MORPHOLOGICAL AND BEHAVIOURAL FEATURES OF BeWo CELLS GROWN ON MATRIGEL OFFERS A MODEL FOR HUMAN CYTOTROPHOBLAST CELLS DURING EARLY IMPLANTATION

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ABSTRACT
In order to observe the morphological changes that occur in cell-cell and cell-matrix interactions among trophoblast cells and other neighbouring cells during human implantation, BeWo cells were cultured in a 3-dimensional artificial extracellular matrix (Matrigel) using a double-chamber system. These cultures were then processed for light and electron microscopical examination. Results obtained show that the morphology and behavioural pattern of BeWo cells grown on Matrigel is similar to that reported for in vitro normal human cytotrophoblast cells on extracellular matrix (ECM). These results suggest that culture of BeWo cells on extracellular matrices may be useful for the study of some of the early embryonic events leading to human implantation, especially during the period when trophoblastic cells interact with and erode the uterine epithelium and ECM.

Keywords: BeWo cells, Trophoblast, Morphology, Matrigel, Implantation

INTRODUCTION

The trophectoderm is the first element of the morula to differentiate as it develops into a blastocyst and it forms an encircling layer around the blastocyst (Loke and King, 1995; Wang *et al.*, 2004). The *in vivo* attachment of the blastocyst to surface epithelium remains to be unequivocally described in humans. However, syncytial trophoblast formation is not thought to occur in preimplantation human blastocysts (Loke and King, 1995; Wang *et al.*, 2004; Valsamakiset *et al.*, 2006). It has been suggested that it is the multicellular syncytium (Hertig *et al.*, 1956; Weitlauf and Suda, 1988) that invades and degrades endometrial tissue during the first few days of implantation (Vicovac *et al.*, 1995; Roh *et al.*, 2005). There is still a lot that is not known about the factors that control trophoblast differentiation and invasion. However it appears that changes must occur in cell-cell and cell-matrix interactions among trophoblast cells and other neighbouring cells. These changes might involve altered patterns of cell surface expression or activation of molecules involved in the recognition of extracellular matrix components and/or other cells.

By the 8th day after fertilization the trophoblast has differentiated into an outer multinucleated syncytiotrophoblast and an inner layer of mononuclear cytotrophoblast cells (Pijnenborg *et al.,* 1980). Very little has been reported of the events during the first few weeks of gestation in humans. However it appears that the mononuclear cytotrophoblast cells divide and fuse with the overlying syncytium to form villi and expand the surface area of the developing placenta (Aplin, 1991). Two types of villi have been observed in first trimester placentae; free (floating) and anchoring villi (Benirschke and Kaufman, 1990). Floating villi do not contact uterine tissue directly while anchoring villi connect the embryo to the uterus. This connection is established by proliferating cytotrophoblast cells protruding and breaking through the syncytiotrophoblast to form solid cores of mononuclear cells (cell columns) which fix the trophoblast to the endometrial stroma (Aplin, 1991). Once the anchoring villi are formed, some cytotrophoblast cells of these villi acquire a transiently invasive phenotype and migrate into the decidualized endometrium. These motile and invasive cells are referred to as extravillous/intermediate trophoblast cells while the cytotrophoblast cells, which remain attached to the villous basement membrane (BM), are known as villous cytotrophoblast cells (Bischof *et al.,* 1995; Vicovac *et al.,* 1995). Thus cytotrophoblast cells follow one of two differentiation pathways. (i) Villous cytotrophoblast cells form a monolayer of polarised epithelial stem cells some of which terminally differentiate to form a syncytial layer which covers the entire villous surface (Kao *et al.,* 1988; Hemmings *et al.,* 2001). (ii) Cytotrophoblast cells of anchoring villi either form syncytia or break through the syncytium at selected sites to form multilayered columns of non-polarized cytotrophoblast cells which are motile and highly invasive (Kao *et al.,* 1988).

Most of the *in vivo* information about these processes in humans, has been obtained from morphological observations of human implantation sites and first trimester placentae recovered surgically (Damsky *et al.,* 1992; Aplin, 1993). Additional information has been obtained using *in vitro* models of isolated cytotrophoblast cells and villous tissue explant culture (Bischof *et al.,* 1991; Genbacev *et al.,* 1993).

