Histol Histopath (1989) 4: 367-374

Histology and Histopathology

Early human trophoblast cell cultures. A morphological and immunocytochemical study

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Summary. Rapidly growing cytotrophoblasts were isolated from early human chorionic villi and the Papanicolaou method was used to characterize their cytology and transformation into syncytiotrophoblasts. Cytotrophoblasts fused and formed binucleated cells or mononucleated intermediate cells. Syncytial cells were formed by fusion of small cytotrophoblasts or intermediate cells and cytotrophoblasts. Glycosaminoglycans were produced in cytotrophoblasts and released extracellularly. Here they were accumulated and/or diffused into a continuous layer covering the cells. Glycosaminoglycans in syncytial cells were contained in well defined membranous sacs. Cytotrophoblasts only grown beyond confluence differentiated into villi with a villus-like histology.

Key words: Trophoblast, Cell culture, Extracellular matrix, Immunocytochemistry

Introduction

Human trophoblast cell cultures have had a rather limited experimental application because of difficulties with the short survival of the trophoblasts, failure of the cells to replicate and overgrowth of stromal fibroblasts (Stromberg et al., 1978; Stromberg, 1980; Niazi and Loeffler, 1981). While most attempts to culture placental cells have produced mixtures of fibroblasts, syncytiotrophoblasts and cytotrophoblasts (Thiede, 1960; Soma et al., 1961; Lajos et al., 1967; Taylor and Hamcock, 1973; Roy et al., 1976; Stromberg et al., 1978), Cotte and coworkers (1980) succeeded in preparing monolayer cultures of human trophoblasts. Several reports have later described morphological and immunocytochemical features of cultured trophoblastic cells but have paid very little attention to the extracellular matrix (ECM) produced by these cells (Aladjem and Lueck, 1981; Butterworth and Loke, 1985; Morgan et al., 1985; Loke et al., 1986; Nelson et al., 1986).

We have shown that malignant bladder cell cultures produce an ECM which inhibits cell proliferation and enhances tumour nodule formation (Logothetou-Rella et al., 1988). The present study aimed to evaluate observations in embryonic cells, and trophoblasts were therefore cultured and studied to reveal any resemblance to malignant urothelial cells.

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Materials and methods

Tissues

Placental chorionic villi from five pregnancies of 10 weeks gestation were collected for prenatal diagnosis of genetic disorders. Each chorionic villus biopsy was obtained transcervically, under the guidance of a real-time ultrasound scanning, by aspiration using a 20 ml syringe attached to a 17 cm 16-gauge cannula.

The material obtained was examined in the operating theatre by phase contrast microscopy for identification of chonionic villi.

Primary cell cultures

The villi were placed in complete medium (CM) RPM I - 1640 (Gibco), supplemented with 15% foetal bovine serum (Gibco), penicillin (100 U/ml, Gibco), streptomycin (100 μg/ml Gibco), and incubated at 37° C until processing.

The time between villi tissue collection and processing varied from 2 to 24 hours. Half of the tissue collected was used for chromosome analysis while the rest was used for cell culture establishment.

The villi were sliced and washed twice in CM in a small Erlemeyer flask. The CM was removed and replaced

with a mixture of trypsin (0.25% in Ca^{++} and Mg^{++} free PBS, Gibco) - 0.1% collagenase (Type A1, Sigma C - 9891) and incubated at 37° C for 10 min, being shaken gently. The enzyme mixture was then replaced by a fresh one and the villi fragments were incubated at 37° C for another 20 min, being shaken gently.

This step was repeated once more for 20 min but the enzyme mixture was replaced with trypsin (0.25%) solution only. At the end of the incubation period the villi cells were confirmed to be loose under the microscope. Trypsin solution was removed and CM was added. This suspension of villi - loose cell fragments was pipetted in and out gently several times, and the single cell suspension obtained was washed twice in CM by centrifugation at 400 g for 10 min.

