The role of matrix contact and of cell-cell interactions in choriocarcinoma cell differentiation

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Cell differentiation is supported much better by gels of extracellular matrix than by the same matrix provided as a rigid substrate. Many cell types including normal and malignant trophoblast cells, however, form multicellular multilayered aggregates on matrix gels with increased cell-to-cell contacts as compared to regular monolayers on rigid matrix substrates. In such cultures, it remained open, so far, whether stimulated expression of differentiation markers is caused by enhanced cell-to-cell communication or is displayed only by cells in direct contact to the gel. Therefore, choriocarcinoma cells (BeWo) were grown as aggregates: (*a*) on gels of the basement membrane-like Matrigel, (*b*) on plastic coated with poly-HEMA, or (*c*) as aggregates (spheroids) in suspension culture. Production of the differentiation marker chorionic gonadotropin was stimulated significantly in aggregates attached to gels of Matrigel or to the poly-HEMA substrate but not in suspended spheroids. With respect to cell-cell communications, however, expression of E-cadherin mRNA was not altered in any type of aggregates, as compared to control cultures on plastic. The expression of connexin43 mRNA (not of connexin26) was increased only in suspended spheroids, while microinjection of the fluorescent dye Lucifer Yellow suggested that cell communication via gap junctions was absent from cells grown as monolayers and was not induced in any type of aggregate. When cells were grown on gels of Matrigel, the relevance of direct cellular contact to the substrate for differentiation was analyzed by immunohistochemistry. Trophoblastic differentiation markers (chorionic gonadotropin, placental lactogen, placenta-type alkaline phosphatase, and pregnancy-specific glycoprotein β1) as well as the proliferation marker Ki-67 were not preferentially expressed in cells that were in contact with the gel. Similar random distributions of all these markers were also observed in spheroids cultured in suspension. The distributions of several matrix molecules and of different integrins were comparable between aggregates on matrix gels and those in suspension culture. According to these data, cell-cell communication appears to play a subordinate role for cytodifferentiation in cell aggregates on matrix gels, so that substrate anchorage and physical properties of the substrate may be the decisive factors. Interestingly, however, direct contact to the substrate does not seem to be essential for the stimulation of differentiation in cells on matrix gels. The results are discussed in the context of the "tensegrity"[2]-model for cell-matrix interactions in which proper mechanical properties of the substrate are important for the regulation of cell differentiation by allowing a balanced integrity of external and cell-internal tensile forces.

Introduction

Differentiation of cells grown on substrates of extracellular matrix (ECM) components is, on one hand, directed by specific signalling via interactions between matrix molecules and their cell surface receptors [38]. On the other hand, differentiation is stimulated much better if the matrix is provided as a flexible/malleable gel such as gels of collagen or of the basement membrane-like Matrigel [9, 29, 31, 44, 64]. Since, on matrix gels, increased expression of differentiation markers is associated with a switch in cell morphology from flattened cells in monolayers on rigid substrates to more or less rounded polygonal cells, it has been proposed that cell shape plays a crucial role in differentiation [3, 5, 21, 30, 66]. However, concomitant with these changes in cell shape, the cells necessarily establish increased areas of cell-cell contact, while contact to the substrate is reduced: Mammary epithelial cells on matrix gels, for example, form organotypic alveoli of simple (unilayered) cuboidal or columnar epithelia with increased lateral cell contact, while all cells are polarized and still in contact with the gel [9, 44, 61, 64]. Other types of cells, like normal as well as malignant trophoblast cells, establish multilayered aggregates of polygonal cells when growing on matrix gels. In this case contact to the gel is restricted to the basal layer of aggregates and not possible in upper layers while cell-cell contacts are increased in all cells [29, 31, 32]. Therefore, the question arises whether the observed effects of ECM gels may be mediated by increased cell-cell interactions.