The invasion of the endometrium that occurs during implantation appears to be due to an active invasive process and not simple passive growth pressure. Cells are considered to be invasive partly by virtue of their ability to produce enzymes (Bischof and Martelli, 1992). Invasion is based on a series of complex interactions between the invading cell and various types of matrix components (Liotta, 1984; Bischof and Martelli, 1992). The possibility that trophoblast invasiveness might be associated with its proteolytic activity was proposed by Mossman (1937) several decades ago. It has been demonstrated that trophoblast cell cultures produce plasminogen activator (PA) which digests a fibrin matrix (Martin and Arias, 1982). In addition cytotrophoblast cells have been found to secrete metalloproteinases (MMPs) that degrade ECM components (Librach *et al.,* 1991). The invasion of the endometial ECM appears to be mediated by several classes of proteinases whose interaction initiate a series of proteolytic events that result in matrix degradation. Knowledge of the normal process of invasion is mainly from *in vitro* studies and the study of abnormal pregnancies resulting from both "over-invasion" as in placenta accreta and "under-invasion" which is associated with pre-eclampsia (Zhou *et al.,* 1993). There appears to be no animal model which can be used to study trophoblast invasion into the human uterus as no other species shows the same pattern or degree of invasion.

Choriocarcinoma cell lines (CCLs) are believed to be the invasive malignant counterpart of the trophoblast (Hohn *et al.,* 1992). The invasiveness of the CCLs (BeWo, Jeg-3 and JAr) grown as spheroids was tested in the Mareel-assay (Grummer *et al.,* 1989). In a later study by Grummer *et al.* (1994) the same cell lines were proven to be invasive in a general invasion assay using embryonic chick heart fragments, with JAr spheroids being

the most aggressive. On radiolabelled PF HR9 extracellular matrices, the degradation of the matrix and the release of radioactivity by BeWo cells was reported to be comparable to that of second trimester human cytotrophoblast cells (Fisher *et al.,* 1990).

In vitro studies on such human trophoblastic cell lines may provide some information about the early stages of human implantation. While there are a large number of reports dealing with the hormonal secretions of BeWo cells there appears to be little published information on the ultrastructure and growth characteristics of these cells. Therefore in order to provide a better understanding of the morphological characteristics of BeWo cells and any changes that may occur during growth on extracellular matrix and to differentiate these changes from changes that may occur during co-culture with human endometrial tissue one of the aims of this study was to characterize the histological and ultrastructural features of BeWo cells using qualitative methods. In the present study, BeWo cells were cultured in a 3-dimensional artificial extracellular matrix (Matrigel) using a double-chamber system (Hill *et al.,* 1994) to examine the histological and ultrastructural characteristics of BeWo cells grown on the artificial basement membrane gel, Matrigel and any apparent interaction between BeWo cells and between BeWo cells and matrix.

MATERIALS AND METHODS

BeWo cells on matrigel

Half millilitre aliquots of the BeWo cell (BeWo, Human Choriocarcinoma Cell Line (CCL), American Type Culture Collection; Pattillo and Gey, 1968) suspension were seeded into each Matrigel (Colloborative Biomedical Products, Bedford, USA) coated millicell-CM insert (12mm in diameter; 0.4mm pore size, Millipore Products Division, Bedford, USA) which were placed in a standard 24-multiwell cell culture plate (Costar, USA). Half a millilitre of DMEM-F12 was placed around the millicell insert and the entire plate was placed in an incubator at 37^{o}C in an atmosphere of 95% air and 5% carbon dioxide. Aliquots of the same cell suspension were seeded on 13 mm diameter glass coverslips placed in a multiwell cell culture plate and grown under the same conditions. The cells were cultured for various lengths of time (1-8 days) and the media was changed every two days. Samples were taken daily for morphological evaluation.

Preparation of BeWo cell spheroids

For initiating spheroid culture, growing cells were harvested by using 0.5% trypsin/EDTA at room temperature (as above). The cells were suspended in DMEM-F12 culturing media and approximately 7 x 10^5-1 x 10^6 cells in 10-15mls of DMEM\F12 were added to non-tissue culture grade sterile Petri dishes (90mm in diameter) and placed in an incubator at 37^{O}C in an atmosphere of 5% carbon dioxide and 95% air for 16-24 hours. The media was changed every 48 hours. Large inocula resulted in a more rapid development of spheroids while using lower numbers took longer for spheroids to grow.

BeWo cell spheroids on matrigel

Samples of the spheroids prepared from suspension culture were placed on Matrigel-coated cell inserts with 8-10 spheroids per insert and cultured under the same conditions as the monolayer of BeWo cells grown on Matrigel. Samples were taken daily for confocal, light and electron microscopy. Some human endometrial epithelial cells were grown on Matrigel and used as controls

Spheroid growth studies

To assess the ability of spheroids to attach and grow out onto a surface, some spheroids were placed in 35 mm plastic culture dishes in 5 millilitres of DMEM-F12 culturing medium and others were grown on 13 mm diameter glass coverslips placed in a multiwell cell culture plate in half a millilitre of the same culture medium. The dishes were placed in a 37^{o}C incubator gassed with 5% CO_2 and 95% air, for 7 days. The culture medium was changed every 2/3 days. At 24-hour intervals samples were fixed for confocal and scanning electron microscopy.

