E-cadherin expression during the differentiation of human trophoblasts

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Summary

The morphologic and functional differentiation of human trophoblast cells culminates in the formation of the terminally differentiated multinucleated syncytiotrophoblast. In culture, isolated mononuclear cytotrophoblasts aggregate and then fuse to form syncytia, recapitulating the in vivo process. In the present studies, we investigated the expression of the Ca2+-dependent cell adhesion molecule (CAM), E-cadherin, during the morphologic differentiation of trophoblastic cells. Cytotrophoblasts were isolated from human chorionic villi, and JEG-3 and BeWo choriocarcinoma cells, cytotrophoblastic cell lines which under standard culture conditions are not fusion competent, were obtained by dispersion of ongoing cultures. Cultures were terminated at timed intervals and E-cadherin was analyzed by immunocytochemistry and electron microscopy using specific antibodies. In addition, E-cadherin expression was investigated by western and northern blotting. During the aggregation of cytotrophoblasts, E-cadherin was localized on the cell surface at points of cell-cell contact and could not be demonstrated following cellular fusion. In contrast, it remained on the surface of aggregated JEG-3 and BeWo cells throughout the duration of culture. Western blot analysis revealed a time-dependent increase in E-cadherin (120 × 103 M) which coincided with maximal aggregate formation at 24 h in both normal trophoblasts and JEG-3 cells. In fusing cytotrophoblasts, a marked reduction in E-cadherin was subsequently observed as syncytial trophoblasts became the predominant cellular form in culture. Exposure of the normally non-fusing BeWo cells to 1.5 mM 8-bromo cyclic AMP induced cellular fusion and syncytium formation. This process was accompanied by a disappearance of E-cadherin from the cell surface as assessed by immunocytochemistry and western blotting and a parallel reduction in the abundance of the E-cadherin mRNA. Immunoneutralization experiments using an antiserum directed against the extracellular domain of cadherins inhibited syncytium formation in normal trophoblasts compared to an antiserum against the E-cadherin cytoplasmic tail, which had no effect upon aggregation and fusion of these cells. We conclude that E-cadherin exists in a dynamic state in fusion-competent cytotrophoblasts and that down regulation of its gene expression coincides with cellular fusion. In addition, this process appears to be cyclic AMP-mediated in BeWo choriocarcinoma cells. The results suggest that the temporal and spatial regulation of expression of this cell adhesion molecule may regulate, in part, the processes of aggregation and fusion of these cells during their differentiation into syncytiotrophoblasts.

Key words: trophoblasts, E-cadherin, cell fusion, cell adhesion molecule, CAM, human.

Introduction

Knowledge of the molecular processes involved in the morphogenesis of the human embryo and extraembryonic tissues such as the placenta is scant. Understanding the adhesive interactions between cells, which result in normal cell differentiation and development of the placenta is vital in delineating the events leading to the successful establishment and maintenance of pregnancy. The placenta is derived from cells of the embryonic trophectoderm (Boyd et al. 1980; Cunningham et al. 1989). Upon implantation, cytotrophoblastic cells proliferate and differentiate to form the outer cell layers of chorionic villi (Kaufman, 1985). The inner layer of the mature villus consists of mononuclear cytotrophoblasts associated with each other and with the overlying syncytiotrophoblast. This cell-cell interaction has been shown to be mediated, in part, by
desmosomes (Beham et al. 1988). The mononuclear cytotrophoblasts are mitotically active and are considered the trophoblastic stem cells, while the syncytial trophoblasts are the terminally differentiated cells, which form through fusion of cytotrophoblasts (Pierce and Midgley, 1963). This morphogenetic process results in the functional differentiation of these cells, which, following fusion, can synthesize and secrete the characteristic protein hormones of pregnancy.

Work in this laboratory has characterized an in vitro model, which recapitulates the in vivo morphogenetic process and allows for the study of the coupled events of morphologic and functional differentiation of these cells. Mononuclear cytotrophoblasts isolated from human placenta attach to the culture substratum, aggregate and then fuse to form syncytiotrophoblast (Kliman et al., 1986, 1987). During this process of morphologic differentiation, functional differentiation of these cells with respect to their endocrine activities also occurs: whereas the freshly isolated cytotrophoblasts do not express the characteristic endocrine function of syncytiotrophoblast (e.g. chorionic gonadotropin, placental lactogen and SP1 production), the syncytiotrophoblast, which form in vitro by fusion of the mononuclear cells, do. In addition, this trophoblast–trophoblast interaction is a cell specific event in that trophoblasts do not aggregate and fuse with non-trophoblastic cell types such as fetal hepatocytes or kidney cells. Further, using a suspension culture system, we have shown that cytotrophoblast aggregation is a Ca^2+- and protein synthesis-dependent process and is associated with desmosome formation (Babalola et al. 1990). Therefore, it is logical to postulate that Ca^2+-dependent membrane proteins involved in cell adhesion are required for normal trophoblast differentiation to occur.

