Establishment and characterization of a cytotrophoblast cell line from normal placenta of human origin

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A cell line has been established from human placenta at the first trimester of normal pregnancy. The cell line was obtained by culture of purified cytotrophoblast cells in serum-free medium supplemented with epidermal growth factor, insulin, dexamethasone and 0.1% bovine serum albumin. The cells can be subcultured for >30 passages in one to three splits. All the cells were mononuclear epithelial-like cells positive to cytokeratin 18, gonadotrophin-releasing hormone (GnRH), neuropeptide Y, neurotensin, leucine-enkephalin, dopamine and 5-hydroxytryptamine immuno-cytochemical staining. The cells secreted GnRH, progesterone and oestriadiol (in the presence of testosterone) but little human chorionic gonadotrophin and no β-endorphin.

The cell line showed human karyotypes and had a population doubling time of 48 h in serum-free medium. However, the cells would stop growing in the medium containing fetal bovine serum. A normal cytotrophoblast cell line established in serum-free medium will be particularly useful in the study of cytotrophoblast cell proliferation and differentiation.

Key words: cell proliferation/growth factors/human placental cytotrophoblast/neurotransmitters/sex steroids

Introduction

Cytotrophoblast cells are the proliferating mononuclear trophoblast cells in human placenta. The differentiation of cytotrophoblast cells into syncytiotrophoblast cells has been characterized by morphologically forming multinucleate syncytium and biochemically producing a high concentration of chorionic gonadotrophin (Ringler and Strauss, 1990). When cultured in the presence of serum, cytotrophoblast cells differentiate spontaneously into multinucleate cells (Kliman et al., 1986; Yagel et al., 1989). The degree of proliferation and differentiation of cytotrophoblast cells in culture varies largely with the batches of serum. Therefore it is unlikely that a pure culture of cytotrophoblast cells free of syncytiotrophoblast cells or a reproducible culture with certain proportions of cytotrophoblast and syncytiotrophoblast cells can be obtained (Goustin et al., 1985; Yagel et al., 1989). In addition, serum contains mitogenic factors which contaminate fibroblast cells and which often overgrow the cytotrophoblast cells in culture (Cotte et al., 1980; Bax et al., 1989; Yagel et al., 1989).

Our approach to obtaining pure, proliferating cytotrophoblast cells is to grow the cells in a defined condition, free of serum, which strictly selects for cytotrophoblast cells. Non-trophoblast cells, such as fibroblast cells, will die and disappear from culture because of the lack of essential growth factors (Li and Zhuang, 1991). Biochemically differentiated trophoblast cells will be eliminated from culture because they neither proliferate nor arise from the differentiation of the propagated cytotrophoblast cells. Here we report a normal cytotrophoblast cell line established in a defined medium.

Materials and methods

Human placental villi collection and primary culture of cytotrophoblast cells

First trimester (6-8 weeks gestation) placental chorionic villi were obtained after electronic curettage from the maternity ward of Haidian Hospital, Beijing, China and rinsed thoroughly in cold phosphate-buffered saline (PBS) solution. Cytotrophoblast cells were isolated by a method adapted previously for serum-free culture (Li and Zhuang, 1991). The chorionic villi were dissected from the membrane under a dissecting microscope and minced into small fragments. The fragments were washed twice with PBS and resuspended in ice-cold 0.25% trypsin (Sigma Chemical Co., St Louis, MO, USA) solution with 15 IU/ml DNase I (Gibco BRL, USA) at 7-9°C for 45 min. Trypsinization at a low temperature can inhibit the internalization of trypsin and reduce damage to the cytotrophoblast cells by the protease. This technique can reduce the requirement for serum proteins in serum-free culture (McKeehan, 1979). Trypsinization was stopped by adding two volumes of Ham's F-12/Dulbecco's modified Eagle's medium (DMEM). The cell suspension was centrifuged at 120 g for 5 min. After the supernatant was aspirated, the pellet was resuspended in 50 ml Ham's F-12/DMEM and isolated by gentle pipetting with a Gilson 1 ml pipeteman. The dispersed cells were filtered through a 75 Nytecx (nylon sieve) to remove the gross villous core residues. After centrifugation (120 g for 5 min), the cells were washed once with the same medium, resuspended in 1 ml Ham's F-12/DMEM and added slowly to the top of bovine serum albumin (BSA) gradients (prepared by sequentially adding 3 ml of 3, 2 and 1% BSA in Ham's F-12/DMEM to a 15 ml centrifuge tube). The cells were sedimented for 1 h at unit gravity and cytotrophoblast cells were collected from the bottom of the tube. The purified cytotrophoblast cells were seeded in collagen-coated 24-well plates (Corning Inc., Corning, NY, USA) at 105 cells/well with 1 ml Ham's F-12/DMEM 1:1 (FD medium; Gibco BRL) containing 10 ng/ml epidermal growth factor (EGF; Collaborative Research Inc., Lexington, MA, USA), 10 μg/ml insulin (Sigma Chemical Co.), 0.1% BSA, 2 mM glutamine (Dongfong Chemical Co., Shanghai, China), 1.75 mM HEPES (Sigma Chemical Co.,
Immunocytochemistry