The cell suspension derived was used to seed small glass petri-dishes which were sealed in a modular incubator chamber (Flon) containing 3 open petri-dishes with sterile distilled water for proper humidity, gassed with 90% N_2, 5% CO_2 and 5% O_2, and incubated in a large CO_2 - humidified - 37^2 - incubator. Falcon flask stock cultures were used for serial subcultivations, one subcultivation every 7 days, by the traditional trypsin treatment. The cell culture medium was changed every three days. Trypan blue dye exclusion was used for viability testing. The cell culture was confirmed to be trophoblastic by human chorionic gonadotropin (HCG) measurements in the medium by EIA method (Tietz, 1986).

Primary cell cultures of 2, 7, 15 and 30 days old were rinsed twice with PBS and then fixed in 50% ethanol and stained by Papanicolaou, PAS and Alcian blue methods.

Histology

One confluent 75 cm^2 Falcon flask stock cell culture, full of villi, after 8 subcultivations (2 months old), was fixed in formalin. The villi were collected gently with fine forceps and embedded in paraffin. Thin serial sections were stained with hematoxylin and eosin.

Electron microscopy

Pellets of trypsinized primary 15-day-old continuous cell cultures were fixed in phosphate buffered 1% glutaraldehyde, postfixed in buffered 1% osmium tetroxide, dehydrated in graded ethanols and embedded in Epon 812.

Semithin sections were cut with glass knives, stained with toluidine blue and used for light microscopical orientation. Ultrathin sections were cut with diamond knives, contrasted with uranyl acetate and lead citrate and examined under the transmission electron microscope.

Immunocytochemistry

Primary 15-day-old petri dish cell cultures were fixed in acetone (-20°C) for 10 min and used for immunocytochemical studies. The peroxidase-antiperoxidase method was applied (De Lellis et al., 1979), using the antisera and conditions shown in Table 1.

Control studies included: a) absorption of the primary antibody with the homologous antigen; b) replacing the primary antibody with non-specific serum and c) relevant positive controls.

Chromosome analysis

The direct method of chromosome preparation from villi was applied (Simoni and Sabro, 1986). This method gave 20 metaphases and the chromosomes were studied with Giemsa staining.

Results

All cell cultures grew rapidly and consisted mainly of variously sized small trophoblasts for the first week. After 15 days intermediate elongated fibroblast-like cells with one large nucleus appeared. Thirty-day-old cultures consisted of mononucleated or binucleated cytotrophoblasts, intermediate trophoblast cells,

Table 1.

Antiserum against	Dilution of primary antiserum	Incubation time	Temperature	Source/Code
Keratin	1:40	2 hours	Room t.	Dako Corp/A575
Carcinoembryonic antigen (CEA)	1:60	"	"	" A115
Protein S-100	1:200	"	"	" Z311
Neuron Specific Enolase (NSE)	1:100	"	"	" A589
Human Chorionic Gonadotropin (HCG)	1:200	"	"	" A231
Lysozyme	1:100	"	"	" A099
$Alpha_1$antichymotrypsin	1:100	"	"	" A022

Fig. 1. Small mononucleated and binucleated cytotrophoblasts with overlapping, foamy cytoplasm and ill defined borders. Intracytoplasmic accumulation of glycosaminoglycans is present (arrow). Papanicolaou stain. × 1,250

