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PHYSIOLOGY

Capacity for Hormone Production of Cultured Trophoblast Cells Obtained from Placentae at Term and in Early Pregnancy

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Problem: *There is an increased doubt about the identity of isolated cytotrophoblast cells at term. Therefore, we compared pregnancy serum levels of three hormones [human placental lactogen (hPL), human chorionic gonadotropin (hCG), and leptin] with the capacity for hormone production of early placentae [EP; 8–13 weeks of gestation (WG)] and term placentae (TP; 38–42 WG).*

Methods: *Serum levels of these hormones were determined in 15 paired maternal (7–41 WG) and fetal (37–41 WG) samples. Cytotrophoblast cells were isolated from term (TP; 38–42 weeks) and early (EP; 8–13 weeks) placentae by enzymatic digestion and subsequent purification on a Percoll gradient. These cells were cultured for 6 days. The production of the hormones hPL, hCG, and leptin was determined as release during culture + cell content after culture − cell content before culture.*

Results: *Serum levels (mean ± SD;* n = *15) at 7–12 and 37–41 WG were 89,652 ± 21,431 and 13,620 ± 5854 mIU/ml for hCG, 400 ± 182 and 7088 ± 2030 ng/ml for hPL, and 12,675 ± 4266 and 32,236 ± 10,961 pg/ml for leptin, respectively. For cultured cells from EP and TP, hCG and hPL showed different patterns of release during the first 2–3 days. While the release of these two hormones by EP cytotrophoblast cells continued during 6 days in culture, their concentrations reached a plateau for TP cytotrophoblasts between 4 and 6 days. Leptin was undetectable (<15 pg/ml) in TP cell cultured media, while for EP all three hormones showed the same release profiles. Production calculated for 30,000 TP trophoblast cells cultured for 6 days (*n = *8) was 2–31 mIU for hCG and 0.5–2 ng for hPL. For EP (*n = *11), it was 50–1070 mIU for hCG, 15–323 ng for hPL, and 137–580 pg for leptin. Net synthesis of hCG and hPL for TP was >10-fold and <1-fold, respectively. In contrast, the production of all three hormones for EP was at least 100 times the initial cell content.*

Conclusions*: These results demonstrate that trophoblasts from early pregnancy show much higher production rates of hCG, hPL, and leptin than at term. However, the in vitro findings are difficult to be reconciled with the different serum concentrations of the two hormones hPL and leptin observed during the course of pregnancy.*

KEY WORDS: hPL; hCG; leptin; production; human; serum; first trimester; third trimester; trophoblast culture.

INTRODUCTION

Two major protein hormones of the human placenta, human chorionic gonadotropin (hCG) and human placental lactogen (hPL), show different concentration patterns in maternal serum throughout pregnancy. hCG reaches peak concentrations between 8 and 12 weeks of gestation, followed by a decrease during the second trimester, with a plateau in the third trimester (1). In contrast, hPL increases continuously from the third week of gestation, reaching the highest concentration during the third trimester (2).

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Leptin is a circulating hormone produced by adipose tissue and involved in the regulation of energy expenditure, food intake, and adiposity (3,4). In human pregnancy leptin, as a novel placenta-derived hormone, showed an increase in serum levels during the second trimester (160% of the first trimester) and remained high during the third trimester (5). The increased level of leptin in the serum of pregnant women is due mainly to the placenta because the gene expression of leptin throughout gestation remained at a high level, and at term its expression was similar to that in adipose tissue (6), and plasma leptin levels postpartum (24 hr after placental delivery) were reduced below those measured during the first trimester (5).

These observations indicate that the synthesis of these hormones is independently regulated in the first- and the third-trimester trophoblast.

The current study was undertaken to evaluate the differences in hormone production between early and term trophoblasts and their relevance to in vivo observations. The release and production of hCG, hPL, and leptin by cultured cytotrophoblast cells were determined.