It is well accepted that morphogenesis and cell differentiation depend, in part, on the regulated expression of cell surface glycoproteins, which, through their connections to the cytoskeleton, result in altered gene expression (Edelman, 1988). Some of these adhesion molecules have been well-characterized including the Ca^2+-dependent cell-CAM 120/80, variously referred to as E-cadherin, uvomorulin, or L-CAM, depending on the species of origin (Damsky et al. 1984; Takeichi, 1991; Kemler et al. 1989).

E-cadherin, a 120×10^3 M_r transmembrane glycoprotein, is of particular interest with regard to embryonic development and placental morphogenesis because antibodies to E-cadherin inhibit compaction of preimplantation mouse embryos, a developmental event known to involve blastomere adhesion and formation of intercellular junctions (Hyafil et al. 1980; Damsky et al. 1983; Vestweber and Kemler, 1984). In addition, E-cadherin has been demonstrated on the surface of cytotrophoblasts in situ, but not on the surface of the encompassing syncytiotrophoblast (Eidelman et al. 1989). The differential expression of E-cadherin implies that the function of this molecule may be confined to one, but not the other of the two morphologic trophoblast forms and its role in cellular adhesion may contribute to the functional differentiation of these cells.

In the present report, we investigated the expression of E-cadherin during the differentiation of human trophoblastic cells in vitro. E-cadherin in normal, differentiating trophoblasts was compared with that of JEG-3 and BeWo choriocarcinoma cells, cytotrophoblast-like cells, which aggregate but under standard culture conditions do not fuse to form syncytiotrophoblast. A clonal line of BeWo cells can be induced to fuse by the addition to the culture media of cyclic AMP analogues or agents which increase intracellular cyclic AMP levels (Wice et al. 1990). Thus, these three cell types, normal trophoblasts, JEG-3 and BeWo cells, have provided us with the cell models to study the dynamics of E-cadherin expression during cell aggregation and fusion. Elucidation of the molecular mechanisms of this morphogenetic process can help us understand epithelial cell fusion and in particular provide us with further insight into the differentiation of the human trophoblast.

Materials and methods

Reagents and chemicals

All reagents were of analytical grade and were obtained from Sigma Chemical Company (St Louis, MO), unless otherwise stated.

Cell preparation and culture

Cytotrophoblasts were prepared from term human placentae as previously described (Kliman et al. 1986). Briefly, chorionic villi were minced and then digested with trypsin and DNAse in calcium- and magnesium-free media followed by centrifugation on a 5–70% Percoll gradient. Cells banding at a density of 1.048–1.065 g ml^-1 represent a highly enriched (~95 % pure) and viable preparation of cytotrophoblasts. This population of cells was collected and used in the present studies.

JEG-3 choriocarcinoma cells (American Type Culture Collection, Rockville, MD) and the 630 clone of BeWo cells (kindly provided by Dr A. L. Schwartz, Washington University, Saint Louis) were harvested from on-going cultures with 0.1% trypsin in EDTA buffer.

Cells were seeded onto glass coverslips (2×2 cm), placed in 6-well plates (Nunclon Delta, Denmark) at a density of 5×10^5 cells/26 mm well (cytotrophoblasts) or 1.0 to 2.5×10^6 cells/26 mm well (JEG-3 cells) for indirect immunofluorescence studies. For immunoblotting, cells were cultured in 100 mm culture dishes (Nunclon Delta) at densities of 10 to 15×10^6 cells/dish (cytotrophoblasts) or 3.0 to 5.0×10^6 cells/dish (JEG-3 and BeWo cells). Culture media (Dulbecco’s modified Eagle’s medium containing 25 mm glucose, 25 mm Hepes and 50 μg ml^-1 gentamicin) was supplemented with 10% heat inactivated fetal calf serum (FCS).