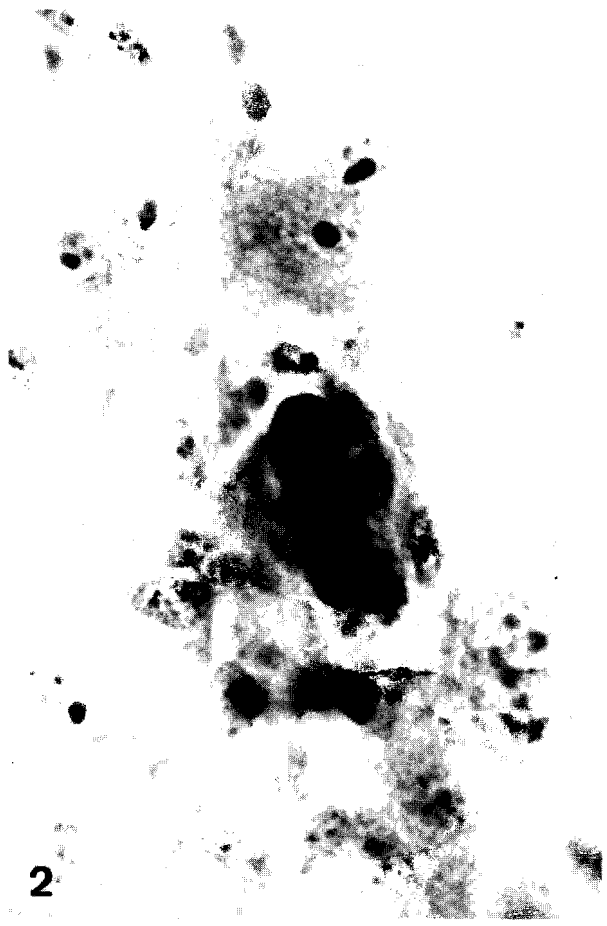
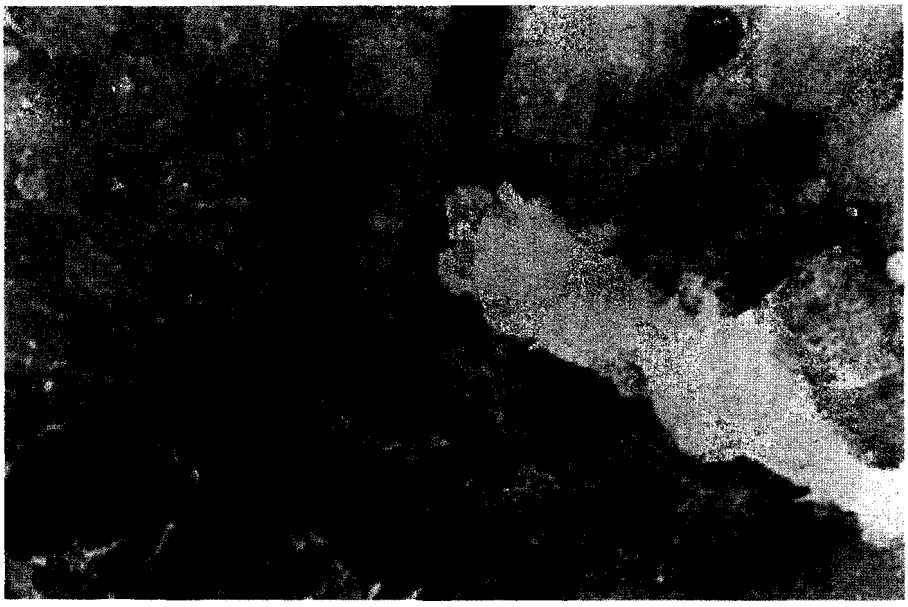
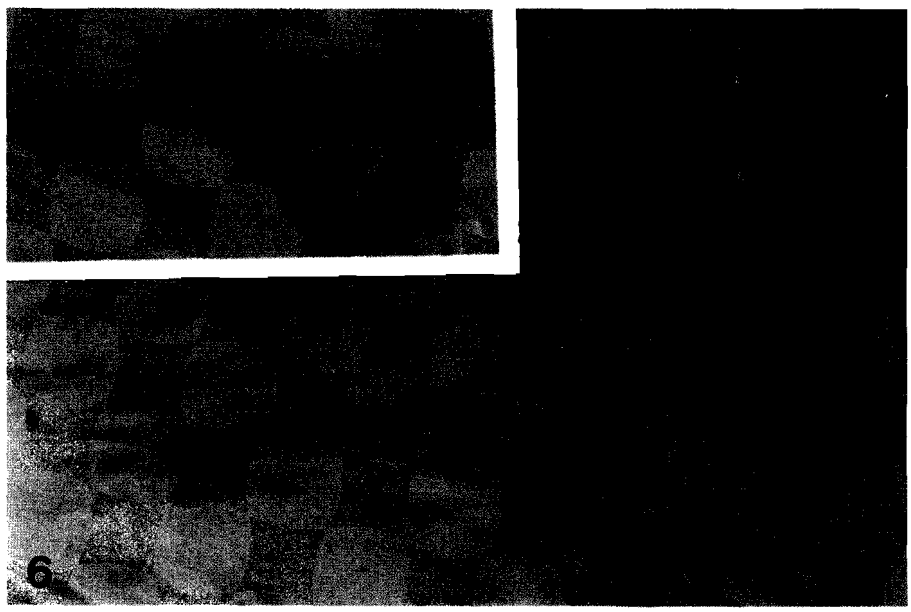


