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Characterization and differentiation of human first trimester placenta trophoblastic cells in culture

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Summary — A preparation of highly enriched isolated cytotrophoblasts was obtained from first trimester placenta using dispase incubation of villous tissue at 4 °C, followed by a spontaneous cell release at 37 °C. After 24 h of culture, 90–95% of the cells were immunostained by anticytokeratin antibody, showing their epithelial characteristic. After 48 h of culture, these cells differentiated into syncytiotrophoblast, as shown by optic and electron microscopic study. The secretion of hCG, and of its free α and β subunits, and the secretion of hPL were studied as a function of cell culture time. While the level of secreted hCG and its free subunits was stable during 72 h of culture, the hPL level was undetectable during the first 48 h of culture, increasing continuously afterwards. Addition of dibutyryl cAMP from the start or after 96 h of cell culture induced an increase of hCG production and of its free subunits and also stimulated the secretion of hPL. This suggests that these cells maintained the capacity to respond to stimuli which increased intracellular cAMP level. Such a cell culture is of interest in further determining the mechanisms of early gestation involved in the differentiation and growth of placental cytotrophoblasts, and in the regulation of their endocrine functions.

cytotrophoblast / syncytiotrophoblast / placental secretion *in vitro* / human early placenta

Résumé — Caractérisation et différenciation *in vitro* de cellules trophoblastiques du placenta humain du premier trimestre. Une préparation hautement enrichie de cellules cytotrophoblastiques est obtenue à partir des villosités chorales du placenta humain du 1^{er} trimestre par incubation à la dispase à 4 °C suivie d'une dispersion spontanée des cellules à 37 °C. Après 24 h de culture, 90 à 95% des cellules sont marquées par un anticorps anticytokeratine révélant leur caractéristique épithéliale. Après 48 h de culture, ces cellules se différencient en syncytiotrophoblastes comme le montrent les études en microscopie optique et électronique. La sécrétion de gonadotrophine chorionique (hCG) et de ses sous-unités libres α et β ainsi que la sécrétion d'hormone lactogène placentaire (hPL) ont été étudiées en fonction du temps de culture. Tandis que la sécrétion d'hCG et de ses sous-unités est stable durant les 3 premiers jours de culture, la sécrétion d'hPL est indétectable pendant les premières 48 h et augmente ensuite progressivement.

L'addition de dibutyryl cAMP en continu ou après 96 h de culture induit une augmentation de la sécrétion d'hCG et de ses sous-unités libres α et β et stimule aussi la sécrétion d'hPL. Un tel modèle de culture cellulaire sera utile pour étudier les mécanismes impliqués dans la croissance et la différenciation des cellules cytotrophoblastiques du 1^{er} trimestre et dans la régulation de leur fonction endocrine.

cytotrophoblaste / syncytiotrophoblaste / sécrétion placentaire *in vitro* / placenta humain précoce

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INTRODUCTION

The placenta performs many important functions during pregnancy. It plays an active role in the transport of nutrients and immunoglobulins from the maternal to the fetal circulation and prevents the rejection of the fetus by the mother (Wild, 1983). Moreover, the placenta is an endocrine organ, secreting steroid and protein hormones (Simpson and Mac Donald, 1981). The regulation of these different activities is poorly understood, and most *in vitro* studies have principally been performed on term placenta. However, first trimester placenta has invasive (Glass *et al*, 1983), immunological (Chaouat, 1987) and specific endocrine (Lachlan and Lopata, 1988) functions, suggesting that a better understanding of placental biology would be gained by studying the mechanisms necessary for its growth and differentiation, and its hormonal synthesizing capacities in early pregnancy.

Over the past years, many attempts have been made to isolate, purify and maintain human trophoblast cells in culture (Loke, 1983; Zeitler *et al*, 1983; MacQueen *et al*, 1987). Recently, Kliman *et al* (1986) reported a new procedure for trophoblastic cell purification based upon percoll gradient separation after treatment of human term placenta villous tissue by sequential Trypsin and DNase incubations.