Indirect immunofluorescence

Cells grown on coverslips were washed 3 times in phosphate-buffered saline (PBS), fixed for 5 min at −20°C in 100% methanol. Fixed cells were washed in PBS and non-specific reactions were blocked by incubating with 5% normal goat serum. Some coverslips were exposed to the rat monoclonal antibody E9, directed against the 80×10^3 M_r fragment of
E-cadherin (Wheelock et al. 1987), at a dilution of 1:10 for 1 h at room temperature. Cells were subsequently incubated with fluorescein-conjugated anti-IgG antibody (Jackson Immunoresearch Laboratories, PA). Coverslips were mounted on glass slides and cells were visualized with a Zeiss microscope.

Electron microscopy

Electron microscopy (from Collaborative Research, Bedford, MA) Cyclopeg membrane inserts (Becton Dickinson Labware, Lincoln Park, NJ). At 36h of culture, the inserts were transferred to multwell culture plates containing PBS at room temperature and the cells were washed to remove the culture medium. The cells were fixed with 1% glutaraldehyde, 0.2% picric acid in 154 mM NaCl, 50 mM phosphate buffer, pH 7.4 (BGPA) for 3 h at 4°C. The cells/membrane inserts were briefly washed in 50% ethanol and centrifuged at 10,000 rpm for 20 min and the supernatant was aspirated. The blots were incubated in this antibody and then incubated in 'blotto' (5% non-fat milk in TBS). Hybridoma supernatant and centrifuged at 10,000 rpm for 20 min and the supernatant was aspirated.

Western blotting

Cells in monolayer cultures in 100 mm dishes were placed on ice and washed 3 times with PBS containing 1.0 mM phenylmethyl sulfonyl fluoride (PMSF). After complete removal of the wash solution, the cells were incubated in 100-200 µl of cell-lysis buffer (Tris-HCl, pH 7.5 containing 0.5% NP-40, 0.5 mM CaCl₂ and 1.0 mM PMSF) at 4°C for 20-30 min on a rocking platform. The cell lysates were scraped off the plate and centrifuged at 10,000 g for 20 min and the supernatant used in the Western blot analyses.

Aliquots of cell lysates equivalent in total protein content in all experimental groups (60-200 µg/lane) were subjected to polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (Laemmli, 1970). The proteins were electrophoretically transferred to nitrocellulose paper (BA-S NC; Schleicher and Schuell, Keene, NH) using the procedure of Towbin et al. (1979). The blots were washed 2 times with TBS (Tris-buffered saline) for 10 min each and then incubated for 30 min in ‘blotto’ (5% non fat milk in TBS). Hybridoma supernatant (the E-cadherin monoclonal antibody E9) was diluted 1:5 in blotto. The blots were incubated in this antibody and then exposed to peroxidase-conjugated anti-IgG (Jackson Immunoresearch Laboratories) for 2 h at room temperature and the peroxidase color reaction developed with 4-chloro-naphthol as substrate (Sigma Chemical Company, St Louis, MO). Blots were scanned using a Shimatsu densitometer to assess relative changes in E-cadherin.

RNA isolation, electrophoresis and northern blotting

Poly(A)-tailed RNA from cultured human cytotrophoblasts was isolated by the oligo(dt)-cellulose column method after first isolating total cellular RNA with GTC/CsCl method (Maniatis et al. 1982). Total cellular RNA was isolated then dissolved in DEPC-treated water, denatured at 65°C, then adjusted to 0.5 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA, pH 7.5. The RNA was then loaded onto disposable column with washed pre-resuspended oligo(dt)-cellulose. The flow-through was collected and re-denatured, run through the same column again by gravity. The column was then washed once with two column volumes of 0.5 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA, pH 7.5. Poly(A)-tailed RNA was then eluted by 10 mM Tris-HCl, 1 mM EDTA, pH 7.5.

Poly(A)-tailed RNA from cultured cell lines (JEG-3 and BeWo) was isolated by the oligo(dt)-cellulose/proteinase K method. Cells were collected and resuspended in lysis buffer (0.5 mM NaCl, 10 mM Tris, 10 mM EDTA, 1% SDS), followed by careful shearing through needles. Proteinase K was then added at the concentration of 0.3 mg/ml⁻¹. After the suspension has been incubated at 37°C for 3 h, it was added to washed, pre-resuspended oligo(dt)-cellulose. The mixture was then incubated overnight with gentle rocking at room temperature. Finally the mixture was loaded into disposable column and washed with 0.1 M NaCl, 10 mM Tris, 0.1 mM EDTA, 0.2% SDS. Poly(A)-tailed RNA was then eluted by 10 mM Tris, 0.1 mM EDTA, 0.2% SDS.