The highly purified cytotrophoblast cells were cultured in 24-well multiplates with FD medium containing 10 ng/ml EGF, 10 μg/ml insulin, 10^-6 M dexamethasone (Sigma Chemical Co.), 30 nM sodium selenite (Collaborative Research Inc.), 0.1% BSA and 2 mM glutamine. After the cell colonies had grown up in the primary culture, subcultures were performed by cold trypsinization. Cells were re-plated in 35 mm collagen-coated dishes (Corning Inc.). Serial passages were made at near confluence culture in one to three splits.

Serial passages

The highly purified cytotrophoblast cells were cultured in 24-well multiplates with FD medium containing 10 ng/ml EGF, 10 μg/ml insulin, 10^-6 M dexamethasone (Sigma Chemical Co.), 30 nM sodium selenite (Collaborative Research Inc.), 0.1% BSA and 2 mM glutamine. After the cell colonies had grown up in the primary culture, subcultures were performed by cold trypsinization. Cells were re-plated in 35 mm collagen-coated dishes (Corning Inc.). Serial passages were made at near confluence culture in one to three splits.

Immunocytochemistry

The cytotrophoblast cultures of different passages were characterized by immunocytochemical or immunofluorescence staining for numerous markers with specific antibodies. Antibodies employed for the determination of the intermediate filament proteins were the monoclonal antibodies against human cytokeratins 13 and 18 (K 8.12 and CY-90 respectively; Sigma Chemical Co.) and human vimentin (VIM-13.2; Sigma Chemical Co.). Antibodies for the determination of the cell functions were the antibodies against human chorionic gonadotrophin (HCG) and gonadotrophin-releasing hormone (GnRH; raised in this laboratory), the antibodies against β-endorphin, neuropeptide Y, neurotensin and dopamine (generously donated by Prof. C.L.Zhang, Institute of Zoology, Chinese Academy of Sciences, Beijing, China), the antibody against 5-hydroxytryptamine (seratonin; generously donated by Prof. W.Q.Huang, The Fourth Military Medical University) and the antibody against leucine-enkephalin (generously donated by Prof. X.Z.Zhu, Institute of Materia Medica, Chinese Academy of Sciences).

Cells were fixed with 0.125% glutaraldehyde for 30 min at room temperature. After several washes with PBS, the fixed cells were sequentially incubated at room temperature with 0.3% hydrogen peroxide in methanol for 30 min at room temperature, 1% NH4HCO3 and 1% gelatin in PBS for 1 h and 10% fetal calf serum in PBS for a further 1 h. The pretreated cells were then incubated with the primary antibodies at appropriate dilutions in a refrigerator (7-9°C) overnight. Binding of the primary antibodies was visualized by peroxidase-anti-peroxidase (PAP; anti-horseradish peroxidase (HRP) antibody raised in this laboratory) or HRP-conjugated secondary antibody, followed by 3,3′-diaminobenzidine-H2O2 (prepared from Sigma peroxidase substrate kit) or fluorescein isothiocyanate conjugated CY-90 (Sigma Chemical Co.).

Hormone content analysis

The cytotrophoblast cells of different passages were incubated in regular media for 24 h or for 4 h with bacitracin (Sigma Chemical Co.; for a GnRH radioimmunoassay). The media were collected and stored at -20°C for a hormone radioimmunoassay. The HCG content of the culture medium was determined using a secondary antibody precipitation radioimmunoassay method (Research Group of Endocrinology, 1977; [125I]HCG was purchased from 401 Institute, Beijing, China and the goat anti-rabbit immunoglobulin G antiserum was raised in this laboratory). Oestradiol (in the presence of 10^-7 M testosterone as substrate) and progesterone were assayed by a radioimmunoassay according to Jones et al. (1982). Both steroid antibodies were raised in this laboratory; [3H]progesterone and [3H]oestradiol were purchased from Amersham, UK. The radioimmunoassay of GnRH ([125I]Na was purchased from DuPont USA) was carried out as described by Arimura et al. (1973). The number of cells in each culture was counted with a Coulter counter (Coulter Electronics Inc., FL, USA; model ZF) after trypsin dispersion. Data are expressed as mean ± SE.