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[2] "Tensegrity" is a contraction of *tensional integrity*. The term was created by B. Fuller for certain physical principles in architecture [23]. Later on, in the years 1981 to 1985, D. E. Ingber introduced this term to cell biology in order to explain how cells and tissues are constructed and how physical forces (in particular internal and external tensional forces) can be instructive in cyto- and histodifferentiation (reviewed in [33–35]).

Direct cell-to-cell interactions may involve adhesion molecules or communication via gap junctions. In general, cell adhesion molecules like cadherins are thought to take part in signalling pathways [4]. In addition, communication between cells through gap junctions (which may be pronounced in three-dimensional cultures) may modulate differentiation [6]. So far, however, there is little direct evidence for the role of such interactions in cell shape-dependent differentiation: When mammary epithelial cells grown within ECM gels [62] or hepatocytes on artificial substrates [59] are kept spatially isolated they still express increased levels of differentiation markers (as long as they are permitted to maintain a roundish cell shape) implying that cell-cell contact and cell-cell communication are not required for differentiation. When cells were grown in multilayered aggregates on matrix gels it was also not shown clearly, so far, whether only cells in direct contact with the gel or also those in upper layers express higher levels of differentiation markers.

In this communication, we have compared the expression of differentiation markers by BeWo choriocarcinoma cells in three different types of cell aggregates: cell clusters growing *a)* either on matrix gels or *b)* on a synthetic non-natural substrate, or *c)* as spheroids kept in suspension culture. In these three settings cell morphology was basically similar, i.e. cells assumed a more rounded shape associated with increased cell-to-cell contact areas as compared to monolayer cultures. The expression of the trophoblastic differentiation marker chorionic gonadotropin (hCG) was stimulated only in either type of attached aggregates but not in suspended spheroids. In order to obtain direct evidence for the roles of cell-cell contact and of cell-cell communication in the control of differentiation we have analyzed, in these three types of aggregates, the expression of the epithelial adhesion molecule E-cadherin and of different gap junction proteins (connexins) as well as cell-to-cell transfer of the fluorescent dye Lucifer Yellow and compared these data with the production of hCG. Differential expression of hCG, however, was neither correlated with cell-cell contacts (expression of E-cadherin or of connexins, cellular communication via gap junctions) nor with deposition of ECM components or expression of integrins within the different aggregates suggesting that these factors are not decisive for shape-dependent differentiation in this system. In addition, while anchorage to a substrate appears to be essential for differentiation (provided that cells are permitted to take up a roundish morphology), immunohistochemical analysis suggests that in multilayered aggregates not every cell needs to be in direct contact to the substrate. This apparent paradox will be discussed in the context of modern concepts on structural forces that may be involved in signal transduction.

Materials and methods

Cell cultures

BeWo human choriocarcinoma cells (American Type Culture Collection, Rockville, MD/USA; Cat. No. CCL 98) were maintained in Ham's F12 (Gibco, Heidelberg/Germany) containing 15 % fetal bovine serum (Gibco). They were subcultured once a week using trypsin-EDTA (Gibco) to suspend the cells. For experiments, cells from subconfluent monolayers were replated at a density of 15 000 cells/cm^2 and incubated at 37 °C in a moist atmosphere of air with 5 % CO_2. The medium was exchanged every day.

The cells were either grown on plain tissue culture plastic (Falcon, Becton Dickinson, Heidelberg/Germany), on gels of the complex basement membrane type matrix Matrigel ([39]; purchased from Collaborative Research, Bedford, MA/USA) or of 1.5 mg/ml collagen type I (Vitrogen, Collagen Corporation, Palo Alto, CA/USA) or on plastic coated with 0.1 µg/cm^2 poly-HEMA (polyhydroxyethylmethacrylate, Aldrich, Steinheim/Germany) according to Folkman and Moscona [21]. Poly-HEMA was dried down to the bottom of 24-well plates from a solution in 95 % ethanol overnight under a laminar air flow. Gels of Matrigel or of collagen type I were reconstituted at neutral pH in 96-well plates under aseptic conditions by incubating 35 µl of either matrix per well at neutral pH at 37 °C in humidified air for 1 h.