20 µl of 1 mg ml⁻¹ glycogen in every 1 ml eluent was added as carrier in standard ethanol precipitation. Poly(A)-tailed RNA was then resuspended and quantitated by absorbance at 260 nm. It was then denatured in MOPS/formaldehyde, electrophoresed in 0.8% agarose/formaldehyde gels and transferred to Nytran membrane (Schleicher and Schuell, Keene, NH). Hybridizations with nick-translated cDNA probes were performed as previously described (Ringer et al. 1989). After hybridization, the filters were blotted, wrapped in plastic and placed with X-ray film (Kodak X-Omat, Eastman Kodak, Rochester, NY), for autoradiography at -80°C. The cDNA for human E-cadherin was generously provided by Dr R. Kemler (Max-Planck-Institute for Immunobiology, Freiburg, Germany; Mansouri et al. 1988) and the cDNA for the human β-chorionic gonadotropin by Dr J. Fideles (California Biotechnology, Mountain View, CA, USA).

Antibody experiments

Cytotrophoblasts from normal placental were isolated as described above and plated in 4-well culture plates (Nunclo Delta, Denmark) coated with fibronectin (50 µg ml⁻¹; Collaborative Research, Bedford, MA) in serum-free media (DMEM, 25 mM glucose, 25 mM Hepes and 50 µg ml⁻¹ gentamicin). It has been previously shown that under serum-free conditions, a fibronectin-coated surface can support trophoblast attachment, migration, aggregation and fusion (Kao et al. 1988). At time 0 h, a total of 5 x 10⁵ cells/well were incubated in the presence of anti-cadherin antibodies at final concentrations of 0.5 mg ml⁻¹ or 1 mg ml⁻¹. At 24 h, the cultures were washed with warmed media to remove non-adherent cells and fresh media and antibodies were added. The cultures were terminated at 72 h from the initiation of the
experiment (a time when over 70% of nuclei are found in syncytia; Kliman et al. 1986). The cells were washed with PBS (three times) at room temperature, fixed with Bouins for 10 min, washed again and stained with hematoxylin. An inverted Diaphot-TMD Nikon microscope was used to count the total number of syncytia and the number of nuclei per syncytium per well. Approximately 500 high power fields (400x) were counted per culture condition. Two rabbit anti-cadherin sera, L2 and L4 were used in this series of experiments. Their preparation and characterization has been described previously (Blaschuk and Farookhi, 1989). L2 is directed against the extracellular domain of cadherins and has been shown to be capable of disrupting cadherin-mediated cell adhesion (Pouliot et al. 1990). L4 recognizes the intracellular domain of these cell adhesion molecules and was prepared against the peptide DYLNEWGNRFKKLAD (mouse E-cadherin cytoplasmic tail domain). The immunoglobulins were isolated from the antisera by affinity chromatography on a Protein A column (BioRad) according to the manufacturer’s instructions. The purified antibodies were then extensively dialyzed against 10 mM ammonium bicarbonate, lyophilized and reconstituted in media to give the appropriate immunoglobulin concentrations in the cultures.

Results

Distribution of E-cadherin in cultured trophoblastic cells

Using indirect immunofluorescence, we first examined the distribution of E-cadherin in isolated cytotrophoblasts during their morphologic differentiation into aggregates and syncytia. Mononuclear cytotrophoblasts at 3h after isolation showed either no reaction or limited intracellular fluorescence. As aggregates formed, E-cadherin localized at points of cell–cell contact and was not found on outer cell borders which were not in contact with other cells (Fig. 1), indicating, as expected, spatial specificity of E-cadherin in cell–cell interactions. Immunoelectron microscopy showed clusters of E-cadherin to be present in areas where microvilli came in contact with adjacent cells (Fig. 2). Controls, using normal rat IgG were negative. As the cells fused to form multinucleated syncyia, E-cadherin immunostaining was markedly reduced, being present only at points of cellular contact between unfused mononuclear cells and the larger syncytial trophoblasts (Fig. 1 D and E). At no time after the fusion of cells was E-cadherin detectable on the cell surface or intracellularly. Mononuclear cells frequently migrated on top of the large flattened cytoplasmic expanse of syncytial trophoblasts so that the immunolocalization of E-cadherin en face could be observed. This revealed a reticular, honeycomb pattern of distribution of E-cadherin on apposing cell surfaces.