Fig. 2. Various sized mononucleated and binucleated cytotrophoblasts around glycosaminoglycan mass. Papanicolaou stain. × 500

Fig. 3. Mixed culture of syncytiotrophoblasts, cytotrophoblasts and spindle-shaped intermediate cells. Glycosaminoglycan sac with long extension is present (arrow). Two binucleated cells at the bottom right, one containing 2 equal small sized nuclei, and the other 2 equal larger nuclei. Papanicolaou stain. × 125





syncytiotrophoblasts and many villi. HCG was detected in the medium from 3 days after subcultivation (21 mIU/ml).

Cytological features

The cytotrophoblasts consisted of epithelial cells of various sizes and were often arranged in sheets (Figs. 1, 2). The cells had a clear to faintly eosinophilic cytoplasm and ill-defined boundaries. Perinuclear vacuoles were occasionally seen. The large, round to oval-shaped nuclei were of different size, hyperchromatic, and with prominent nuclear membranes. One large, compact to five smaller rod-shaped nucleoli were present in each nucleus.

The intermediate trophoblast cells were larger than the cytotrophoblasts. They possessed single but double-sized nuclei and abundant eosinophilic cytoplasm with long projections (Figs. 3, 10).

Cytotrophoblasts and intermediate trophoblast cells grew mixed and not in the separate groups that normal fibroblasts and epithelial cells usually grow in a mixed cell culture (Fig. 3).

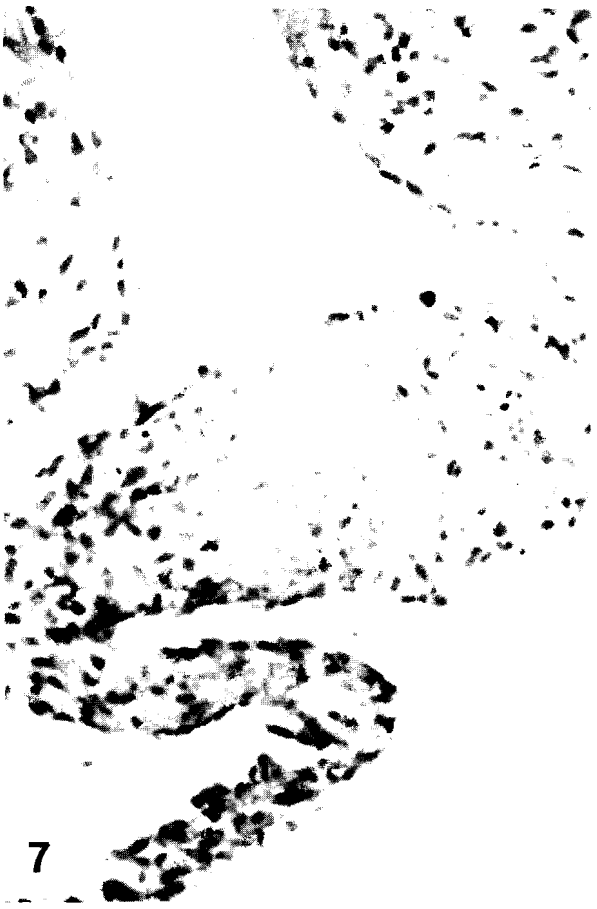
Binucleated cells as a result of merged cytotrophoblasts and/or intermediate trophoblast cells were also observed. Fusion of intermediate trophoblast cells with cytotrophoblasts resulted in anisokaryotic syncytio, while fusion of small cytotrophoblasts resulted in isokaryotic syncytio (Fig. 3).

