Establishment and Characterization of a Tumorigenic Trophoblast-like Cell Line from a Human Placenta

Chi-Kuan Ho, Hung Chiang, Shuan-Yow Li, Chiou-Chung Yuan, and Heung-Tat Ng

ABSTRACT

A trophoblast-like cell line, TL, was established from a normal-term human placenta. The TL cells were epithelial in morphology with relatively large vesicular nuclei, prominent nucleoli, and numerous microvilli on the cell surface. Cytoplasmic organelles were generally sparse but mitochondria and polyosomes were abundant. The cells grew as compact sheets with close membrane approximation interconnected occasionally by desmosome-like junctions. TL cells contained placental alkaline phosphatase, a placenta-associated antigen, cytokeratin, and prekeratin, but not keratin. In parallel, they were negative for factor VIII, vimentin, and fibronectin. Population doubling time was estimated to be about 34 h. TL cells were tumorigenic in nude mice and an increase in tumorigenicity was observed after a certain number of passages in vitro. Chromosome analysis revealed that TL cells were highly heterogenous and had a female aneuploid karyotype with a hypotriploid mode. Unlike trophoblastic cell lines established from neoplastic tissues, TL cells did not synthesize human choricgonadotropin or other gonadal hormones, and only a small amount of ferritin (40 ng/10^6 cells) could be detected in the cell supernatant and cell extract. Based on various morphological and histochemical criteria, we suggest that the TL cells are derived from the Langhans cells (villous cytotrophoblast), and due to their special features, the cells may be valuable for the study of the differentiation and tumorigenesis of trophoblastic cells.

INTRODUCTION

The placenta is regarded as a temporary endocrine organ that nourishes the fetus with necessary gonadal hormones, allows appropriate physiological exchanges between mother and fetus, and provides a barrier from the maternal immune system. The trophoblast consists of two layers, the syncytiotrophoblast and the cytotrophoblast. Indirect evidence suggests that syncytiotrophoblastic cells are derived from the cytotrophoblastic cells which gradually diminish in number and biological activities towards the maturation of the conceptus (1-3). Furthermore, GTDs presumably originate from trophoblastic cells which undergo neoplastic transformation at certain stages of differentiation (4). Despite the important role of the placenta, not much is known about the differentiation profile of the trophoblastic cells and the same is true for the etiology and tumorigenesis of GTDs.

A number of human choriocarcinoma cell lines have been established (5-8) and have provided valuable information about the nature of this endocrine tumor. However, models suitable for the study of the biological and functional properties of normal and preneoplastic trophoblastic cells are not yet available.

The present report describes the establishment and characterization of a trophoblast-like cell line (TL) from apparently normal placental tissue. This cell line will provide important information in respect to the functional properties and the differentiating characteristics of trophoblastic cells and may be a valuable tool for the study of the tumorigenesis of GTDs.

MATERIALS AND METHODS

Placental Tissue. Human placentas were obtained as soon as feasible after normal-term delivery (usually within 3 h) from apparently healthy women admitted to the department of Obstetrics & Gynecology, Veterans General Hospital. A tissue fragment of about 6 cm^2 was excised with aseptic scalpels from the trophoblastic portion of each placenta for primary culture and, a total of over 30 placentas have been used for the present study.

Tissue Culture. The amniotic and chorionic membranes of the excised placental tissues were carefully removed under sterile conditions and were divided into 2-mm^3 pieces with a pair of surgical scissors. The fragments were then extensively washed with sterile HBSS to remove contaminating RBC. The fragments were then suspended in 100 ml of 0.25% trypsin (Difco;1:250) in Ca++ and Mg++-free HBSS containing 0.025% EDTA and constantly stirred by a magnetic stirrer at 37°C. At 30-min intervals, disaggregated cells in the supernatant were harvested by centrifugation and undigested tissues were subjected to further trypsinization. Harvested cells were washed twice with HBSS and then cultured in α-MEM supplemented with 10% FBS (Gibco Co.), 5% pooled human AB serum, 2 mm glutamine, 25 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and antibiotics (20 μg/ml of gentamicin, 100 units/ml of penicillin, and 100 μg/ml of streptomycin) in 100-mm tissue culture plates (Costar No. 3100) at 37°C in a humidified 5% CO_2 incubator. Fresh medium was fed as necessary when pH began to drop, and serial subculturing of cells was performed by trypsinization with trypsin-EDTA.