PATIENTS, MATERIALS, AND METHODS

Serum levels of hormones were determined in 15 paired maternal and fetal samples collected as described previously (7). Consecutive maternal vein (MV) blood samples from a peripheral vein were collected at approximately 10, 20, and 30 weeks of gestation and at term. Fetal samples were collected from the umbilical vein (UV) and umbilical artery (UA) at term within 5–10 min of delivery. All deliveries ($n = 15$) were at term and resulted in a single neonate with an appropriate birth weight for gestational age without any sign of infection.

Isolation of Trophoblast Cells

Trophoblast cells were isolated from first-trimester placental tissue obtained from women undergoing elective therapeutic abortion between 7 and 13 weeks of gestation under informed consent, and from term placentae following uncomplicated cesarean section, and collected within half an hour of delivery. A modification of the method of Kliman *et al.* was used (8). The villous tissue was separated from the decidual plate and fetal membranes, minced and washed thoroughly with cold saline, and digested with trypsin (Sigma T-8918) in a shaking water bath at 37°C for 1.5 hr. After this period DNase I (Sigma) was added and the incubation continued for a further 30 min. The dissociated cells were collected by filtration through four layers of surgical gauze and centrifuged at 1000*g* for 10 min. The resulting cell pellet was washed with HBSS, layered onto 10 ml of fetal calf serum (FCS), and centrifuged at 1000*g* for 10 min. The cells were collected, washed with HBSS, and then layered onto a discontinuous (5% steps) Percoll (Pharmacia) gradient made up of 8-ml steps from 50 to 25% and centrifuged for 30 min at 1200*g*. The appropriate cell bands were collected and washed in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL). The viable cell number was determined using trypan blue exclusion in a standard hemocytometer.

Cell Culture

Previously, we were able to isolate three cell bands from term placentae, with densities of 1.051, 1.058, and 1.063 g/ml, which were postulated to be predominantly cytotrophoblast cells at different stages of development (9). As these cells mature their cytoplasmic volume increases and thus their density decreases. The cytotrophoblast cells with the highest density (1.063 g/ml) show a maturation process in culture, with a decrease in their density as evidenced by reapplication onto a Percoll gradient. We assumed that the cytotrophoblast cells isolated from the Percoll gradient fraction with $D = 1.063$ g/ml are at the earliest stage of development. In addition, these cells have shown the highest rate of hormone release in culture compared to the other cells isolated at a lower Percoll density. The same characteristics were found for the cytotrophoblast cells isolated from first-trimester placentae. Therefore, in the current study only the cells with the highest density (1.063 g/ml) were used.

After isolation, 2×10^5 viable cells per well were plated in 24-well dishes in 1.8 ml of medium. The culture medium used was DMEM containing 2% Ultroser-G (Gibco-BRL), gentamycin (50 μg/ml), streptomycin (100 μg/ml), and penicillin (100 U/ml). Cells were cultured for 6 days at 37°C in humidified 5% CO_2/95% air. Samples of spent culture medium (0.25 ml) were collected every 24 hr. At the end of the culture period the cells were solubilized in 5% Triton X-100 in DMEM for 30 min. The lysates collected were centrifuged for 10 min at 1200*g* and the supernatant was stored at −20°C until assayed.

Analyses

To determine the homogeneity of the cells obtained, immunocytochemistry was performed with an anticytokeratin antibody (1:2; clone CAM5.2; Becton & Dickinson). Primary antibody binding was detected using an appropriate biotinylated secondary antibody (1:200; Dako) followed by an avidin–biotin–phosphatase complex (ABC Kit; Dako). Phosphatase activity was detected using a New Fuchsin Substrate solution (10) and counterstained with hematoxylin. Immunostaining specificity was confirmed by the appropriate negative controls (i.e., replacing the primary antibody with nonimmune IgG).