The ill-defined, irregular multinucleated syncytiotrophoblasts had a fibrillar cytoplasm with some granularity in the perinuclear regions. A few cytotrophoblasts and syncytiotrophoblasts contained a semi-transparent substance, which stained

Fig. 4. Higher magnification of another intracellular glycosaminoglycan containing sac with long extensions. Papanicolaou stain. × 1,250. Inset: Propagation of membrane extensions and glycosaminoglycans across the cytoplasm of syncytiotrophoblasts. Papanicolaou stain. × 200

Fig 5. Trophoblast cell culture covered with diffused PAS-positive glycosaminoglycans. The thin layer formed is broken during rinses and cell parts underneath it become exposed. PAS. × 500

Fig. 6. Overview micrograph of cell culture with PAS-positive material. PAS × 200. Inset: PAS-positive material surrounded by trophoblasts. PAS × 400



light blue-green with the Papanicolaou staining method (Fig. 4). A similar substance was observed extracellularly as big masses and as diffuse covers of cell groups (Fig. 5). It prevented hypo-osmotic lyse effects on the cells. Twenty-day-old primary cell cultures did not lyse when exposed to distilled water for 15 minutes, while normal urothelial control cells lysed in 3 minutes. The material was PAS-positive and constituted the major component of ECM (Fig. 6).

Cytotrophoblasts initially formed small nodules and villus-like structures were later visible in the culture with the naked eye. Only cytotrophoblasts formed villus-like structures (Fig. 7).

Histology

Parallel as well as circular arrangements of cytotrophoblasts around the eosinophilic, centrally-located masses of PAS-positive material was the prominent feature. Various shapes and sizes of nuclei and cells were observed (Fig. 7).

Electron microscopy

Two cell types were observed, small cytotrophoblast and intermediate trophoblast cells. Both cell types had irregularly-shaped nuclei and prominent nucleoli. Abundant lipid droplets and exocrine granules (containing GSGs) were present in cytoplasm (Figs. 8, 9). Cytofilaments were mainly accumulated in the cell periphery. Poorly-developed junctions occasionally linked cells together. Intermediate, spindle-shaped cells

Fig. 7. Hematoxylin and eosin-stained section of villus-like structure from cell culture. Hematoxylin and eosin. × 200