Cytotoxicity Studies. TL cells were grown on sterile glass coverslips (20 x 20 mm^2) until confluency and then fixed with either acetone or 10% neutral formalin at 4°C for 5 min. The cells were then stained with Giemsa, hematoxylin-eosin, and periodic acid-Schiff stains by conventional methods. The presence of cell-associated keratin (Dako No. M615), cytokeratin (Dako No. M717), prekeratin (Dako No. A622), vimentin (Dako No. M725), fibronectin (Green Cross, Osaka, Japan), placental alkaline phosphatase (Green Cross No. 6150), and placenta antigen (Dako No. A146) were detected by the peroxidase-antiperoxidase method, and factor VIII was assayed by the method described by Sehested and Hou-Jensen (9). The secondary antibodies used in the peroxidase-antiperoxidase assay were preabsorbed with a mixture of acetone-fixed placenta and spleen powder before use. Mouse TL tumor fragments and normal placental tissues embedded in paraffin were studied in similar manners. The specificity of the various antisera was confirmed using tissue sections from human epidermis (for keratin, cytokeratin, and prekeratin), fibrosarcoma (for vimentin and fibronectin), placental tissue and products of conception (for placental common antigen and placental alkaline phosphatase), and capillary hemangioma (for factor VIII), and when the same tissue sections were used as negative controls, no cross-reactivity was observed.

Inoculation into Nude Mice. Confluent monolayers of TL cells were scraped off the culture plates with the aid of sterile rubber policeman. The cells were washed twice in sterile phosphate-buffered saline at pH 7.4 and resuspended in the same buffer, and 0.2-ml aliquots of cells (1 x 10^6 cells) were then injected s.c. into 5-week-old BALB/c nude mice in groups of 2-5. The animals were examined twice weekly for the development of palpable tumors.

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The abbreviations used are: GTD, gestational trophoblastic disease; hCG, human chorionic gonadotropin; HBSS, Hanks' balanced salt solution; MEM, Eagle's minimum essential medium; FBS, fetal bovine serum; TC, trophoblastic cell.
Chromosome Analysis. Cells at the exponential phase of growth were treated with Colcemid (0.6 μg/ml), and the G-banding pattern of the cells was obtained using the method of Seabright (10). The number of chromosomes of 81 metaphase cells was scored.

Electron Microscopy. TL cells were grown to confluency and then fixed in situ with 2.5% glutaraldehyde in 0.2 M cacodylate buffer for 1 h at room temperature and were then scraped off the culture plate. The cells were then pelleted, postfixed in 1% osmium tetroxide, dehydrated, and then embedded in Epon. Ultrathin sections (60–80 nm) were cut, stained with uranyl acetate and lead citrate, and examined under a Zeiss 10C electron microscope. Mouse TL tumors and normal placental tissues were treated in the same manner for comparison.

Estimation of Doubling Time. Cells were seeded at an initial concentration of 2 x 10^5 cells/ml in 24-well microtiter plates (Linbro No. 76-065-05) and cultured in α-MEM supplemented with 10% FBS and antibiotics. At various time intervals, the cell number of duplicate cultures was estimated either by the trypan blue exclusion test on trypsinized cells or by the uptake of a neutral red solution according to the method of Finter (11).

Preparation of Cell Extract and Supernatant. Cells were grown to confluency in 100-mm tissue culture plates, and the spent medium was then replaced by media with or without 10% FBS. The cells were further incubated for 72 h under normal culture conditions, and the supernatants were harvested for assay of hormones and various antigens. The remaining cells were dislodged by trypsin and pelleted by centrifugation. Cell extract was prepared by sonification using the method of Sekiya et al. (8).

Biochemical Studies. Cell supernatants, cell extract, and sera from tumor-bearing nude mice were assayed for the level of hCG, estradiol, prolactin, α-fetoprotein (Serono Diagnostics SA, Aubonne, Switzerland), human placental lactogen (Pharmacia Diagnostics-AB, Uppsala, Sweden), estradiol (New England Nuclear, DuPont Co.), progesterone (Sorin Biomedica, Saluggia, Italy), ferritin (Gamma-Dab; Travenol-Land), human placental lactogen (Pharmacia Diagnostics-AB, Uppsala, Sweden), and carcinoembryonic antigen (International CIS, Cedex, France) by standard radioimmunoassay according to procedures recommended by the suppliers.

RESULTS

Establishment of the Cell Line TL. The trophoblast-like cell line designated TL was established in vitro from a placenta of normal-term delivery. The patient was a 29-year-old woman, J. L. J., who was admitted to the Veterans General Hospital on March 21, 1983 and gave birth to a 3850-g female baby on the following day. The woman had no previous record of mole or other obstetric abnormality. Prenatal examination revealed that both mother and infant were in good health.