Since first trimester placenta villous tissue has different properties and is less fibrous, such trypsin treatment produces some deleterious effects on cytotrophoblast viability and recovery, and increases mesenchymal cell contamination. We report a new method to obtain highly enriched cytotrophoblast preparations using Dispase incubation at 4 °C, followed by a spontaneous cell release at 37 °C overnight. The isolated cell was characterized

by using optic microscopy, electron microscopy, and by studying cell capacity to secrete placental hormones, human chorionic gonadotropin (hCG) and human placental lactogen (hPL) during 7 d of culture.

MATERIALS AND METHODS

First trimester placentas were obtained from patients undergoing voluntary elective abortion at 6–12 weeks of amenorrhea. Placental villous tissues were dissected free of chorionic membranes and incubated at 4 °C for 1 h in phosphate buffer containing 100 µg/ml of gentamycin and 2.5 µg/ml of amphotericin.

Cell isolation and culture

The villous tissue was then decanted and incubated with a solution of dispase (1 ml/gram of villous tissue at 2.4 U/ml, a neutral protease from *Bacillus polymixa* (dispase II, EC 3-4-24-4-) in Puck' solution (Boehringer) for 3 h at 4 °C. The dispase solution was then discarded and the tissue incubated overnight in medium A: DMEM containing 25 mM glucose, 4 mM glutamine, 25 mM HEPES, 100 µg/ml gentamycin, 2.5 µg/ml amphotericin (Seromed Biochrom, Lyon, France) and 20% of heat-inactivated fetal calf serum (Biological Industries) in 5% CO₂-95% air at 37 °C.

During this step, cells were spontaneously released into the medium either as single cells, or aggregated cells. Cells were separated from intact villous core by filtering through a sterile nylon gauze (150 N nylon mesh). In order to increase cell recovery, intact villous tissue on the gauze was washed by medium A (3 x 25 ml). Cells were collected by centrifugation at 1000 x g for 10 min and resuspended in medium A (2.5 x 10⁵ cells/ml). Cell viability, estimated by trypan blue exclusion, was 80 ± 10%. Cell recovery was routinely 1 x 10⁶ cells/wet gram of placental villous tissue.

Cells were plated either at 2.5 x 10⁵ cells/ml into 35-mm plates coated with human fibronectin (1 mg/ml, Sigma) for hormonal secretion

studies, or at 0.5×10^5 cells/ml into 1.5 mm wells coated with human fibronectin for immunohistochemical studies. After 2 h, the medium was removed and replaced with new medium A; this step eliminates non-adherent cells and red blood cells. Efficiency of cell plating was routinely 65%.

Immunohistochemical staining of cultured isolated cells

Immunohistochemical studies were performed at 24 h of cell culture. Cells were fixed directly in the wells by formaldehyde 3.7% (vol/vol-Merck) for 15 m at room temperature. We used monoclonal antibodies against cytokeratin, undiluted (Pan-cytokeratin, Amersham, France and anti-vimentin, undiluted (Amersham).

Peroxydase/anti-peroxydase detection was performed according to the manufacturer's instructions (Amersham, France).

Electron microscopy of cultured isolated cells

After 96 h of culture, the cells were fixed directly in the plates at 4 °C for 30 m with 2.5% of glutaraldehyde (Sigma) in 0.1M cacodylate buffer pH 7.4, and postfixed with 1% osmium-tetroxide for 30 min at 4 °C. After ethanol dehydration, the cells were embedded in araldite. For ultrathin cross-section, *in situ* embedded material was cut into small pieces and re-embedded in araldite. Ultrathin sections were stained with uranyl acetate and lead citrate.

Secretion of hCG and hPL by cultured isolated cells

Cells were cultured for 7 d in medium A with epidermal growth factor added (100 ng/ml, Collaborative Research). Dibutyl cAMP (mM) was added either immediately, or after 4 d of culture. The medium was changed daily and stored at -80 °C until analyzed.

Analytical methods

Mab-based specific immuno-radiometric assays (IRMAs) were used for the determination of hCG and of free α and β subunits of hCG levels in cell culture media. The production of Mabs with defined epitope specificity, the development of multisite IRMAs and the testing of their specificity have been described previously (Bellet *et al*, 1986; Ozturk *et al*, 1987). The level of hPL secreted in the culture medium was determined by hPL IRMA kit (Amersham).