Tissue processing for electron microscopy

The cultured cells were washed with pre-warmed (37^{o}C) 0.1M phosphate buffer and fixed by adding pre-warmed (37^{o}C) 3% glutaraldehyde in 0.1M phosphate buffer solution at a pH 7.4 into the multiwell culture plates, left overnight in fixative at 4^{o}C and then rinsed two times in phosphate buffer. The cells were postfixed in 2% aqueous osmium tetroxide for two hours and then rinsed in distilled water. They were dehydrated in increasing concentrations of ethanol.

After dehydration, the bases of the cell inserts (containing the cells) were gently and carefully cut out with a sharp scapel. The tissue was then placed in pots and passed through two changes of propylene oxide (1.2-Epoxypropane; Fisons Scientific Equipment, Loughborough, England) and left overnight in a 50/50 mixture of propylene oxide and Epon resin (Agar Scientific Limited, Essex, UK) on a rotating mixer. This was followed by two changes of freshly prepared Epon resin mixture, three hours each change. The layers of Matrigel from the inserts were then cut into smaller pieces.

Glass knives were made using a LKB 7800 knifemaker (LKB Bromma, Sweden) and serial semithin sections (about 0.5 mm thick) were cut from Epon blocks using a Reichert (OM U3 Austria) ultramicrotome. The sections were picked up onto clean microscope slides (Chance Propper Limited, Warley, England) using a dissecting needle, numbered in sequence, dried on a hotplate at 60^{o}C and stained with 1% Toluidine blue in 1% borax for approximately 30 seconds (until a gold ring was formed around the drop of stain). They were then washed with distilled water, differentiated with 50% ethanol, dried in air for a few minutes or on a hotplate for about 5 seconds and mounted with No. 1 coverslips using DPX and examined by light microscopy.

In addition, silver-gold interference coloured ultra -thin sections (about 50-70 nm thick) were cut from the same blocks as those described above using the same ultramicrotome and collected on 3.05 mm 200 mesh copper (for standard staining) (Agar Aids, Essex, UK). Sections on copper grids were stained with a saturated solution of uranyl acetate in 50% methanol for 15 minutes in the dark, washed gently with a stream of distilled water from a dropping pipette and then stained with lead citrate for 5 minutes in a carbon dioxide-free atmosphere. The grids were then washed, dried on filter paper in a covered Petri dish and examined with the electron microscope. The grids were scanned for technically good sections and 10 electron micrographs per sample were taken in a systematic random manner using a Philips 301 transmission electron microscope (Philips, Holland) at an accelerating voltage of 60 kv. A grating replica of 2,160 lines per mm (Agar Ltd, Essex, UK) was used as a magnification standard on each negative. Negatives were developed in a dark room and viewed using a projection microscope (Carl-Zeiss, Germany).

Cytokeratin and vimentin immunostaining method (alkaline phosphatase-anti-alkaline phosphatase (apaap) technique)

BeWo cell cutures on coverslips in multiwell dishes were washed with prewarmed 0.1M phosphate buffer to remove all cell debris and then fixed in 50:50 methanol:acetone fixative for one and a half minutes. The cells were gently washed in Tris buffered saline (TBS) (pH 7.6) for five minutes within the culture dishes. The culture dishes containing cells were placed in staining jars with moistened cotton wool and covered to provide the humidified condition necessary for staining. Approximately 30 ml of either anti-cytokeratin (Monoclonal Anti-Pan Cytokeratin: Sigma Immunochemicals, Uk) at a dilution of 1:100 in Tris buffer or anti-vimentin (Monoclonal Anti-Pan Vimentin: Sigma Immunochemicals, UK) at 1:50 dilution in tris buffer was applied for two hours and then washed in TBS for 5 minutes. The cells were incubated in 30 ml of rabbit anti-mouse polyclonal antibody (RAM) (Rabbit Anti-Mouse Immunoglobulins, Dako A/S, Denmark) at 1:20 dilution for 30 minutes, washed for 5 minutes

in TBS and then incubated in mouse monoclonal alkaline phosphate-anti-alkaline phosphatase complex (APAAP: Sigma Immunochemicals, UK) at a dilution of 1:40 in Tris buffer for 30 minutes. The cells were then washed in TBS for 5 minutes. To amplify the reaction, the RAM and APAAP steps of the procedure were repeated. Two mililitres of Substrate-Fast Red solution was applied to the cells for 20 minutes. The cells were washed with distilled water, counterstained with haematoxylin for one minute, rinsed gently in distilled water and mounted with glycerine (Dakopatts, Dako Corporation, Carpinteria, CA).