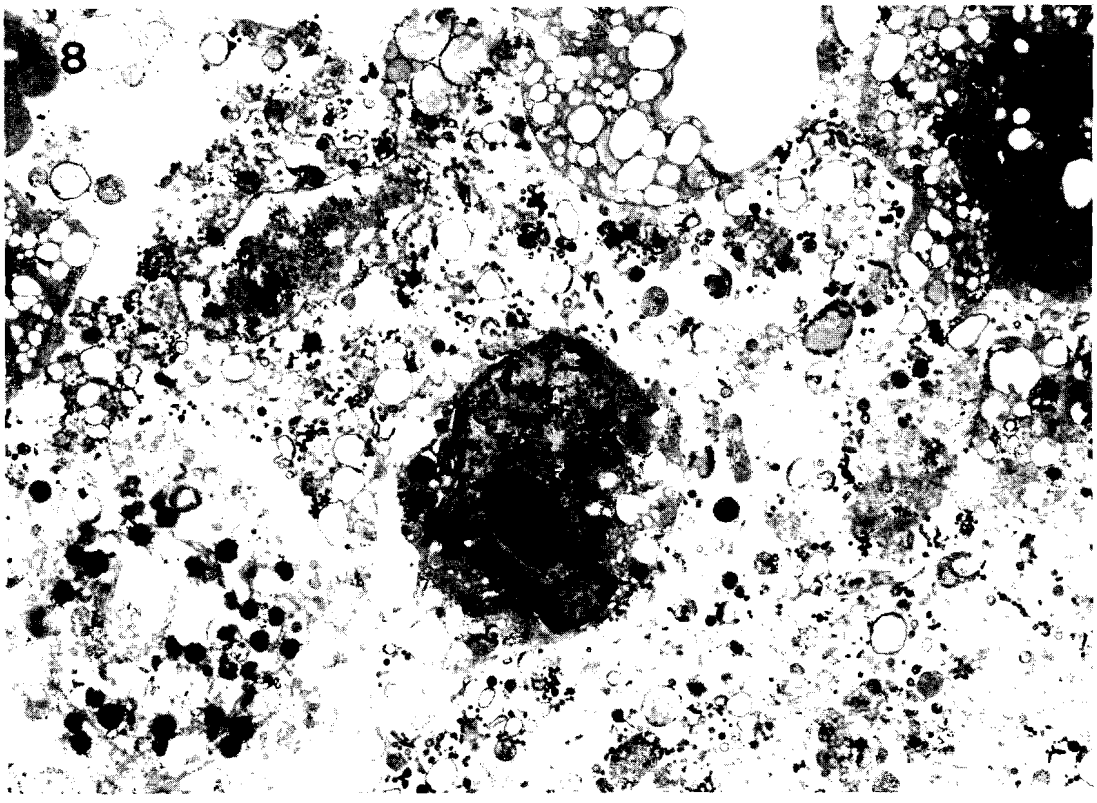


Fig. 8. Cytotrophoblasts with numerous lipid droplets and excretory vacuoles. Uranyl acetate and lead citrate. × 3,960

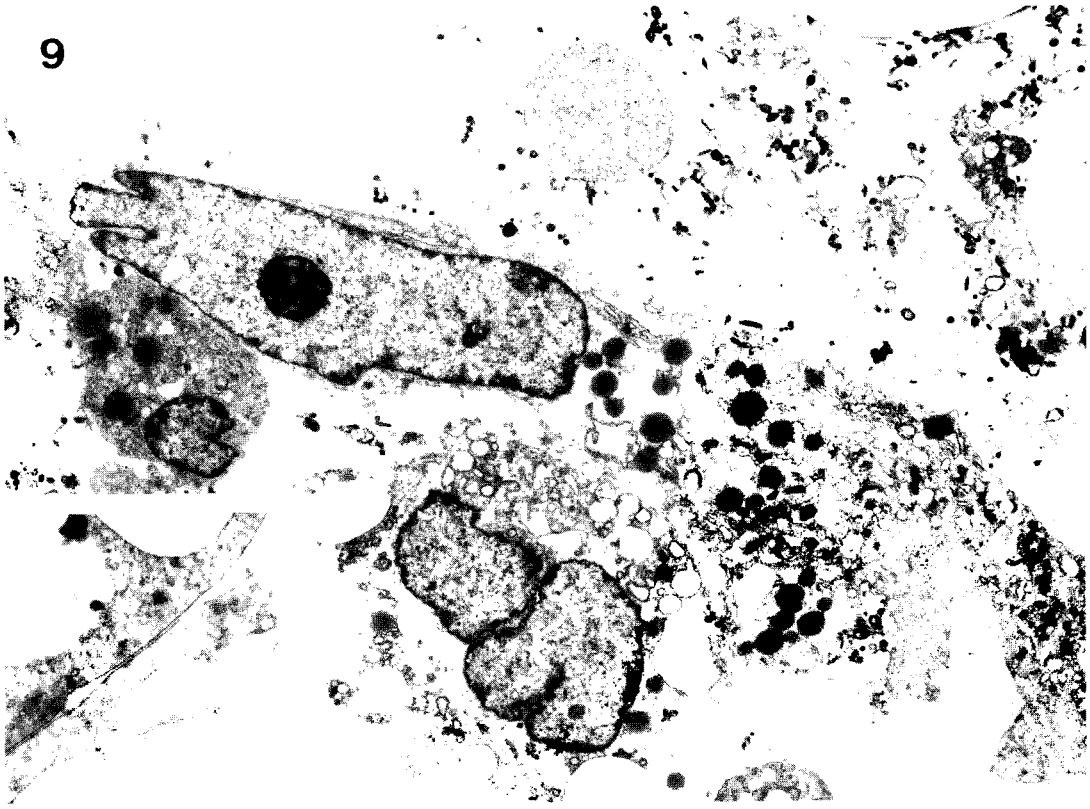


Fig. 9. Intermediate spindle shaped cell next to cytotrophoblasts. The cells contain well developed rough endoplasmic reticulum and abundant lipid droplets. Uranyl acetate and lead citrate. × 3,960. Inset: Higher magnification of two cytotrophoblasts. No junctions are present in the area. Bundles of fine filaments are located between the accumulation of excretory vacuoles and the cell membrane. Uranyl acetate and lead citrate. × 13,200

Fig. 10. Keratin-positive cytotrophoblasts and intermediate cells. Antikeratin. × 200.

possessed double sized nuclei and more cytoplasm as compared to those of cytotrophoblasts (Figs. 8, 9).