Cell spheroids in suspension culture were generated as described previously [24] by aliquoting single cell suspension into Erlenmeyer flasks at a concentration of 6×10^5 cells in 6 ml and growing them on a gyratory shaker at 70 rpm in 5 % CO_2/95 % air at 37 °C. After three days, spheroids of uniform medium size (average diameter about 300 µm) were selected for continuing culture.

Cellular hexosaminidase, estimation of total cell protein

Since Matrigel substrates contain exogenous protein, an indirect estimate of total cell protein was chosen as described previously [31] measuring the activity of the ubiquitous lysosomal enzyme, hexosaminidase, according to Landegren [40] and calibrating it against total cell protein.

Determination of chorionic gonadotropin

The accumulation of secreted human chorionic gonadotropin (hCG) in the culture medium during the last period of 24 h was determined on day 4 of culture by an established radioimmunoassay (Hybritech™ Inc., Köln/Germany).

Immunohistochemistry

Rabbit polyclonal antibodies against hCG, human placental lactogen (hPL), pregnancy-specific glycoprotein β1 (SP1), placenta-type alkaline phosphatase (P1AP), human fibronectin (FN), a polyclonal goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC), a polyclonal goat anti-mouse IgG conjugated with rhodamine, a goat non-immune complete immunoglobulin fraction, the peroxidase-antiperoxidase immunocomplex (PAP), and a monoclonal antibody against human collagen type IV (CIV) were obtained from Dako GmbH (Hamburg/Germany). Mouse monoclonal antibodies to the proliferation-associated nuclear antigen Ki-67 and polyclonal rabbit anti-rat IgG conjugated with FITC were from Dianova (Hamburg/Germany). A mouse monoclonal antibody against human laminin (LN) was purchased from Sigma (Deisenhofen/Germany). A rabbit polyclonal antibody against human collagen type I (CI) was from Paesel und Lorei (Frankfurt a.M./Germany). Commercial antibodies against different integrin subunits were a polyclonal rabbit anti-α1-integrin (Chemicon, Temecula, CA/USA), and mouse monoclonal antibodies against α5-integrin or β4-integrin, respectively (both from Biomol, Hamburg/Germany) and to the β1-integrin (Dianova). Rat monoclonal antibody to α6-integrin was a generous gift from Dr. A. Sonnenberg (The Netherlands Cancer Institute, Amsterdam/The Netherlands).

Immunohistochemical staining for Ki-67, ECM molecules, and integrins as well as double immunofluorescence for Ki-67 and hCG was carried out on 10 µm thick cryostat cross sections mounted on glass coverslips, air-dried, fixed in ice-cold methanol for 5 min, collected in phosphate buffered saline (PBS), and then preincubated for 2 h with goat non-immune serum diluted 1:50 in PBS containing 1 % bovine serum albumin (BSA; Sigma). The sections were incubated with the first antibody (diluted in PBS/BSA) for 2 h at room temperature in a humid atmosphere. After 3 rinses with PBS, the FITC- or rhodamine-conjugated second antibody (diluted 1:100 in PBS/BSA) was applied for 2 h at room temperature in a humid atmosphere in the dark. After 3 rinses with PBS, the sections were mounted on glass slides with glycerol containing 0.1 % paraphenylenediamine to avoid photobleaching. As a standard procedure to localize hCG, hPL, SP1, or P1AP, 6 µm paraffin sections of material that had been fixed in 3.5 % paraformal-

dehyde for 1 h at room temperature were rehydrated and subjected to the same protocol except that the primary antibodies were detected with the PAP method using 3,3-diaminobenzidine (Sigma). Then the sections were studied and photographed with a Zeiss Axiophot microscope equipped for epifluorescence. Two sets of control sections were treated with either of the antibodies alone.