Statistical Analysis

Statistical analysis was carried out using the Minitab computer package available on the University of Sheffield Main Frame network. Individual values were pooled within groups and means and standard errors were calculated for each group. N values are given in the results tables. Volume fraction data and nuclear profile axial ratios were logarithmically transformed to make them suitable for statistical analysis while data on nuclear profile diameters, were tested directly. Data were analysed using a paired or unpaired Student's t test and one-way analysis of variance as appropriate to compare differences between the groups.

RESULTS

Behaviour of cells in different culture conditions

In all cases the BeWo cells adhered to the different substrata within 12 hours of culture. On both glass and plastic, the cells flattened out and grew as continuous layers. However on Matrigel, the cells initially spread out as a sheet, but after 36-48 hours, small spaces began to form between the cells. As the interstices enlarged, some of the cells aggregated into groups interconnected by strands of cells forming a net-like pattern (Fig. 1). High seeding densities resulted in a more rapid development of cell "islands". After 72 hours the areas between the groups of cells were clear of Matrigel and they occupied a significant portion

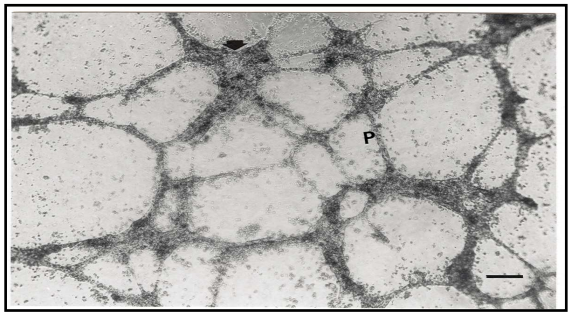
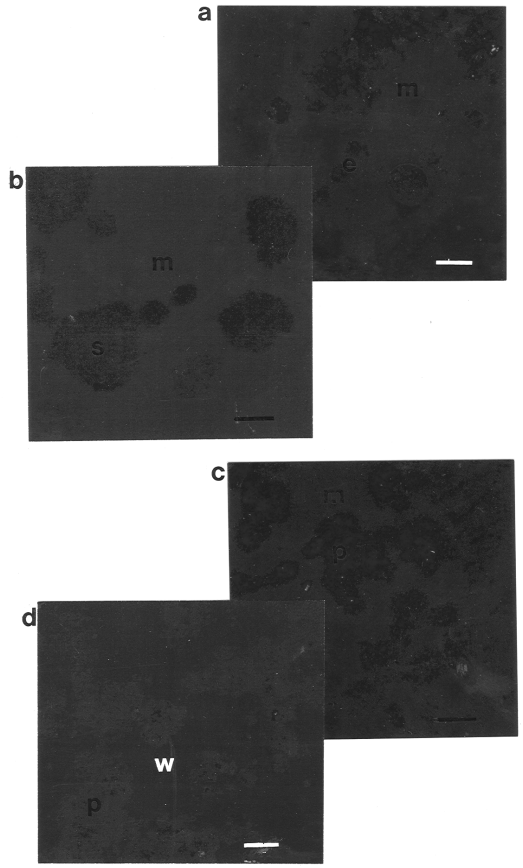


Fig. 1: *Bewo cells were plated either as a dispersed monolayer or as spheroids on cell inserts coated with matrigel. After 24 hours the monolayer of cells formed discrete multicellular aggregates (arrow) that were often connected by cellular processes (P) forming a 'net-like' pattern.*

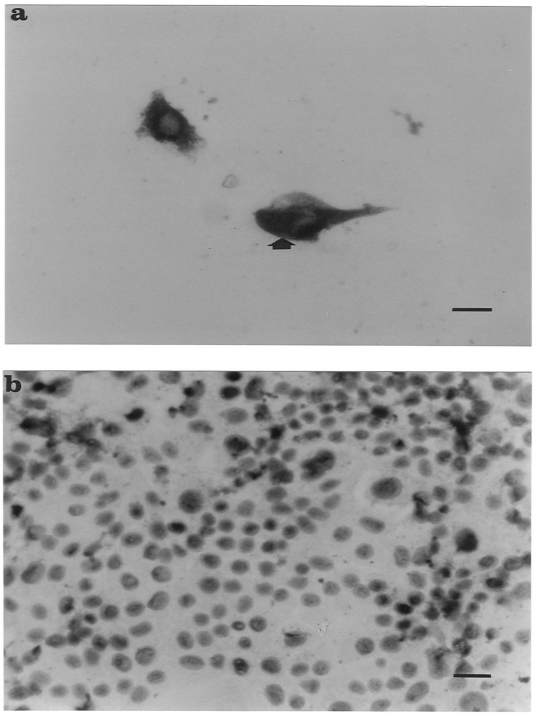
of the cell insert (Fig. 2d). The cell-free zones remained unstained with aqueous osmium tetroxide suggesting that those portions were devoid of tissue and Matrigel possibly due to digestion of the matrix in those areas. The absence of matrix between the cell "islands" was confirmed by confocal microscopy. To examine the nature of the interaction of the BeWo cell "islands" with the underlying Matrigel, the layers of Matrigel together with the cells were sectioned. Semi-thin sections showed that some of the cells had penetrated the underlying matrix. It appeared that cells which remained above the matrix surface were morphologically different compared with those which had penetrated the Matrigel.