JEG-3 choriocarcinoma cells soon after harvesting and plating also had little E-cadherin or sparse punctate collections distributed within their cytoplasm. Similar to the mononuclear cytotrophoblasts, upon aggregate formation, E-cadherin was localized at cell–cell borders (Fig. 1 C). Interdigitations of cell membranes were also evident through the immunocytochemical distribution of E-cadherin on the cell surfaces. No cytoplasmic staining was apparent within the aggregated cells. Once
established, this pattern of distribution of E-cadherin in JEG-3 cells did not change up to 120 h of culture. E-cadherin distribution was similar in non-fusing BeWo cells up to 96 h (Fig. 3). The addition of 1.5 mM 8-bromo cAMP to the culture media induced BeWo cell fusion, which was initially observed at 48 h and was extensive by 72–96 h. E-cadherin was initially present at cell–cell borders, but its staining pattern became discontinuous and fragmented as cells started to fuse prior to its complete disappearance (Fig. 3). Addition of the cAMP analog to normal cytotrophoblasts or JEG-3 cells had no effect on cell aggregation and fusion and did not alter E-cadherin immunolocalization.

**Immunoblot analysis of E-cadherin**

Western blotting was performed to establish the time course of both the appearance of E-cadherin after isolation of normal cytotrophoblasts, JEG-3 and BeWo choriocarcinoma cells and its expression during the in vitro differentiation of trophoblastic cells. In both normal cytotrophoblasts and JEG-3 choriocarcinoma cells, E-cadherin increased during the initial 24 h following plating coinciding with the time course of formation of cellular aggregates (Fig. 4). In agreement with the immunocytochemical observations, E-cadherin in normal cytotrophoblasts increased during the initial 24 h of culture (Fig. 4A), but decreased by 82% between 24 h and 96 h of culture coincident with the marked decrease in the number of cellular aggregates and the parallel increase in the formation of multinucleated syncytia (Fig. 5A). In contrast, E-cadherin levels remained constant in non-fusing JEG-3 cells from 24 h to 96 h (Fig. 5B). 8-bromo cAMP-treated BeWo cells showed a progressive disappearance of E-cadherin, which at 96 h was decreased by 88% over control, non-treated, non-fusing BeWo cells (Fig. 6A).

**Northern blot analysis of E-cadherin**

Northern hybridization suggests that the mechanism of the reduction of E-cadherin observed in fusing cells by indirect immunofluorescence and immunoblot analyses is through down regulation of E-cadherin gene expression. Northern blot analysis was conducted on nitrocellulose filters blotted with equal amounts of poly(A)-RNA from cytotrophoblasts cultured up to 96 h. 32P-labeled human E-cadherin cDNA hybridized with a band of approximately 4.5 kb (Fig. 7). There was a gradual decrease in E-cadherin mRNA levels, which at 96 h were at 16% of the 0 h level. This result parallels the formation of syncytia and the reduction of E-cadherin observed by both immunofluorescence and western blotting. In contrast to the down regulation of E-cadherin gene expression, the 1.05 kb βCG mRNA increased significantly demonstrating the endocrine functional differentiation of these cells. The non-fusing JEG-3 cell mRNA levels of both E-cadherin and βCG did not change throughout the duration of the experiment (data not shown), while 8-bromo cAMP treatment of BeWo cells markedly reduced E-cadherin mRNA and increased the βCG 1.05 kb transcript (Fig. 6B). Actin mRNA has been shown to be down regulated in time course experiments of aggregating and fusing cytotrophoblasts and by cAMP analog treatment, while the βCG transcript is upregulated (Queenan et al. 1987; Ringler et al. 1989). Thus, the βCG cDNA probe was used in the present study in
order to both validate the E-cadherin mRNA reduction and to demonstrate the endocrine functional differentiation of the cells.