Chromosome analysis and tumorigenesis test in nude mice

For karyotyping, cells at the exponential phase of growth were arrested by adding 0.03 μg/ml colchicine for 6 h, treated with a hypotonic solution (75 mM KCl) at pH 8.0 for 30 min and then fixed twice in Carnoy's fixative (methanol/acetic acid) for 15 min. The G-band pattern was obtained using the trypsin method. Chromosomes of 100 cells were analysed. For the tumorigenesis test, the cells of the 25th passage were injected s.c. into nude mice (10^7 cells/injection). The animals were killed after 2.5 months and examined for generated tumours.
Figure 3. Typical human karyotype of the normal placental cytотrophoblast (NPC) cell line. Most NPC cells had a diploid karyotype with a chromosome number of 46, including the X and Y chromosomes (original magnification ×400).

Table I. Functional characterization of the primary cytотrophoblast and normal placental cytотrophoblast cell line (NPC) cells

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Primary cytотrophoblast</th>
<th>NPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gonadotrophin-releasing hormone</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Neuro peptide Y</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Neurotensin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cholecystokinin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Leucine-encephalin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dopamine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5-HT</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Progesterone</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Human chorionic gonadotrophin</td>
<td>+</td>
<td>Weak</td>
</tr>
<tr>
<td>β-Endorphin</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Vimentin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cytokeratin peptide 18</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Cytokeratin peptide 13</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Estimation of doubling time

Cells were seeded at an initial concentration of 2×10⁴ cells/ml in Corning collagen-coated 24-well multilizes and cultured in regular medium for 6 days. The medium was refreshed after 3 days. At various intervals, the cell number of triplicate cultures was counted by a Coulter counter after incubation with trypsin solution.

Results

Establishment of a human normal placental cytотrophoblast (NPC) cell line and cell morphology

In primary culture, cytотrophoblast cells began to attach within 2 h after plating. They spread and showed a monolayer epithelial cell morphology after 24 h (Li and Zhuang, 1991). During the first week of culture, cells actively migrated and re-aggregated. Most cell re-aggregates were detached and lost from the culture after medium changes. Few mitotic cells were observed. During the second week, small colonies of epithelial cells could be identified in the culture. Mitotic figures were observable. Cell colonies continued to grow slowly to form large cell colonies in 4–6 weeks. For subculture, individual cell colonies were pooled or picked up individually with cloning rings by cold trypsinization. After subculture, cell proliferation became accelerated and culture was carried on continuously for >30 passages before senescence. Multinucleation was not observed throughout the culture period. The NPC cells grew as monolayers with an epithelial-like general morphology (Figure 1). The cells were mononuclear and each contained one to four nucleoli. Lipid droplets were scattered in the cytoplasm and the number of lipid droplets was increased markedly in serum-supplemented medium. The NPC cells migrated actively. When the cells were inoculated and attached as single cells, they were able to migrate to form monolayer aggregates. At a low culture density, the cells became more elongated and extended fibres were found to be communicating between cells at a distance.

Growth characteristics

The NPC cells grew well in FD medium supplemented with EGF, insulin, dexamethasone and BSA on a collagen matrix, and had a population doubling time of ~48 h, as estimated by the direct viable cell count (Figure 2). Optimal cell growth was obtained with an initial inoculum of 2×10⁴ cells/ml. The cells became senescent after extended passages beyond 30 passages. When cultured in 5% fetal bovine serum-supplemented medium, the cells soon flattened out, accumulated many lipid droplets in the cytoplasm and ceased to proliferate. No tumour was observed for 2.5 months after injection in the nude mice injected after the 25th passage of NPC cells.

Chromosome analysis

A chromosome analysis revealed that ~95% of NPC cells had a diploid karyotype with a chromosome number of 46, including the XY sex chromosomes (Figure 3). Some other cells had karyotypes with a few chromosomes more or less than 46. Few cells had >100 chromosomes.
Immunocytochemical studies

As indicated in Table I and Figure 4, ~98% of NPC cells of different passages showed a positive staining of cytokeratin peptide 18, GnRH, neuropeptide Y, neurotensin, leucine-enkephalin, dopamine, serotonin and cholecystokinin but showed negative staining of β-endorphin, cytokeratin peptide 13 and vimentin. As for HCG, <2% of NPC cells stained weakly with anti-HCG.