Excretory granules and a medium electron-dense material were present in between the cells.

Immunocytochemistry

All cell types, syncytiotrophoblasts, cytotrophoblast, and intermediate cells were strongly immunostained for keratins (Fig. 10), lysozyme and alpha-1-antichymotrypsin. The same cells showed moderate to strong immunostaining for HCG, with the strongest reaction in binucleated cytotrophoblasts (Fig. 11). A weaker immunoreactivity was exhibited for NSE, protein S-100 and CEA.

Incubation with non-specific serum as first layer

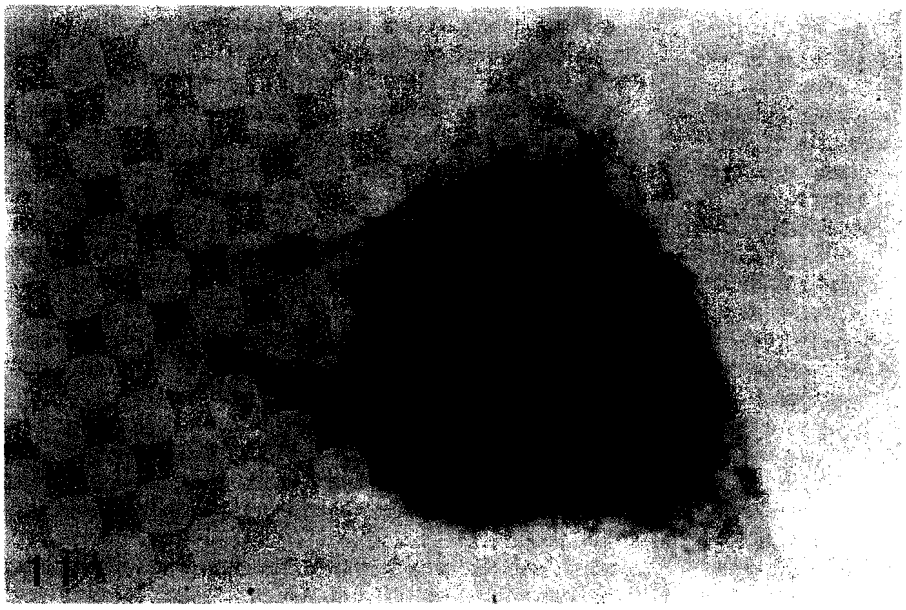


Fig. 11. HCG immunoreactivity in binucleated cytotrophoblast. Anti HCG. × 1,250

instead of specific primary antibody resulted in no staining of the cells. Control cultures immunostained with the relevant antibodies showed satisfactory results.

Karyotype

The karyotype of all villi cells processed was normal.

Discussion

The transformation of cytotrophoblasts into syncytiotrophoblasts has been difficult in vitro because of the lack of suitable cell preparations and cell systems (Feinman et al., 1986). Thiede (1960) described three types of cells, epitheloid, multinucleated and fibroblasts, while Valenti (1963) reported that both epitheloid and syncytial cells disappeared as a result of stromal overgrowth. With our technique, which is a modification of the successful method described by Cottè and co-workers in 1980, we did not have this problem for the 5 trophoblast cell cultures presented in this report. Trophoblast cell growth was good enough for the present cytological, immunocytochemical and ultrastructural study. It is difficult to compare our observations with those of previous workers (Lueck and Aladjem, 1980; Cottè et al., 1980), since their morphological observations were based on phase-contrast microscopy of live cell cultures. Phase-microscopy cannot distinguish a fibroblast from a fibroblast-like epithelial cell. Papanicolaou-staining of our cell cultures allowed cytological details and comparison between the different cell types while immunostaining confirmed their origin.