Inhibition of syncytium formation by function perturbing antibodies

The L2 antibody inhibited syncytial trophoblast formation in a dose-dependent manner. The L2-treated cultures had increased numbers of single cells and a striking paucity of large, multinucleated syncytia (Fig. 8). There were a total of 1679 syncytia in control, L4-treated cultures compared to 1200 and 878 syncytia when the L2 antibody was used at final concentrations of 0.5 mg ml\(^{-1}\) and 1 mg ml\(^{-1}\) respectively. This represented a 28\% and 48\% inhibition of the total number of syncytia formed when the function-perturbing antibody was used. Further, the number of nuclei per syncytium was dramatically reduced in the L2-treated cells, as demonstrated by a 75\% reduction in the number of syncytia containing more than 15 nuclei (Table 1). There were no syncytia with greater than 30 nuclei in the L2-treated cells. As a control, the L4 antibody against the cadherin cytoplasmic tail was used at a final concentration of 1 mg ml\(^{-1}\) and had no effect compared to untreated cultures during the same time period. Use of the E9 antibody, (which is directed against the human E-cadherin extracellular domain, but has no function perturbing activity), in a parallel experiment had no effect, suggesting that the L2 result
E-cadherin in human trophoblasts

Fig. 4. Time course of the appearance of E-cadherin in cytotrophoblasts (A) and JEG-3 choriocarcinoma cells (B) as assessed by western blotting. Cells were isolated as described in the text, cultures were established and terminated at the indicated times. Little E-cadherin was detectable at 0h and its levels gradually increased reaching a plateau between 12 and 24h of culture in both cytotrophoblasts and JEG-3 cells.

Fig. 5. Levels of E-cadherin in normal cytotrophoblasts (A) and JEG-3 choriocarcinoma cells (B) from 24 to 96h of culture as assessed by western blotting. Note the gradual decrease in the levels of E-cadherin in the fusing cytotrophoblasts coinciding with cellular fusion and the predominance of syncytial trophoblasts at 96h of culture. In contrast, non-fusing JEG-3 cells maintained a constant E-cadherin level through the duration of the experiment.

Table 1. Effect of immunoneutralization of E-cadherin on syncytial trophoblast formation

<table>
<thead>
<tr>
<th>Nuclei/syncytium</th>
<th>Control (L4)</th>
<th>L2 (% inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5–10</td>
<td>1222</td>
<td>712 (42%)</td>
</tr>
<tr>
<td>11–15</td>
<td>273</td>
<td>120 (56%)</td>
</tr>
<tr>
<td>16–20</td>
<td>96</td>
<td>25 (74%)</td>
</tr>
<tr>
<td>21–30</td>
<td>66</td>
<td>31 (68%)</td>
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<tr>
<td>&gt;30</td>
<td>22</td>
<td>0 (100%)</td>
</tr>
</tbody>
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represents functional inhibition of the differentiation process rather than a non-specific effect.

Discussion

Using an in vitro system, which recapitulates some of the in vivo processes involved in the morphologic and functional differentiation of human trophoblasts, we demonstrated that trophoblastic cell aggregation is associated with the intercellular accumulation of E-cadherin and with subsequent loss of this component of the adhesion complex upon fusion of the cells to form syncyta. The presence of E-cadherin on normal cytotrophoblasts (Fisher et al. 1989) as well as choriocarcinoma cell lines such as JAR cells (Wheelock et al. 1987), has been previously described. The present study is the first to follow the distribution of this cell adhesion protein during the morphological differentiation of cytotrophoblasts into syncytial trophoblasts, demonstrate its presence at points of cell–cell contact by electron microscopy and confirm these descriptive results by western blot analysis. Further, this report shows that immunoneutralization inhibits syncytial trophoblast formation and that the loss of E-cadherin upon cell fusion is, in part, due to the down regulation of E-cadherin mRNA, which, in BeWo choriocarcinoma cells is a cAMP-mediated process. These observations demonstrate a specific temporal and spatial regulation of expression of E-cadherin during the morphologic differentiation of normal human trophoblasts and suggest a role for this cell adhesion molecule in this morphogenetic process.

It is clear that the enzymatic digestion required for isolation of trophoblastic cells either from placenta or from ongoing cultures of choriocarcinoma cells, cleaves cell surface determinants that are critical to the aggregation process. This hypothesis is corroborated by
Fig. 6. (A) Effect of 8-bromo cAMP on E-cadherin levels assessed by western blot analysis of cultured BeWo choriocarcinoma cells. Cells were exposed to control medium or 1.5 mM 8-bromo cAMP for 96 h, harvested and E-cadherin protein was analyzed as described in the text. Note that the 88% decrease observed in the cAMP analog-treated cellular E-cadherin coincided temporally with the extensive cell fusion and formation of syncytia seen in this group of cells (Fig. 3). (B) Northern blot analysis of E-cadherin mRNA in the absence (−) or presence (+) of 1.5 mM 8-bromo cAMP at 96 h of culture of BeWo choriocarcinoma cells. As a control, the same blot was probed with a cDNA probe of the β subunit of human chorionic gonadotropin (βCG). Actin mRNA has been shown to be down regulated by cAMP analog treatment, while the 1.05 kb βCG transcript is upregulated in trophoblastic cells (Ringler et al. 1989).