Samples were assayed for hCG, hPL, and leptin using standard enzyme-linked immunosorbent assay (ELISA) methods developed in our laboratory as described previously for hCG and hPL (11). For leptin, the method was as follows: microtiter plates (Maxisorp; Nunc, Denmark) were coated with monoclonal anti-OB (leptin) (clone 44802; R&D Systems, USA) at 2 μg/ml in PBS. The plates were incubated overnight at 4°C, and the excess protein binding sites were blocked, after aspiration of the coating solution, with PBS containing BSA (Fluka, Switzerland; 0.5%, w/v) for 2 hr at ambient temperature. Then the wells were washed with PBST, i.e., PBS containing Tween-20 (Sigma, USA; 0.1%, v/v). One hundred microliters of standards or samples, diluted in Blotto (Pierce, USA), was added in duplicate to the wells and the plate was incubated at 30°C for 2 hr, with a shaking speed of 500 rpm. The standard was recombinant human leptin (R&D Systems), and the final concentrations of between 1000 and 15.6 pg/ml were prepared by serial 1:2 dilution in seven steps. The blank was Blotto alone. The plates were then washed three times with PBST. The second (detection) antibody was biotinylated anti-human leptin (clone 44804; R&D Systems), used at a concentration of 250 ng/ml in Blotto (100 μl/well). Incubation conditions were as above, followed by a wash step with four cycles of PBST. Then the conjugate horseradish peroxidase–streptavidin (Southern Biotechnology Associates, USA; diluted 1:4000 in Blotto) was added and the plate incubated for 45–60 min under the same conditions and washed as above (four cycles). For the development, 100 μl of ready-to-use TMB substrate solution (Zymed Inc., USA) was used in a timed sequence. After 15–30 min of incubation in the dark at room temperature, the reaction was stopped by the addition of 100 μl 2 *M* hydrochloric acid, the plates were mixed on a shaker, and the optical densities were determined, using a two-channel microplate reader (Bio-Rad 550), at 450-nm against a 590-nm reference. This assay is highly specific due to double monoclonal antibody use; the sensitivity is 10 pg/ml or less, and the intra- and interassay coefficients of variance at 125 pg/ml are 2.74 and 8.89%, respectively.

The production of hCG, hPL, and leptin was calculated as the difference between the sum of the total release into the medium during culture plus the cell content after culture and the cell content before culture.

Serum Hormone Levels

The release capacity into the fetal circulation was calculated as the difference between the umbilical vein and the umbilical artery level, expressed as a percentage of the maternal vein level. They were determined as an in vivo control parameter in 15 paired maternal and fetal samples.

Statistics

The Mann–Whitney test was used to estimate the statistical significance of the differences between groups.

RESULTS AND DISCUSSION

Serum Study

Maternal hormone levels of hCG, hPL, and leptin showed significant changes throughout gestation (Table I), with a drop in serum concentrations for hCG and an increase in levels for hPL and leptin, which are in line with previous observations (1–4). In addition, there were differences in the placental release capacity into the fetal circulation; hCG (0.13%) < hPL (0.36%) < leptin (22%). These observations were similar to those seen recently under in vitro conditions in placental perfusion (12). Regarding the release by placental tissue of leptin, which is much higher than for the other two hormones, a specific mechanism has to be involved because all three hormones are considered to be produced at the same placental site (syncytium), and, moreover, there is considerable structural similarity between hPL and leptin, in their molecular size and absence of glycosylation. In addition, when the umbilical vein–umbilical artery difference was

Table I. Serum Level of Hormones[a]