Northern blot analysis

For Northern blot analysis total cellular RNA was isolated by the guanidinium isothiocyanate/cesium chloride method [53] and quantified spectrophotometrically at 260 nm. RNA samples were separated electrophoretically on 1.2% agarose gels containing 2.2 M formaldehyde. The amounts of ribosomal RNA in each lane were visually compared after staining the gels with ethidium bromide. The RNA was blotted onto nylon membranes (Hybond-N, Amersham-Buchler GmbH, Braunschweig/Germany) and incubated at 80 °C for 2 h. cDNA probes were labeled with [α-^{32}P]dCTP using Klenow DNA polymerase and random oligonucleotides as primers [18]. After prehybridization at 42 °C for 3 h in a solution containing 55% deionized formamide, 1 M NaCl, 1% sodium dodecyl sulfate (SDS), 10% dextran sulfate (500 kDa), and 100 µg/ml salmon sperm DNA, hybridization was carried out with 25 ng labeled DNA at 42 °C for 24 h using the same buffer.

The following DNA fragments were used for hybridization: *a)* 1.1 kb cDNA corresponding to part of the coding region of rat connexin26 gene (clone 26-1 [68]), *b)* 1.4 kb cDNA corresponding to the coding region of rat connexin43 gene (clone G2B [7]), *c)* 386 bp cDNA equivalent to part of the coding region of the human E-cadherin gene (clone HC6-1 [22]). Blots with specific probes were washed once in 2 × SSC (1 × SSC is 0.15 M NaCl with 0.015 M sodium citrate) containing 1% SDS at 60 °C for 5 min, once in 1 × SSC with 0.1% for 1 h, and in 0.5 × SSC, 0.1% SDS, for 30 min. The membranes were exposed to Kodak XAR-5 films at –80 °C with intensifying screens. For consecutive hybridization, the gene probe was removed from the membrane by immersing the membranes into a boiling solution of Tris-HCl (1 mM, pH 7.5) and 0.1% (w/v) SDS and cooling down to room temperature. Each blot was rehybridized with a human β-actin specific probe [43].

Dye coupling

For detection of dye coupling according to [37, 60], glass microelectrodes (Hilgenberg, Malsfeld/Germany) were backfilled with a 4% solution of the fluorescent dye Lucifer Yellow CH (457 daltons; Sigma) in 1% LiCl. Electrodes were positioned with a Leitz micromanipulator, and the spheroids were injected with the dye by applying a negative current step of up to 20 nA for 10 s. Microinjection was monitored with a Zeiss photomicroscope equipped with epifluorescence, and photographs were taken 2 min after injection had started. For each type of culture investigated at least 20 aggregates or 20 cells at different locations in monolayers were analyzed.

Histology

After culture, the cells were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 30 min, postfixed in 2% (w/v) osmium tetroxide, dehydrated in ethanol and embedded in Epon/Araldite. Semithin sections (0.75 µm) were stained with 0.5% Toluidine Blue and 0.5% (w/v) pyronin in 0.5% (w/v) sodium borate. The sections were examined and photographed in an Axiophot microscope (Zeiss, Köln/Germany). Thin sections were contrasted with uranyl acetate and lead citrate and studied with a Zeiss EM 10 electron microscope.

Samples for immunohistochemistry were washed with PBS and either quick-frozen in liquid nitrogen and stored at –21 °C, or fixed in 3.5% paraformaldehyde, dehydrated in graded series of ethanol and embedded in paraffin.

Results

When BeWo choriocarcinoma cells were grown on gels of Matrigel they formed distinct spheroid-like colonies of rounded cells in contrast to the continuous monolayers of flattened cells obtained on tissue culture plastic (cf. [30, 31]). A similar morphology was accomplished when the cells were cultured attached to a rigid surface of plastic coated with poly-HEMA which reduces adhesiveness, or as spheroids in suspension culture. Concomitant with formation of multilayered aggregates and cell rounding, cellular contact areas were expanded in all of these types of aggregates as compared to cells grown as monolayer cultures (Fig. 1). This is particularly prominent in the upper layers of poly-HEMA-attached aggregates (cells in the basal layer are in contact with the substrate and with cells) and even more in suspended spheroids lacking cell-substrate anchorage.