RESULTS

After 24 h of culture, the cells formed islands, but individual cells were still discernable. At 48 hours of culture, the cells were all aggregated and individual islands were linked by cell chains (fig 1A). At 72-96 h of culture, multinucleated cells were formed and the syncytium appeared (fig 1B).

At this time, as studied by electron microscopy, the cell surface of the outer part of the clump exhibited cytoplasmic folds, well developed microvilli, and coated and non-coated vesicles. The surface of cells, at the apposition with culture plates, lacked microvilli. Adjacent surfaces of aggregated cells developed desmosomal connections. Internalized desmosomes were visible in multinucleated cells. Around the nucleus, the usual cytoplasmic organelles, including a well developed rough endoplasmic reticulum and lysosomes were observed. Osmiophilic lipids, electron-dense vesicles, and numerous cytoplasmic filaments were also present (figs 2A and 2B).

After 24 h of culture, 90-95% of cells were immunostained by antibody against cytokeratin, demonstrating the epithelial characteristic of cultured cells (fig 3A), and less than 10% of cells were positively stained by anti-vimentin antibody, showing a low contamination by mesenchymal and

A **B**

Fig 1. Morphological aspect of trophoblastic cells as a function of culture time (x 200). A: Cells after 48 h in culture. B: After 96 h in culture.

endothelial cells (fig 3B). This characteristic was maintained at each time of culture.

These immunohistochemical and electromicroscopic data showed that the isolated cells from placenta villous tissue, after dispase treatment, were essentially a cytotrophoblast cell type. These cells were able to differentiate in culture by aggregating and fusing to form syncytiotrophoblasts.

HORMONAL SECRETION BY TROPHOBLASTIC CELLS IN CULTURE

Figure 4 shows the daily secretion of intact hCG (fig 4A), of the free α (fig 4B) and β (fig 4C) subunits of hCG, and the daily secretion of hPL (fig 4D) by cells cultured for 7 d in the absence, or in the continuous presence, of dibutyl cAMP (mM). In the absence of dibutyl cAMP, the level of secreted intact hCG, and its free α subunit, decreased between the third and fourth d of cell culture and was stable afterwards; the level of free β hCG was low at all times of cell culture. The decrease of secreted hCG was not related to a variation in cell viability, as estimated by cell counting, but probably related to the lack of specific synthesis inducers during cell culture. Indeed, the addition of dibutyl cAMP to the culture medium at the fourth day, induced another increase of secreted intact hCG and its free α and β subunits, as soon as the first following 24 h.

When dibutyl cAMP was present from the start of the culture, the level of intact secreted hCG was maximum on the fourth day (220 ng/ml), and increased 10-fold, as compared to the control; the level of secreted free α subunit also reached a maximum at the fourth day (120 ng/ml), and was increased by 6-fold, compared to the control; the level of secreted free β subunit

was stable during the 7 d of culture (15 ng/ml), and was increased by 6-fold, compared to the control (d 5 of culture).

The secretion of hPL (fig 4D) was detectable after only 2 d of culture and increased linearly afterwards. Since hPL secretion was linked with the appearance of multinucleated cells (syncytiotrophoblast), this result supports the idea that cultured cells are able to differentiate *in vitro* from cytotrophoblasts to syncytiotrophoblasts. In our experimental conditions, the secretion of hPL was stimulated by the addition of dibutyl cAMP.

DISCUSSION

The aim of this work was to obtain a highly enriched cytotrophoblast preparation from first trimester human placental villous tissue. The production of such a cell preparation and the maintenance of these cells in culture should allow further investigation of the mechanisms (hormonal and/or the role of growth factors) involved in the differentiation of the cytotrophoblast into syncytiotrophoblast in the early development of human placenta. Indeed, hormonal secretion, such as hCG (Lachan and Lopata, 1988; Patillo *et al*, 1983), and the presence of growth and oncogenic factors (Adamson, 1987; Rydnert *et al*, 1987) associated with the developmental regulation of placenta, were expressed by high levels during the first trimester of gestation and either decreased, or disappeared afterwards.