	Maternal vein level (MV; $n = 15$)				Fetal level (37–41 WG; $n = 15$)			Release capacity[b]
	7–12 WG	16–21 WG	26–30 WG	37–41 WG	UV	UA	UV − UA	
hCG (mIU/ml)								
Mean	89,652	36,360**	17,049**	13,620**	38	29	9	0.13
SD	21,431	16,190	7,919	5,854	16	15	5	0.13
hPL (ng/ml)								
Mean	400	1,665**	4,494**	7,088**	59	38	22	0.35
SD	182	581	1,354	2,030	29	29	12	0.21
Leptin (pg/ml)								
Mean	12,675	15,891*	21,395**	32,236**	15,772	9,817	5,955	22
SD	4,266	6,173	8,364	10,961	7,850	6,301	2,638	13

[a] WG, weeks of gestation; UV, umbilical vein; UA, umbilical artery. MV groups vs MV (7–12 WG): * $P < 0.01$; ** $P < 0.001$.
[b] UV − UA/ % of MV (37–41 WG).

related to the umbilical artery, values of over 60% were obtained, indicating that most of the umbilical circulating leptin was not of fetal origin.

Cell Culture Study

For first-trimester cytotrophoblasts, hCG, hPL, and leptin showed a biphasic concentration course with an initial release rate (day 1 to day 3) lower than that observed in the following period of culture (day 3–day 6), during which the concentration of all three hormones showed a continuous increase (Figs. 1a–c). In contrast, when cytotrophoblast cells were obtained from term placenta there was a linear increase in hCG and hPL concentration between day 1 and day 4, with a plateau in the last 3 days (Figs. 1d and e), indicating that these cells had stopped releasing these two hormones after 4 days in culture. Leptin could not be detected in the media of cultured term trophoblast cells (<15 pg/ml). In addition to the release of the hormones, their production was quantified by comparing the cell content before and after 6 days in culture with 30,000 first-trimester and term trophoblast cells (Table II). In the first trimester, the cell content of all hormones showed a substantial increase after culture. The production capacity, expressed as the ratio of the production to the initial cellular content, was 101, 105, and 173 for hCG, hPL, and leptin, respectively. In contrast, after 6 days in culture term cytotrophoblast cells showed a substantial drop in cell content. Although term cells showed a capacity for hCG production more than 10-fold above the initial cell content, there was much less such capacity for hPL production. The higher capacity for production of leptin and hPL by first-trimester cells, together with the lower capacity for hPL production than for hCG production by, and the absence of leptin detection in, third-trimester cells, is not in agreement with what would be expected from the serum concentrations of these hormones in early and late pregnancy. A possible explanation for the diminished capacity of term trophoblasts in culture to synthesize these hormones could be the increased presence of syncytial fragments, which has been suggested by Huppertz *et al.,* together with a cytotrophoblast fraction accounting for less than 10% at term (13). However, further morphological as well as

Table II. Cell Content, Release, and Production of hCG, hPL, and Leptin Observed Before and After Culture of 30,000 Trophoblast Cells

	Cell content			
	Before	After	Release	Production
(A) First-trimester culture				
hCG (mIU); $n = 11$				
Mean	4.81	22.6	470.1	487.9
±SD	2.11	24.6	257.1	262.7
hPL (ng); $n = 11$				
Mean	1.23	7.34	123.4	129.5
± SD	0.71	7.12	94.4	99.4
Leptin (pg); $n = 6$				
Mean	1.65	3.81	283	285
±SD	0.71	0.68	83	83
(B) Third-trimster culture				
hCG (mIU); $n = 8$				
Mean	0.90	0.73	9.25	9.08
± SD	0.81	0.69	8.80	8.67
hPL (ng); $n = 8$				
Mean	0.97	0.43	1.45	0.91
± SD	0.37	0.28	0.43	0.39