Electron microscopy demonstrates that cell morphology exhibits no major differences between aggregates maintained on gels of Matrigel or attached to poly-HEMA-coated plastic and spheroids in suspension culture (Fig. 2). In general, electron microscopic characteristics in all aggregate cultures were as described for spheroids [24]. In all types of aggregates, except for superficial cells, the cells in the different layers had a polygonal to rounded morphology. Cells on the surface formed apical microvilli and tended to flatten out covering several underlying cells. Intercellular spaces were mostly narrow. Accumulation of extracellular material in interstitial spaces was not observed. Cell membranes tended to run in parallel over longer stretches establishing primitive adherens type junctions.

The enlargement of cell contact areas in aggregate cultures was not associated with significant changes in the expression

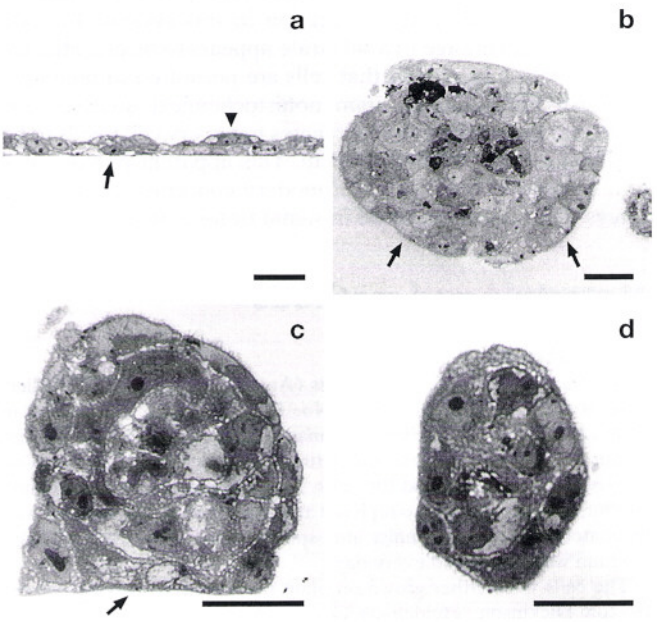

Fig. 1. Morphology of BeWo cell aggregates under different culture conditions as compared to monolayer cultures. Cells were grown for 4 days on tissue culture plastic (**a**), gels of Matrigel (**b**), on plastic coated with 0.1 µg/cm^2 poly-HEMA (**c**), or as spheroids in suspension culture (**d**). While in (**a**) the cells are more flattened (even overgrowing cells are flat, *arrowhead*), they assume a more or less rounded morphology in the aggregates (**b–d**). The plane of contact with the substrate (ECM gel in (**b**) or poly-HEMA/plastic in (**c**)) is indicated by *arrows*. – Bars 20 µm.

of mRNA for the cell adhesion molecule E-cadherin. As demonstrated by Northern blot analysis, E-cadherin mRNA is expressed at comparable levels, in relation to β-actin mRNA, in monolayer cultures and in attached as well as in suspended spheroids (Fig. 3).

Since alterations of the extent of cell-cell contact areas may result in changes of cellular communication via gap junctions, the expression of gap junction proteins was examined by Northern blot assays. While connexin26 was not detected under any condition, the expression of connexin43 was slightly increased in suspended spheroids (Fig. 3). However, dye coupling could not be demonstrated by microinjection of the fluorescent dye Lucifer Yellow, neither in cell monolayers, nor in aggregates on gels of Matrigel or of collagen type I, nor in suspended or attached spheroids (Fig. 4).