Two weeks postinitiation of the placental culture, an epidermoid-like cell colony was observed in a culture plate among scattered colonies of fibroblasts. The medium of the culture was fed daily to maintain a relatively alkaline pH and no attempt was made to remove the fibroblasts. In about 3 weeks, the epidermoid cells replicated rapidly and after 2 passages, fibroblasts were totally eliminated and did not reappear in subsequent passages. The cell line had been maintained continuously for over 3 years, and samples of cells at various passages were stored in liquid nitrogen for future studies.

Growth Characteristics. The cells grew well in either α- or Dulbecco's MEM with high glucose and high bicarbonate plus 5–10% FBS and had a population doubling time of about 34 h as estimated by direct viable cell count or by a neutral red uptake assay (data not shown). Optimal cell growth was obtained with an initial inoculum of 2–5 x 10^4 cells/ml. When cultured in serum-free medium, the cells could be maintained for an extended period of time (1–2 months) without appreciable decrease in cell viability. These characteristics were observed at or about the 120th passage.

Cell Morphology. As shown in Fig. 1, TL cells grew as monolayers with an epithelial-like general morphology. The cells appeared to be heterogeneous and pleomorphic depending on cell density and the culture conditions. TL cells tend to be elongated at low density but will become polygonal or ovoid in shape at confluency or under adverse culture conditions. Electron micrographs of TL cells at the 254th passage revealed that the cells were roughly ovoid in shape with prominent nuclei, sparse chromatins granules, and well defined nucleoli (Fig. 2). Cytoplasmic vacuoles were numerous and finger-like microvilli could be seen at the cell surface and at the intercellular spaces. The cytoplasm was generally sparse in organelles, but mitochondria and free polysome-like particles were abundant and distended membranous structures with or without association with polysomes could sometimes be observed. However, prominent endoplasmic reticulum, Golgi apparatus, and tonofilaments were not detected. Weibel-Palade bodies and bundles of fine filaments which are markers of endothelial cells were also undetectable. Intercellular membranes were aligned in close approximation but interconnection by desmosome-like junctions was scarcely observed (Fig. 2B). Multinucleated giant cells with up to about 20 nuclei could be seen but were very few in number.

Tumor Formation in Nude Mice. When aliquots of TL cells (10^7 cells/mouse) were injected into nude mice, minute palpable tumors (2–3 mm^3) could be seen at the sites of injection in 3–4 weeks. The tumors grew slowly and reached the size of 6–8 mm^3 in about 2 months. The cells were not metastatic and tumor take was about 80% (16 of 20 mice). The same results were obtained with cells at both the 17th and the 186th passages. However, increased tumorigenicity was observed when cells were tested at the 350th passage such that in all mice (6 of 6) given injections of TL cells (10⁷/mouse), nodules (2–3 mm^3) became apparent at the inoculation sites in about 3 days and progressively developed into clusters of multiple tumors (4–6 in number) up to 24 mm^3 in about 4 weeks; all mice eventually died in about 6 weeks. Sections of such tumors revealed similar phenotypic characteristics as those from earlier passages except that necrosis was found in the center of all larger size (about 12 mm^3) tumors (not shown). The morphological features of a TL tumor at the 186th passage are shown in Fig. 3. The tumor cells grew in compact nests or sheets and were aligned in a mosaic pattern resembling that of human

Fig. 1. Morphology of TL cells at passage 156 as observed under a phase contrast microscope. Cells appeared ovoid or polygonal in shape with clearly visible vacuoles, large nuclei and 1–2 prominent nucleoli, and were arranged in close approximation. × 400.
ESTABLISHMENT OF A HUMAN TROPHOBLAST-LIKE CELL LINE

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The cells had relatively large nuclei and prominent nucleoli, and in most cells vacuoles could be seen which sometimes extended to form a continuum of empty space although syncytia formation was uncommon. The vacuoles were not electron dense, not stained with PAS stain, and were presumably empty. Infiltration by mouse lymphoid cells or fibroblasts was inapparent and vascularization was sparse. The electron micrograph of TL tumor cells was very similar to those of cells grown in vitro and to the villous cytotrophoblastic cells of normal term placental tissues (Figs. 2, 4, and 5).

Chromosome Studies. Chromosome analysis revealed that the TL cells were highly heterogeneous and had a human hypotriploid karyotype with a modal number of 65 from a chromosome count of 81 metaphase cells ranging from 52 to over 81 as shown in Fig. 6. The predominant karyotype was as follows: 65 XX, +2 Iso(Xp), -2, +3, +5, +6, +8, +9, -10, -10, +Iso(10p), +11, +12p, +14, -15, -16, +19, +19, +19, +20, +20, -21, +22, plus 5 abnormal marker chromosomes as indicated in Fig. 7. Marker chromosomes could be found in all cells examined with numbers ranging from 2–5. The origins of the markers have not been determined in the present study.