We report here a method of obtaining highly enriched cytotrophoblast preparations from first trimester placental villous tissue. The cell preparations were not contaminated to any significant extent with endothelial or mesenchymal cells, as demonstrated by the high level of cell immunostaining with anti-cytokeratin antibody

and the low staining with anti-vimentin antibody (less than 10%) (Vettenranta *et al*, 1986; Mac Queen *et al*, 1987). Moreover, as early as the first 24 h of culture, 90–95% of cultured cells synthesized α hCG subunit, shown by staining with anti α hCG antibody (data not shown), and correlated by the level of secreted hCG and free hCG α subunit into the culture medium.

When the culture time increased, cells aggregated, and multinucleated cells appeared; the electron microscope, and a study of the secreted hPL level, provided evidence that these cells were able to differentiate into syncytiotrophoblasts *in vitro*, and to synthesize and secrete hPL, a placental hormone found only in the syncytial epithelial layer of villous tissue (Simpson and MacDonald, 1981; Hoshina *et al*, 1982; Hoshina *et al*, 1985). The time required *in vitro*, to observe the formation of aggregated cells and the formation of the syncytiotrophoblast was identical for either first trimester, or term placenta (Kliman *et al*, 1986). As a function of culture time, a parallel study was performed to determine the level of secreted intact hCG and its free α and β subunits, using specific monoclonal antibodies and radiometric assay (Bellet *et al*, 1986; Ozturk *et al*, 1987). In contrast to data obtained from either term placenta cultured cells (Kliman *et al*, 1986), or from first trimester explant cultures (Maruo *et al*, 1987), which reported

either an increase of hCG at 48 h of culture, or a decrease of hCG at each culture time, we observed a stable secretion of hCG during the first 3 d of cell culture. The discrepancy between these data could be due to the different assay used for the evaluation of secreted hCG, to the difference between early and term placenta, or to the difference in the procedure of cell isolation and culture.

Although we observed a decrease in the secretion of hCG and of its free α and β subunits on the fourth day of culture, these secretions could be induced again by the addition of dibutyryl cAMP. This last result, and the increase of secreted hCG in the continuous presence of dibutyryl cAMP, as compared to the control, suggests that these cells possessed the potential for synthesizing hCG, but that specific inductors were missing in our culture conditions. These results are in agreement with the key role of cAMP in the regulation of hCG synthesis (Jameson *et al*, 1987; Zhou *et al*, 1987; Nulsen *et al*, 1988). Moreover, the addition of cAMP stimulated the secretion of hPL by cultured cells, in contrast to previous results obtained for term placenta (Zeitler *et al*, 1983). These results support a stimulatory role of the adenylate cyclase pathway as an intracellular regulator of human trophoblastic endocrine activities, not only for hCG, but also for hPL, in the first trimester placenta.

Fig 2. Electron micrograph of trophoblastic cells after 96 h in culture (x 7400). A: Abundant microvilli (mv), coated vesicles (cv), rough endoplasmic reticulum (rer) and large electron dense vacuoles (v) (x 8800). B: Clump of 4 adherent cells (I, II, III, IV) during cellular fusion showing desmosomes between adjacent cell membranes (d), intermediate filaments (f), lysosomes (ly) and osmophilic lipids (l). The cell surface exhibited microvilli (mv) on the outside of the clump and in the intercellular space between adjacent cells (x 9400).

Fig 3. Immunohistochemical staining of cultured trophoblastic cells. At 24 h of culture, cells were stained for cytokeratin (A), and for vimentin (B).

Fig 4. Hormonal secretion by trophoblastic cells in culture. Cells were cultured, as described in *Materials and Methods*, in the presence or in the absence of dibutyryl cAMP (mM). Dibutyryl cAMP was added either at 2 h, or at 96 h of culture. The media were removed daily and replaced with fresh medium. The results are the mean $\pm$ SD of duplicate incubation from 4 separated incubations. A: ng of intact hCG/ml/24 h; B: ng of free α hCG/ml/24 h; C: ng of free β hCG/ml/24 h; D: ng of hPL/ml/24 h.

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