The synthesis and secretion of hCG has been shown to be a reliable marker for differentiation of BeWo cells [31]. However, an increase in hCG production comparable to that observed after transfer of cells from tissue culture plastic to matrix gels of Matrigel (cf. [31]) was found only in spheroids attached to the poly-HEMA substrate but not in suspended spheroids (Fig. 5).

Since cell communication did not appear to be relevant for differentiation of BeWo cells, we examined the requirement of direct cell-matrix contact for stimulated expression of differentiation markers. After growth for different periods of time on gels of Matrigel or of collagen type I, cells expressing the trophoblast-specific differentiation markers hCG, SP1, hPL or P1AP were localized by immunohistochemistry. On both substrates and at all stages of culture, cells staining positively for any of these antigens were randomly distributed over all layers of the multilayered colonies (Fig. 6). A preference of cells in contact with the substrate was not observed. A similar, i.e. random, distribution of differentiated cells was also obtained in multicellular spheroids grown in suspension (Fig. 7). The much higher number of cells staining positively for hCG seen on matrix gels as compared with spheroids in suspension culture corresponds well with the levels of secreted hCG in both systems (Fig. 5). Proliferation of cells growing on matrix gels of Matrigel or collagen type I was also not restricted to a certain layer in the colonies as demonstrated by immunohistochemical staining of cells expressing the nuclear antigen Ki-67 which is associated with proliferation (Fig. 8a). Random distribution of proliferating cells was also observed in suspended spheroids (data not shown). Double immunofluorescence staining with antibodies against hCG and Ki-67, respectively, distinguished separate subpopulations expressing either epitope for the majority of about 70% of the cells (Figs. 8b, c). For the remaining portion of cells, colocalization of hCG and Ki-67 may have resulted from overlapping parts of proliferating and differentiated cells due to the thickness of the cryostat