Recent studies have revealed that cytotrophoblasts aggregate in culture and then fuse to form syncytiotrophoblasts (Lueck and Aladjem, 1980; Kliman et al., 1986). We observed that syncytial cells consisted of different sized nuclei (anisokaryotic). The large syncytial nuclei were of the same size as those possessed by the intermediate cells, while the syncytial small size nuclei corresponded to the nuclear size of small cytotrophoblasts.

The ultrastructural study further confirmed the nuclear size difference between the small cytotrophoblast and the intermediate spindle-shaped cells. The nuclear size of the cytotrophoblast was half the size of the intermediate nucleus. Isokaryotic syncytial cells possessed small nuclei of the same size as those of cytotrophoblasts. These observations suggest that primary cytotrophoblasts grow rapidly, forming binucleated or mononucleated intermediate cells as well as nodules and villi. Syncytial cells were formed by fusion of intermediate cells with different sized small cytotrophoblasts or by fusion of only small cytotrophoblasts, indicating that there is no cell type specificity on cell fusion. Cells grown beyond confluence differentiated into villi with a villus-like histology, as also described by Nelson and associates (1986) using immunofluorescent, autoradiographic and biochemical techniques.

In the mixed cytotrophoblasts and intermediate cell cultures, the two cell types did not grow in separate groups but made contact with each other. In addition, the two cell types showed the same intracellular matrix, indicating that the origin of this intermediate cell is probably from synkaryon fusion of two cytotrophoblasts, and is certainly not a fibroblastic contaminant. It has been suggested that the fibroblast-like appearance of these cells is due to their active cell movements (Lueck and Aladjem, 1980).

Cyto- and syncytiotrophoblasts as well as intermediate cells expressed immunoreactivity for keratin filaments and CEA, thus confirming their epithelial nature. This is in agreement with recent work which has shown that trophoblasts are keratin positive and negative for vimentin and desmin at all stages of

pregnancy (Khong et al., 1986).

The immunoreactivity for NSE and protein S-100 confirms the endocrine feature of trophoblastic cells.

The different intensity of HCG-immunoreactivity between binucleated cytotrophoblasts and syncytiotrophoblasts supports the theory that these cells undergo both morphological and functional differentiation in culture (Kliman et al., 1986). It may also mean that HCG in syncytiotrophoblasts is synthesized and released continuously whereas it is stored longer in granules in the binucleated cytotrophoblasts. Intermediate cells were HCG-positive in this study, while Tuttle and co-workers (1986) found the same cells to be mainly HCG-negative.

In this study we have shown that trophoblasts are immunostained for lysozyme, which may act as a local protector for the embryo against bacteria.

Glycosaminoglycans (GSG) have previously been detected biochemically in solubilized villus membranes of human placental syncytiotrophoblasts (Okamura et al., 1981) and in the present report attention is drawn to the cellular location and distribution of GSGs. GSG accumulations in our cell cultures were observed in the early nodule formation, which then developed into villi with longer continuous culture incubation.

The fact that the lining cells to GSG accumulations were round may indicate an enzyme activity. The GSG layer prevents water penetration and may inhibit the in vitro cell growth because of insufficient cell nutrition. Thus cell culture subcultivation once a week was found to help cell propagation by breaking the extracellular matrix.

GSGs were produced by cytotrophoblasts and released extracellularly. In syncytiotrophoblasts, GSG accumulations were found in well-defined membranous sacs and looked exactly the same as in malignant bladder cell cultures (Logothetou-Rella et al., 1988 a,b). The cytology of trophoblastic cells presented here is also similar to that of malignant cells, and villi resembled tumour nodules formed by malignant cell cultures (Logothetou-Rella et al., 1988 a,b). Yet, trophoblastic cells show normal karyotype.

Acknowledgements. The authors are grateful to Mr. P. Apostolopoulos for the EIA of the culture medium. This work was supported by the General Secretary of Research and Technology, grant 87EA26, Greece.

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Accepted March 1, 1989