Our previous finding that protein synthesis inhibition with cycloheximide reversibly blocks aggregation of enzymatically dispersed trophoblastic cells (Babalola et al. 1990). The observations of the present study are consistent with the hypothesis that E-cadherin is one such cell surface component mediating this process. Following isolation, few single cells showed intracellular E-cadherin and this was confirmed by the low levels of E-cadherin detectable at zero time in both normal cytotrophoblasts and JEG-3 cells by western blotting. As the cells aggregated in culture, E-cadherin protein levels gradually increased and reached a maximum at 24 h of incubation, which coincided temporally with maximal trophoblastic aggregate formation (Kliman et al. 1986). In parallel, the immunohistochemical studies (fluorescence and electron microscopic) further demonstrated the localization of this CAM at points of cell–cell contact, indicating a close temporal relationship between E-cadherin expression and aggregation. It is attractive to speculate that cell contact induces synthesis and subsequent cell-surface integration of E-cadherin. Subsequently, as fusion of the trophoblastic cells proceeded, E-cadherin disappeared from the cell surface. These results were confirmed by western blotting. The residual E-cadherin seen on western blots at 96 h of incubation may either indicate some low level of E-cadherin expression or represents the continued presence of mononuclear cells, which are still in the process of aggregation and fusion with existing syncytiata. Interestingly, and lending further support for a role for E-cadherin in the process of aggregate formation, JEG-3 choriocarcinoma cells, which aggregate but do not fuse, continued to show intense intercellular localization of E-cadherin and, as shown by western blotting, continued to express this cell surface glycoprotein. These cells therefore, have characteristics of other non-fusing epithelia, which maintain expression of E-cadherin at points of cell–cell contact in confluent cultures (e.g. MDCK cells; for review: Takeichi, 1991; Rodriguez-Boulan and Nelson, 1989).

The results of our study suggest that the disappearance of E-cadherin from syncytial structures is through down regulation of E-cadherin gene expression. Persistence of a low level of the 4.5 kb transcript at 96 h in both normal trophoblasts and cAMP analog-treated BeWo cells represents a combination of a low level expression in syncytiata and the continued presence of mononuclear cells which are still in the process of aggregation and
fusion. Whether the decrease over time of E-cadherin mRNA is a result of transcriptional regulation and/or mRNA stability remains to be elucidated. Further, whether this down regulation plays a role in the fusion process per se or is only a consequence of syncytialization cannot be determined from the present experiments. We can conclude, though, that this represents at least one of the molecular events involved in the remodeling of the adhesion complex which occurs during cellular fusion.

The fate of the pre-existing E-cadherin upon fusion of the cytotrophoblasts is unclear at the present time. In previous studies, it has been shown that one mechanism of the turnover of E-cadherin in vitro is through cleavage of its extracellular domain, which then appears in the culture medium (Wheelock et al. 1987). A recent study indicated that an endogenous proteinase is operative in the turnover of A-CAM (N-cadherin) in lens cells (Volk et al. 1990) and a similar mechanism has been proposed for the fusion of myoblasts during myotube formation (Couch and Strittmatter, 1983 and 1984). Since the antibody employed in our study (E-9) recognizes an epitope on the extracellular portion of the molecule, absence of intracellular E-cadherin following syncytium formation may indicate that a cleavage event may have occurred during trophoblast morphological differentiation, resulting in the loss of the ectocellular domain. Alternatively, protein internalization and rapid degradation to levels below the sensitivity of our detection methods may explain the lack of any intracellular E-cadherin after syncytium formation.

