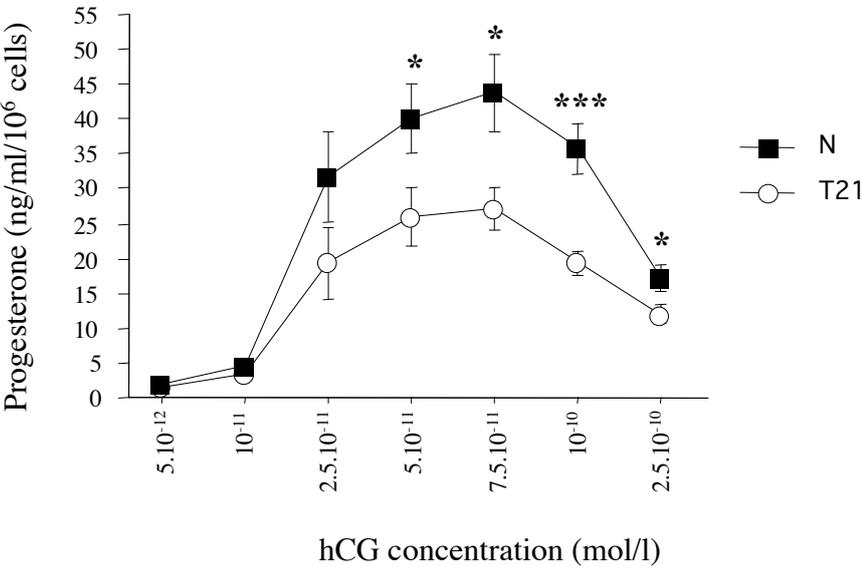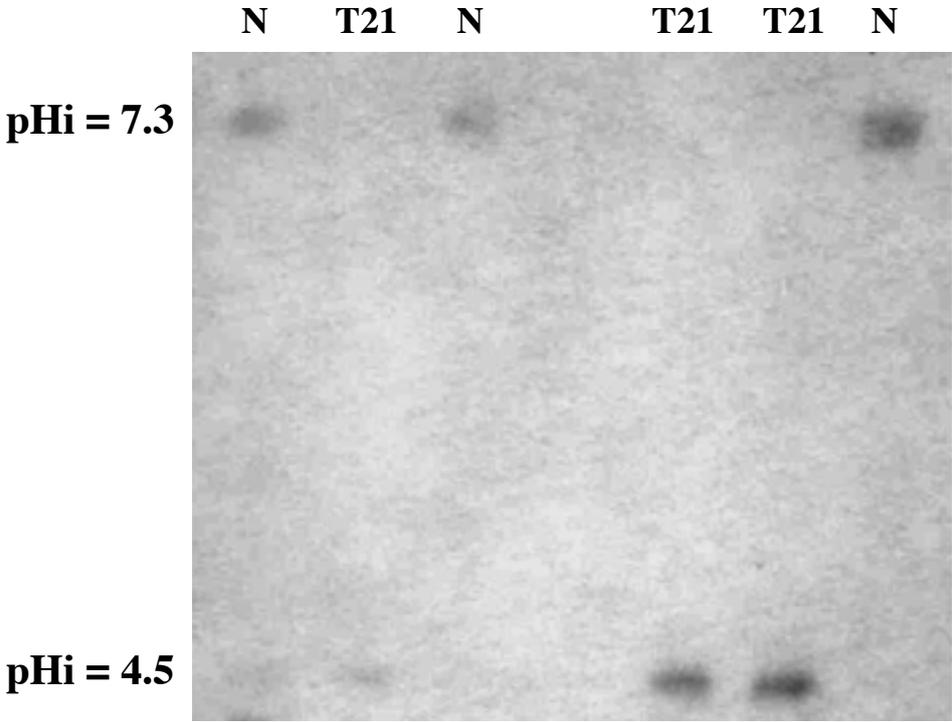
Figure 1

Figure 2

Figure 3

Hours	Control	T21
3	63% ±11 (n = 3)	48% ±9 (n = 3)
6	51% ±17 (n = 5)	45% ±0.03 (n = 3)
12	37% ±12 (n = 8)	28% ±6 (n = 9)
24	22% ±9 (n = 7)	17% ±5 (n = 9)

Table 1. Percentage of hCG in maternal sera at different times after delivery as compared to the values observed 24 h before expulsion.

Lectin	Control	Trisomy 21
Triticum vulgaris	89.5% ±4.8	93.9% ±3.5
Tetragonolobus purpurea	1.4% ±0.5	7.7% ±3.6 *
Ricinus communis	5.7% ±1.4	18.8% ±6.5 *
Concanavalin A	96.8% ±1.8	81.6% ±2 *

Table 2. Glycosylation analysis of hCG in culture medium of 5 different
72-hour trophoblast cell cultures.
Lectin-bound fractions are expressed as a % of the total fraction (mean ± SD).
 * $p < 0.05$