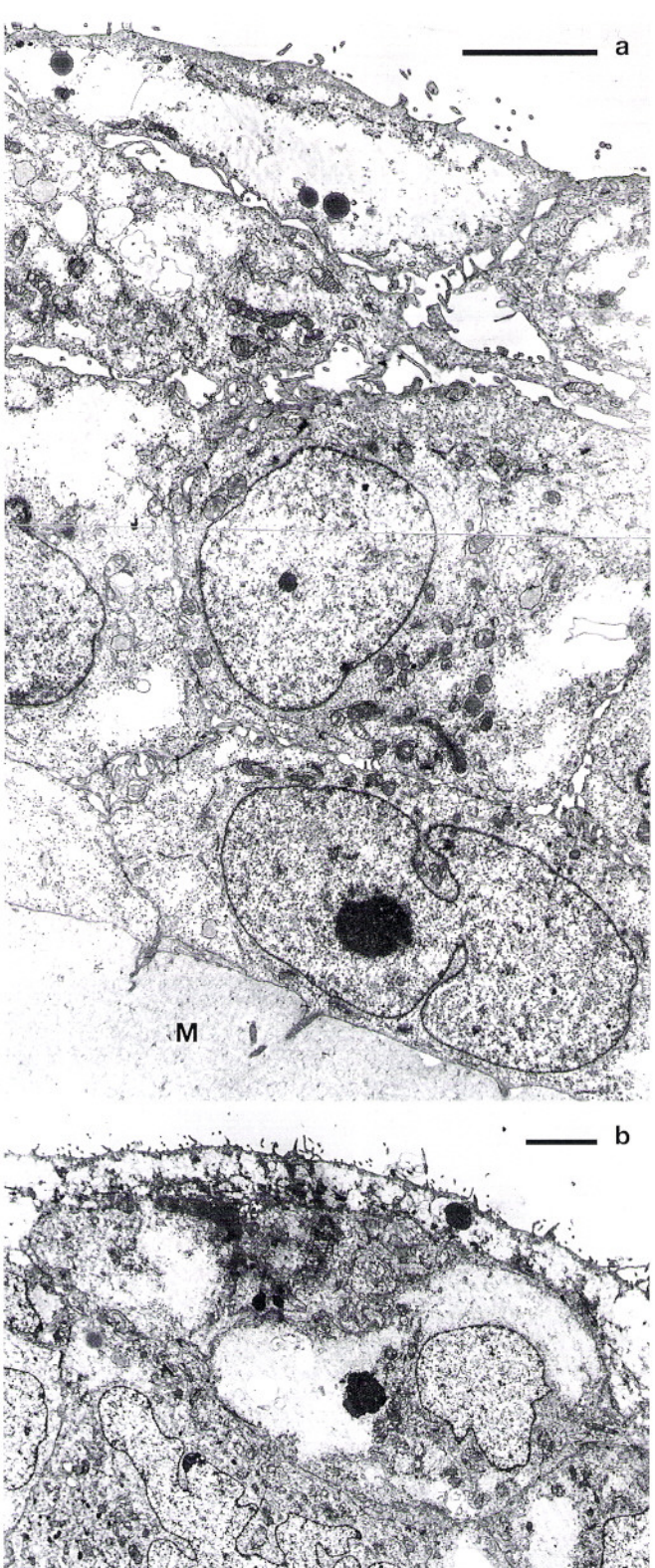

Fig. 2. Transmission electron microscopic images of BeWo cells grown for 4 days on a gel of Matrigel (**a**) or as suspended spheroids (**b**). Cell morphology is comparable in both types of aggregates: Cells in the center have assumed a polygonal to rounded cell shape as have cells in contact with the gel in (**a**). On the surface, cells tend to flatten out and to cover several underlying cells. Microvilli are predominantly formed by superficial cells in both systems. Translucent areas in cells are typical for trophoblastic cells and result from elution of glycogen stores during fixation and dehydration protocols. – M Matrigel. – Bars 5 μm.

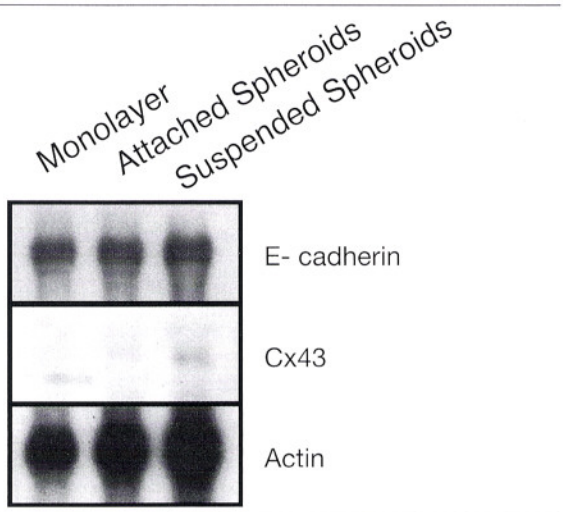

Fig. 3. Expression of E-cadherin and of connexin43 in BeWo cells grown for 4 days in monolayer culture on plastic (*left lane*), as aggregates attached to plastic coated with poly-HEMA (*middle lane*) or in suspension culture (*right lane*). 5 μg of total RNA were separated by gel electrophoresis under denaturing conditions, transferred onto nylon membrane, and hybridized consecutively to cDNA probes for E-cadherin, connexin43, and β-actin. Before rehybridizations were started the previous probe had been removed. No signal was obtained using a probe for connexin26 even with extremely long exposure times (not shown).

sections; however, a coexpression of both markers could not be excluded completely. In a similar way, cells displaying a high signal for hCG in suspended spheroids were also negative for Ki-67 (data not shown).