In contrast when BeWo cell spheroids were cultured on Matrigel (BSM), no holes were seen in the layer of Matrigel even after 7 days in culture (Fig. 2b). Similarly, human endometrial epithelial cells grown on Matrigel and used as controls did not erode the gel although glandular-like structures ("organoids") formed within the Matrigel (Fig. 2a) suggesting that formation of bare areas in the BeWo cell cultures did not result from the mechanical penetration of the gel by the cells.

Cells grown on plastic, glass and in suspension culture showed a high rate of proliferation but on Matrigel they appeared to exhibit a reduced rate of



Figs. 2 a, b, c and d: *Confocal micrographs showing: (a) Endometrial cells on Matrigel after 72 hours in culture; Glandular-like structure (e); (b) BeWo spheroids (s) on Matrigel after 72 hours in culture; (c) BeWo cells (p) on Matrigel after 24 hours in culture, (d) BeWo cells (p) on Matrigel after 72 hours in culture; (W), Areas devoid of Matrigel and cells.*



Figs. 3a, b: *Immunocytochemical staining of BeWo cells after 3 days in monolayer culture: (a) Monoclonal anti-cytokeratin-APAAP, positive anti-cytokeratin staining (arrow); (b) Monoclonal anti-vimetin-APAAP, shows anti-vimentin negative BeWo cells. Bars represent 40**μm***

proliferation. Both monolayers of BeWo cells cultured from isolated cells and spheroids were anti-cytokeratin positive and anti-vimentin negative (Fig. 2a and 2b).

Outgrowth determination

BeWo cell growth was also assessed by the ability of the spheroids to attach and grow out onto culture dishes. Both day one and day seven spheroids attached to culture dishes and began to show cellular outgrowth within 24 hours. This was confirmed by scanning electron microscopy. After 7 days in culture the attached spheroids showed outgrowths which extended to a distance equal to about the radius of the spheroids. The outgrowth usually consisted of more than one layer of cells. Cytoplasmic projections were seen along the edge of the outgrowth and some of the cells appeared to be covered with ‘‘spiky’’ cytoplasmic projections (Fig. 4).

Morphological characteristics of BeWo cells grown on matrigel.

Sections from Matrigel cultures showed sheets or islands of cells. In the early stages of growth (days 1 and 2) a single monolayer of smooth-edged cells

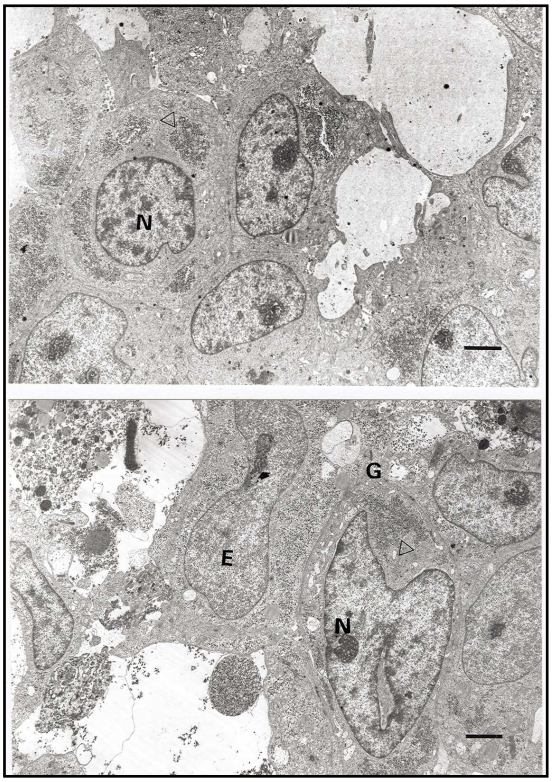


***Figure 4 (Top)**: Transmission Electron micrograh of BeWo cells grown on Matrigel. Cells have regular oval shaped nuclei (N). Glycogen (arrowhead) is seen throughout the cells.*
***(Bottom)**: Electron micrograh of BeWo Sheroids grown on Matrigel showing irregular shaped euchromatic nuclei (E) and prominent nucleoli (N). Golgi bodies (G) glycogen (arrowhead) and thread-like structures (arrow) can be seen. (Uranyl Acetate-Lead Citrate staining; Bar represents 4um*