Hormone secretion

Like primary cultured human cytotrophoblast cells, the established cell line (NPC) showed the constant secretion of HCG, GnRH and progesterone. NPC cells only secreted a very low level of HCG, ~1 ng/10^5 cells/24 h (Table II). However, they secreted as high as 100 ng/10^5 cells/24 h of progesterone (Table III) and 3 pg/10^5 cells/4 h of GnRH (Table IV). In the presence of testosterone, the cell line secreted oestradiol at ~10 ng/10^5 cells/24 h.
Discussion

Previous work on trophoblast tissue culture, cell culture and choriocarcinoma cell lines has contributed to the knowledge of trophoblast endocrine functions (Ringler and Strauss, 1990). Recently, purification techniques for cytotrophoblast cells have become available. Kliman et al. (1986) purified cytotrophoblast cells by Percoll density gradients. However, the purified cells biochemically expressed the phenotypes of syncytiotrophoblast cells and no longer proliferated in culture. More recently, Yagel et al. (1989) and Graham et al. (1993) obtained a pure cytotrophoblast cell culture through the subculture of outgrowth of trophoblast tissue explants in serum-containing medium. They maintained the cells in culture for only 10–12 passages, and these cells produced a large quantity of HCG. Goustein et al. (1985) also reported on their trophoblast cell lines, which expressed the HCG-α subunit and cytokeratin in 95% of the cells and growth was stimulated by platelet-derived growth factor (PDGF). However, the cell lines consisted of both cytotrophoblast and syncytiotrophoblast cells in certain proportions because they were cultured in the presence of bovine serum, as it is known that cytotrophoblast cells can fuse to syncytiotrophoblast cell culture with serum (Kliman et al., 1986). Moreover, it is not easy to eliminate fibroblast contamination completely, which may make experimental results unexplainable. The PDGF receptor and PDGF-stimulated cell growth in trophoblast cell lines may be a case in point (Goustein et al., 1985). In comparison with these cells, NPC cells show advantages such as (i) obtained from a single colony, (ii) grown in defined medium, (iii) non-transformed with a prolonged life span and (iv) maintained most cytotrophoblast functions.

We established serum-free conditions for the long-term culture of human cytotrophoblast cells. These conditions specifically selected for the proliferating cytotrophoblast cells. The cell line so established maintained cytotrophoblast cell identity with a prolonged life span. As the results showed, all cells expressed cytokeratin 18, GnRH, neuropeptide Y, neurotensin, leucine-ε-kiplin, serotonin and dopamine progestesterone. These results are consistent with the cytotrophoblast cells' localization of these peptides in chorionic villi or primary cultured cytotrophoblast cells in the literature (Khodr and Siler-Khodr, 1978; Petraglia et al., 1989; Zhang et al., 1991; Zhuang and Li, 1991; Huang et al., 1993). Moreover, neither syncytiotrophoblast-specific proteins such as endorphin (Laatikainen et al., 1987) or high level HCG (500 ng/2×10⁵ cells/24 h), nor fibroblast marker such as vimentin, was detected in the cells. Taken together, this evidence suggests that the cell line established here represents proliferating cytotrophoblast cells.

Studies on placental functions have traditionally relied on the use of primary placental tissue or enzyme-dissociated placental cells. Cytotrophoblast cell purification techniques have become available only recently (Kliman et al., 1986). However, purified cytotrophoblast cells did not proliferate in culture with serum. Instead, they differentiated morphologically and biochemically into syncytiotrophoblast cells (Kliman et al., 1986). The syncytiotrophoblast differentiation also persisted in long-term cultures of cytotrophoblast cells derived from placental explants (Goustein et al., 1985; Yagel et al., 1989).

However, we found in our previous report that trophoblast cell fusion was inhibited in serum-free medium (Li and Zhuang, 1991). Long-term culture of cytotrophoblast cells in serum-free medium consistently resulted in the establishment of a pure proliferating cytotrophoblast cell line.

NPC provides a unique in-vitro model for the study of the function of replicating cytotrophoblast cells. In the past, cell lines derived from malignant trophoblast tumours have been commonly used for the studies of cell proliferation and endocrine functions of replicating cytotrophoblast cells. These cell lines have some similarities to cytotrophoblast cells, but because they are derived from tumours there are also critical differences (Ringler and Strauss, 1990). Recently, cytotrophoblast cell lines have also been established by viral gene (SV40) transformation. These cell lines resemble the invasive cytotrophoblast cells (Chou, 1978; Logan et al., 1992; Graham et al., 1993). However, SV40-transformed cells produce HCG and prolactin-like choriocarcinoma cells. The properties of these cell lines are under the influence of the viral antigen by unknown mechanisms (Graham et al., 1993), so the results obtained from these cell lines may not reflect the actual properties of normal cytotrophoblast cells. NPC is from non-transformed cells and therefore NPC cells are appropriate for a study of cytotrophoblast cell proliferation and endocrine control. This has been demonstrated by the neuroendocrine activities of NPC characterized here. Moreover, serum-free culture provides additional advantages in that all factors in the culture medium are precisely defined without interference from unknown factors which may be present in serum.

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References


Human normal placenta origin cytotrophoblast cell line


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