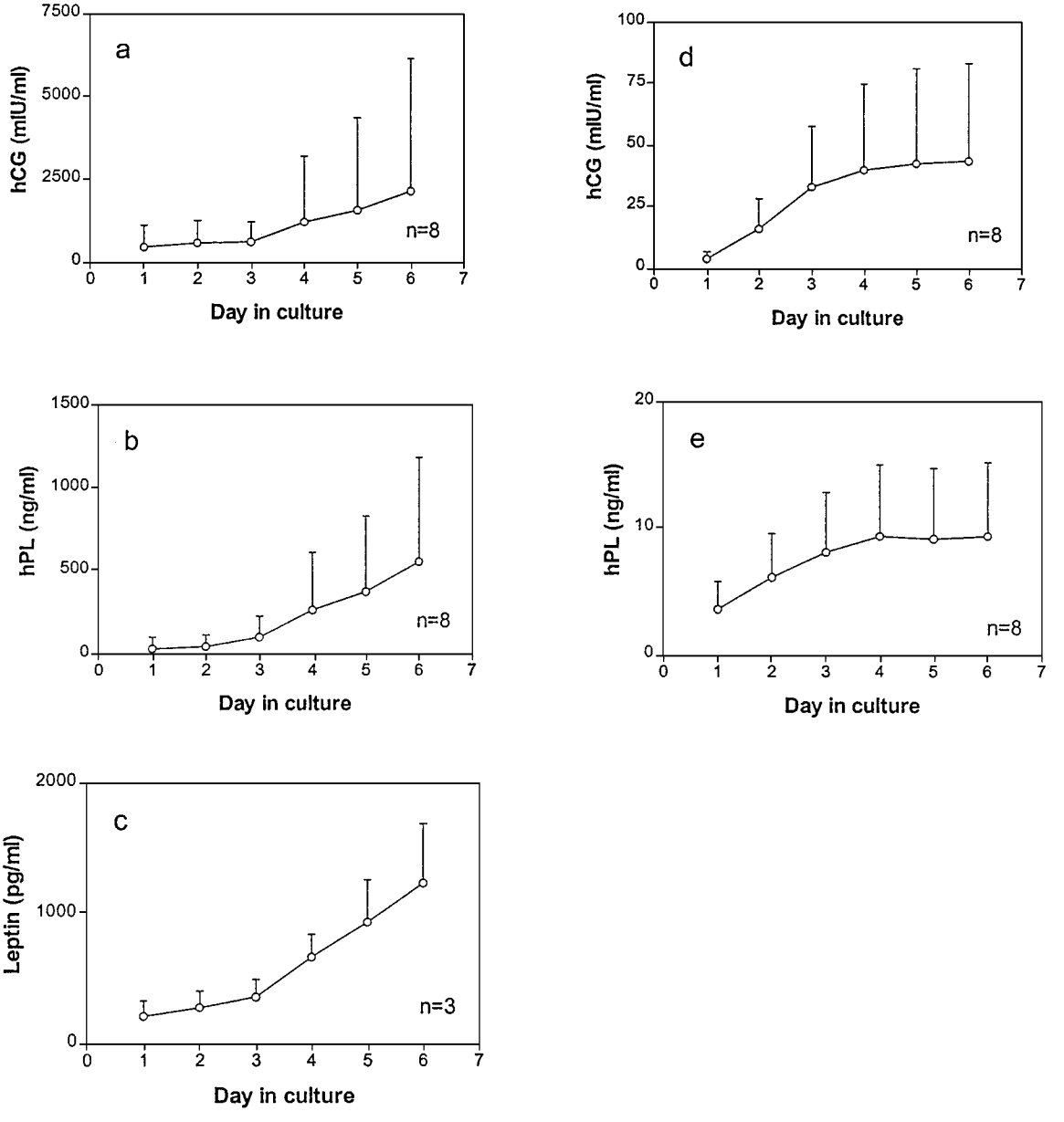

Fig. 1. Time course of protein concentration reflecting secretion during cytotrophoblast culture of 2×10^5 cells in 1.8 ml of medium. Values are means ± SD of first-timester (a–c) and third-timester (d, e) cultures. Please note the difference in scale for hCG and hPL; leptin in the medium of the third-trimester culture was below the detection limit.

biochemical investigations will be required to clarify this point.

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