formed. After three to four days, multilayering began and the basal surface of the islands became irregular and oriented downward from the basal surface of the island into the Matrigel. Also the basal surface of the cells had processes which penetrated into the matrix. At certain points of contact with the processes, Matrigel showed signs of disruption that were not visible when cells were absent or when endometrial epithelial cells or stromal cells were cultured on this substrate. Migration of whole cell islands through the full depth of the Matrigel was not observed. However, single cells and some smaller sheets of cells were seen deeper within the ECM. Individual necrotic cells were also seen at all stages in culture. There was no development of central necrotic zones in the cell islands even after 12 days of culture.

Electron microscopic examination of the cells on Matrigel showed that surface cells were polarised with irregular microvilli. All nuclei were large and oval in shape. They were euchromatic with a rim of heterochromatin. Prominent nucleoli were seen and the nuclear envelope was generally smooth but with a few invaginations. Few Golgi bodies were apparent and sparce rough and smooth endoplasmic reticula were seen. Mitochondria were observed throughout the cytoplasm. Patches of glycogen and lipid droplets were seen in the cytoplasm which appeared associated with each other at some locations (Fig. 5). Cytoplasmic projections were restricted to the basal surface of the layer of cells in contact with the Matrigel.

DISCUSSION

In the present study, a three-dimensional cell culture model of isolated cells was used to examine

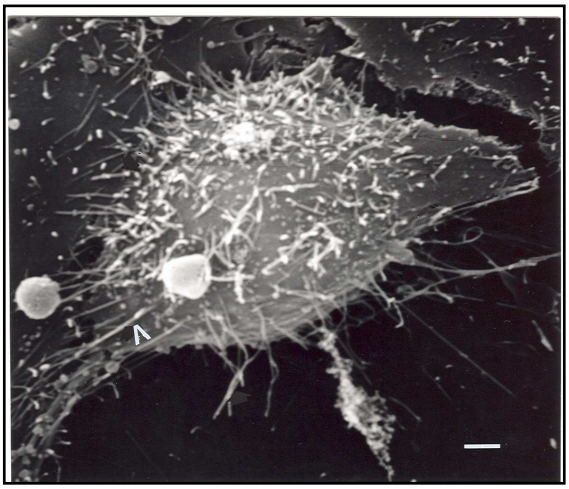


Fig. 5: Scanning electron micrograph of BeWo spheroids grown on coverslips showing spiky processes (arrowhead) on the surface of the cells.
*Bar represents 4**µm***

the morphology of BeWo cells, a human trophoblast cell line (BeWo). Their growth and interaction with Matrigel were also examined in an attempt to model aspects of trophoblast cells thought to be relevant to the attachment and invasion of endometrium around the time of implantation. Qualitative study of BeWo cells on Matrigel (BCM) showed multilayers with polarised cells on the surface of the gel and non-polarised ones within the gel. These cells are comparable to human villous and extravillous cytotrophoblast cells respectively.

The present study has shown that BeWo cells can attach to plastic, glass and Matrigel. The attachment of these cells to plastic and glass was extensive. Aggregates were formed on Matrigel, interconnected by strands of cells forming a net-like pattern, and their number increased with time in culture. Migration of adjacent cells into groups ("islands") may also have contributed to aggregate formation. The formation of BeWo cell aggregates is reminiscent of the formation of cytotrophoblastic columns at implantation (Pijnenborg *et al.,* 1981). These observations are similar to previous reports on BeWo cells and human cytotrophoblast cells. For example, Aplin and Charlton (1990) reported that BeWo cells grow in aggregates which are formed by a combination of cell division and joining up of cells into groups. They also observed that the plating density influenced island formation, as in the present study. In addition Crescimanno *et al.* (1996) showed that BeWo cells formed small rounded clusters with elongated ragged edges and large zones devoid of cells on Matrigel. There are numerous publications indicating that cytotrophoblast cells isolated from first trimester placenta form large aggregates when cultured on Matrigel (Damsky *et al.,* 1994; Hemmings *et al.,* 2001). Bischof and colleagues (1991) demonstrated that cytotrophoblast cells aggregate in a net-like pattern when grown on Matrigel. Librach *et al.* (1991) found that first trimester cytotrophoblast cells plated on Matrigel formed large multicellular aggregates connected by long cellular projections; the cells at the periphery of the aggregates had extensive processes which extended outward as *invadopodia*. It appears therefore that normal and malignant human trophoblast cells behave in a similar manner in culture.