Immunoglobulins purified from the L2 polyclonal antiserum and directed against the cell adhesion domain of cadherins (Farookhi and Blaschuk, 1991; Pouliot et al. 1990; Blaschuk et al. 1990) were inhibitory to the formation of syncytia in comparison to immunoglobulins purified from the L4 antiserum and directed against the cytoplasmic domain of these cell adhesion molecules. The absence of complete inhibition in our studies may be related to the rate of turnover of E-cadherin in these cells, which may not allow for complete immunoneutralization even at an immunoglobulin concentration of 1 mg ml\(^{-1}\). Alternatively, the antibody may have reduced functional activity against the human molecule, thus allowing limited cell aggregation and subsequent fusion. The E9 antibody, which has been generated against the extracellular \(80\times 10^3 M_r\) fragment of the human molecule (Wheelock et al. 1987) and which was utilized in the immunohistochemical and western blot studies described in this report, does not perturb E-cadherin function (Buck and Damsky, personal communications) and thus could not be used

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**Fig. 8.** Phase-contrast photomicrographs at low (A,C,E) and high (B,D,F) power of normal trophoblast cultures at 72 h in the presence of 1 mg ml\(^{-1}\) control antibody L4 (A,B) or 0.5 mg ml\(^{-1}\) L2 (C,D) and 1 mg ml\(^{-1}\) L2 (E,F). Note the large multinucleated syncytia in the control and low dose L2 cultures (arrowheads) and the predominance of single cells in the higher L2 dose culture (E), which contains smaller syncytia with fewer nuclei (arrows). (Bar=50 \(\mu m\)).
to assess the effect of inhibition of this homophilic interaction on trophoblast differentiation. Studies involving the production of function-perturbing antibodies specific for human E-cadherin are currently in progress. None-the-less, the immunoneutralization studies suggest a functional role for cadherins in the morphologic differentiation of human trophoblasts. Since the peptides utilized for the generation of the L2 antiserum contain highly conserved sequences among cadherins (Hatta et al. 1988; Blaschuk and Farookhi, 1989), it is possible, although unlikely, that another cadherin (N or P) plays a role in human trophoblast differentiation. To our knowledge, the only work suggesting the presence of N-cadherin in placenta is by Liaw et al. (1990), who showed the expression of N-cadherin by northern blot analysis of whole placental extracts in the cow. The cell of origin, though, of this molecule by immunocytochemical or in situ hybridization studies was not determined. Moreover, there is no published report demonstrating the presence of N-cadherin or its mRNA in human trophoblasts. In fact, Shimoyama et al. have demonstrated that the major cadherin in human placenta is E-cadherin and that P-cadherin is absent by immunohistochemical examination (1989a) while its mRNA is scarcely expressed (1989b). Therefore, existing entries in the literature in combination with our own observations, strongly support the conclusion that E-cadherin is the main cadherin functionally involved in the process of human cytotrophoblast aggregation and fusion.

Trophoblastic cell aggregation also involves the assembly of desmosomes at points of cell contact (Babalola et al. 1990), which may occur subsequent to the appearance of E-cadherin at the cell–cell interface. Although the precise temporal changes in the distribution of adhesion complex proteins were not analyzed in the present study, evidence for such a hierarchy of cellular interaction has been previously described (for review: Rodriguez-Boulan and Nelson, 1989). For example, antibodies to E-cadherin block desmosome formation in MDCK cells (Gumbiner et al. 1988). The trophoblast adhesion complex described herein, is dynamic in that it undergoes a reorganization during cell fusion. Although the trigger event for fusion of normal cytotrophoblasts to form syncytiotrophoblasts is unknown at the present time, it is quite clear from this study that regulation of E-cadherin expression is part of the remodeling of the cellular adhesion complex associated with this morphogenetic process. The study of cellular aggregation and fusion utilizing the trophoblast model should prove informative for understanding the regulation of expression of genes encoding proteins that confer adhesion and fusion competence as well as the transport, assembly and disassembly of the component parts.

The authors wish to thank Drs Clayton Buck and Steven Albelda for providing them with the E9 monoclonal antibody and for their helpful discussions. The electron microscopic studies could not have been performed without the expertise of Robert M. Smith and Neelima Shah of the core facility of the University of Pennsylvania (supported by NIH grant DK-19525). We also thank Dr R. Klemper for his kind gift of the human E-cadherin cDNA and Dr A. L. Schwartz for the clonal BeWo cell line used in the present experiments.

This work was supported by the McCabe fund (CC), the M.R.C. of Canada (OWB), NIH grant HD-06274 (JFS) and grants from the Mellon and Rockefeller foundations.

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Devi Biol 109, 891–902.


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(Accepted 19 August 1991)