Histochemical and Biochemical Studies. As indicated in Table 1, both in vitro and in vivo passaged cells contained placental alkaline phosphatase, a placental common antigen, cytokeratin, and prekeratin in their cytoplasms but lacked keratin. A similar pattern of reactivity was found on the villous cells (trophoblastic cells) from normal placental tissues except that syncytiotrophoblastic cells displayed a strong reaction to antibody against keratin. Correspondingly, the cells were negative for factor VIII, vimentin, and fibronectin which could be detected on endothelial and stromal cells respectively (Table 1). These results suggest that TL cells are placental epithelial cells in origin and probably at an earlier stage of differentiation.

When culture supernatant, cell extract, and sera from tumor bearing mice were assayed for the presence of different gonadal hormones and fetal antigens, it was found that TL cells were unable to synthesize any of the hormones and antigens tested and only a small amount of ferritin (40.3 ng/10⁶ cells) could be found in the culture supernatant and cell extract.

DISCUSSION

Among the different cell types present in the placenta, TCs deserve special attention, not only because the syncytiotrophoblast is a major source of placental hormones but also because cytotrophoblastic cells are causally related to GTDs (3, 12). Thus far, all
established trophoblastic cell lines are derived from neoplastic tissues and have phenotypic and functional characteristics resembling those of syncytiotrophoblastic cells or the peripheral cytotrophoblastic cells (5-8, 13). However, in order to understand the differentiation profile and functional diversities of the various types of TCs, it is important to study them at the stem cell level.

The present report describes the establishment and characterization of a trophoblast-like cell line from a normal-term human placenta. This cell line, designated TL, has general properties reminiscent of the villous cytotrophoblastic cells. This assumption is based on the following observations: (a) the cell line is derived from placental tissue and bears 2 specific placenta-associated antigens (Table 1); (b) TL cells can be easily identified as immature epithelial cells based on cell morphology, the pattern of cell arrangement, and the interconnection by desmosome-like junctions. Furthermore the cells contain cyto-keratin and prekeratin but lack histological markers normally associated with endothelial cells (factor VIII) and fibroblasts (vimentin and fibronectin); (c) ultrastructurally, TL cells have a simple cytoplasmic structure with no defined endoplasmic reticulum or Golgi but have abundant mitochondria and polysomes (Figs. 2 and 4). This description tends to rule out the association of TL cells with the more mature types of TCs (13). In addition, TL cells are unable to synthesize gonadal hormones; (d) TL tumors are histologically similar to human carcinoma and have ultrastructural characteristics very similar to villous cytotrophoblast from normal-term placental tissues (Figs. 3 and 4).

In the present study, it remains obscure as to when the TL cells are transformed. However, two possibilities may exist. One is that TL cells are spontaneously transformed in vitro and the other is that they are transformed sometime within the gestational period. In view of the relatively short transition (3 weeks) from initiation to the active proliferation phase of the TL primary culture and the fact that neoplastic TC foci have...
establishment of a human trophoblast-like cell line

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* Experiments were performed with cells at passages 9, 105, 233, and 268, and TL tumors were derived from cells at the 17th, 186th, and 350th passages. Similar patterns of reactivity were obtained in all experiments and results were expressed as either positive (+) or negative (−).

been detected in few apparently normal placentas (14), we tend to favor the latter hypothesis. The fact that the patient from whom the placenta is derived is presently free of GTDs does not argue against the possibility that TL cells are transformed in vivo since infiltrated neoplastic TCs may take years to develop into GTDs after the causal pregnancy (14). Furthermore, the probability of selecting the clone(s) of transformed cells from an apparently normal placenta that can grow or can be adapted to grow in vitro must be small since only one other cell line, WISH, is known to have derived from a normal placenta (15) despite the popularity of using placental tissues as research tools.

The expression of the hCG genes by neoplastic TCs has been found to correlate well with their degree of malignancy (16–17), and it is also well established that the level of serum hCG often parallels the prognostic evaluation of GTDs (18–20). Furthermore, recent evidence suggests that tumorigenicity of TCs is linked to the expression of the myc and sis protooncogenes (21–22). All these findings suggest that tumorigenicity of the TL cells have increased following prolonged cultivation in vitro (at or about the 350th passage). This implies that the TL cell line will be a valuable model for the study of the process of malignant changes and the various parameters involved.

To our knowledge, the TL cell line is the only one reported thus far that originates from the chorion of the placenta. The special features of this cell line make it valuable for the study of the differentiation profile, functional properties, and possibly the oncogenesis of TCs.

REFERENCES

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