Observations of the BeWo cells on Matrigel showed that the boundary of the cell islands was polarised, with apical microvilli, whereas the intermediate and basal cells were not. These polarised epithelial-like cells resemble human villous cytotrophoblast cells and the non-polarised ones, extravillous cytotrophoblast cells. Both monolayers of BeWo cells cultured from isolated cells and spheroids were anti-cytokeratin positive and anti-vimentin negative, indicating their epithelial nature.

These findings are in agreement with many other reports. Aplin and Charlton (1990) showed that BeWo cells are morphologically heterogenous, containing both polarised epithelial-like cells and fibroblast-like cells. The latter could adhere and migrate on fibronectin while the former were significantly weaker in these aspects (Aplin *et al.,* 1992). These authors commented that interconversion between the cell forms occurs. From the present work, the epithelial-like cells may represent villous cytotrophoblast while the fibroblast-like cells may be consistent with the extravillous phenotype. It has been reported that during implantation in humans, the trophoblast layer is transformed from a polarised epithelium of villous cytotrophoblast cells to multilayered columns of non-polarised cytotrophoblast cells (Vicovac and Aplin, 1996). Thus it appears that during placental development *in vivo*, a subpopulation of cytotrophoblast cells undergoes an epithelial-to-mesenchymal conversion and loses contact with the villous BM. BeWo cells grown on artificial ECM appear to follow a similar behavioural pattern. Bulmer *et al.* (1988a) reported that choriocarcinoma cells show intense positive cytokeratin staining. The infiltrating trophoblast cells continue to express a repertoire of cytokeratins (Vicovac and Aplin, 1996). Loke and Burland (1988) have demonstrated that cytotrophoblast cells are cy-

tokeratin positive. In addition Genbacev *et al.* (1992) showed that cytotrophoblast cells which proliferate from villi explants *in vitro* also are cytokeratin positive.

In the present study, light microscopy of the cultures on Matrigel showed cells forming layers and spreading. Single cells and some smaller sheets of cells were seen deeper within the extracellular matrix, suggesting that they migrated into it. These migratory cells probably arose by elongation and detachment from the basal surface of the cell islands. The appearance of single cells within the Matrigel in the monolayer BeWo cell cultures raises the possibility that, within the ECM, single BeWo cells could migrate to deeper locations and by division establish new isolated colonies of cells. On the other hand the smaller islands of cells might have migrated as a group into the matrix. The latter seems unlikely. Aplin and Charlton (1990) found single migratory cells in BeWo cultures on substrata containing ECM components found in decidual tissue. Observations in the present study suggest that cells migrate singly into the Matrigel (probably with the aid of MMPs which digest the matrix, see later). This finding is similar to *in vitro* reports **(**Bischof *et al.,* 1991; Genbacev *et al.,* 1993**)** where tissue explants or cytotrophoblast cells from anchoring villi of first trimester human placentae were cultured on Matrigel.

In the present study BeWo cells apparently eroded the Matrigel on which they were grown, leaving large gaps in the gel. This was observed by phase contrast microscopy and confirmed by confocal microscopy. The erosion was not the result of simple mechanical disruption or penetration as human endometrial epithelial cells grown on the same type of Matrigel and used as controls did not erode the gel but formed glandular-like structures within the Matrigel. However even very low doses of collagenase (0.0025%) completely digested the Matrigel suggesting that formation of bare areas in the BeWo cell cultures was probably due to the secretion of enzymes probably MMPs and/or PAs by these cells. PA converts plasminogen to plasmin, an active form of serine protease that can control ECM turnover either directly or indirectly by activating some members of the MMP family. Perhaps the BCMs secrete gelatinases which may be responsible for their invasive behaviour. It is possible that the ECM environment also stimulated increased secretion of MMPs, particularly gelatinases by the BCMs. Alternatively, the level of PA inhibitors and tissue inhibitor of metalloproteinases (TIMPs) was low in these cells. On the contrary spheroids remained rounded even after 7 days in culture and did not invade the underlying matrix. The difference in behaviour of the spheroids and individual cells could be due to a difference in integrin expression (as discussed above) and metalloproteinase secretion. This is of interest because it could reflect the behaviour of cytotrophoblast *in vivo*. The spheroids were surrounded by polarised cytotrophoblast-like cells whereas the individual cells showed a tendency of cell stratification with polarised and non-polarised cytotrophoblast-like cells resembling EVT cells.

Although both embryo and endometrium are sources of MMPs and PAs during early attachment and implantation, it is believed that cytotrophoblast cells are the major source of MMPs and PAs which is used for digestion and penetration of the endometrial ECM (Tabibzadeh and Babaknia, 1995). The endometrial stroma on the other hand provides enzymes, possibly collagenases, for ECM remodelling during the secretory phase of the menstrual cycle. Cultured human trophoblast cells have been shown to secrete the urokinase plasminogen activator (uPA) (Tabibzadeh and Babaknia, 1995).

Results of the present study agree with other studies on human trophoblast cells which show that cultured first trimester cytotrophoblast cells secrete MMPs and digest ECM (Bischof *et al.,* 1991; Librach *et al.,* 1991). In addition Librach *et al.* (1991) showed that the invasiveness exhibited *in vitro* by human cytotrophoblast cells grown on Matrigel was dependent on the production of gelatinase-B. Yagel *et al.* (1988) demonstrated that

trophoblast cells exhibit a degree of invasiveness comparable to that of JAr cells. PAI-1 and PAI-2 have been detected in both *in vivo* and *in vitro* human trophoblast cells (Hofman *et al.,* 1994). The expression of these proteins also coincides with the maximum invasive potential of trophoblast cells *in vivo* which is exhibited in humans in the first trimester of gestation (Librach *et al.,* 1991). Other reports have demonstrated that laminin and Matrigel increase gelatinase A secretion by trophoblast and other cell types (Polette *et al.,* 1994). Therefore it is likely that the matrix induced increased secretion of MMPs in the BeWo cells of the present study.

In contrast to the report by Yagel *et al.* (1988), Fisher *et al.* (1989) showed that human choriocarcinoma cell lines BeWo and JAr adhere to, but do not degrade, the PF HR9 matrix on which they are grown. PF HR9 matrix contains basement membrane specific macromolecules including type IV collagen, laminin and proteoglycans. This finding is also in contrast with the results of the present study which demonstrate that BeWo cells are equally capable of invading and degrading Matrigel. The reasons for these apparent differences remain unclear since the components of the substrates appear to be similar.

Another possible explanation for these results is that there was a high level of tranforming growth factor b_1 (TGF-b_1), tissue inhibitors of matrix TIMPs and/or plasminogen activator inhibitors (PAIs) in the spheroids which prevented the degradation of Matrigel by the MMPs. TGF-b_1 induces TIMP production, reduces the secretion of uPA, increases PAI-1 production and decreases collagenase type IV activity (Graham *et al.,* 1994: Shi-wen *et al.,* 2006). BeWo, JAr, JEG-3 and cultured trophoblast cells secrete TIMPs (Crescimanno *et al.,* 1996). In addition, trophoblast cells produce TGF-b (Tabibzadeh and Babaknia, 1995). It has been demonstrated that control of trophoblast invasion is exerted by TGF -b through TIMPs (Tabibzadeh and Babaknia, 1995). These reports suggest that TIMPs are potent inhibitors of cellular invasion and the proteins secreted by the trophoblast cells can inhibit the activity of other proteins secreted by the same cell which could be responsible for ECM degradation. Therefore it might be speculated that BeWo spheroids used in the present study produce both TIMPs and TGF-b. These speculations would be confirmed following a thorough characterization of TIMPs, MMPs and integrin expression of these cells. Unfortunately such a study was not possible during this investigation.

The origin of BeWo cells as a choriocarcinoma might give rise to the expectation that its invasive behaviour would be more pronounced than that of normal cytotrophoblasts; but this does not seem to be the case since no invasion from the surface of the Matrigel into the base of the cell insert was observed in the present study. Also the results of the present study are in general agreement with other studies which showed that cytotrophoblast cells isolated from first trimester placentae have a higher capacity for infiltration than BeWo cells (Yudate *et al.,* 1996). The invasive capacity of BeWo cells has been reported to be comparable to that of second trimester cytotrophoblast cells (Fisher *et al.,* 1989).

CONCLUSION

Results of the present study show that the morphology and behavioural pattern of BeWo cells grown on Matrigel is similar to that reported for *in vitro* normal human cytotrophoblast cells on ECM. BeWo cells seem to cause "erosion" of Matrigel which may be due to their secretion of MMPs. These results suggest that culture of BeWo cells on extracellular matrices may be useful for the study of some of the early embryonic events leading to human implantation, especially during the period when trophoblastic cells interact and erode the uterine epithelium and ECM. This model may also give further insight into the morphological events of implantation.

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