It is well known that cells can deposit their own extracellular matrix or may alter their pattern of matrix receptors depending on the environment. In order to correlate the differential expression of hCG with possible differences in the distribution of such molecules in different types of aggregate cultures, we localized different matrix components and integrins (those that are differentially expressed in normal trophoblast [13]) in aggregates cultured on gels of Matrigel and in suspended spheroids by immunohistochemistry (Tab. I). In both types of cultures the distributions of matrix molecules and integrin subunits were comparable: Fibronectin, laminin and collagen type I (but not collagen type IV) were detected with a predominantly pericellular arrangement. The integrin subunits tested (except for β4) were predominantly found in the region of cell membranes.

Discussion

Cells growing in vitro on extracellular matrix gels display a much higher (sometimes almost organotypic) degree of differentiation as compared to cell monolayers established on rigid ECM substrates like plastic coated with matrix components [9, 29, 31, 44, 64]. However, this increase in differentiation may not directly depend on matrix recognition but could be mediated by changes in cell-cell interactions, since it has been observed that typically cell-cell contact is enhanced in parallel:

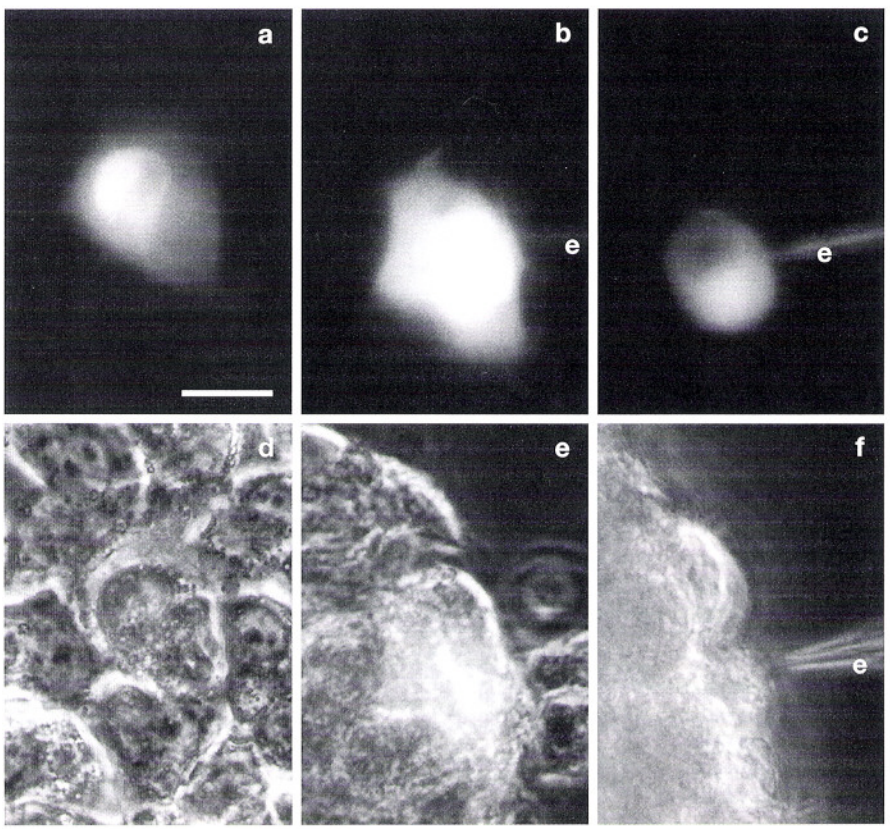

Fig. 4. Dye coupling with Lucifer Yellow (**a–c**) in BeWo cells grown for 4 days as a monolayer on plastic (**a, d**), as aggregates on a gel of Matrigel (**b, e**), or as cell spheroids in suspension culture (**c, f**). Photographs taken 2 min after injection show strongly stained nuclei and diffuse distribution of the dye in the cytoplasm of individual cells. Fluorescence is not transferred to adjacent cells. The same results were obtained with aggregates formed on collagen gels or on poly-HEMA-coated plastic. For each experimental condition, at least 20 cells were injected. – **d to f.** Phase contrast micrographs. – e Electrode. – Bar 20 μm.

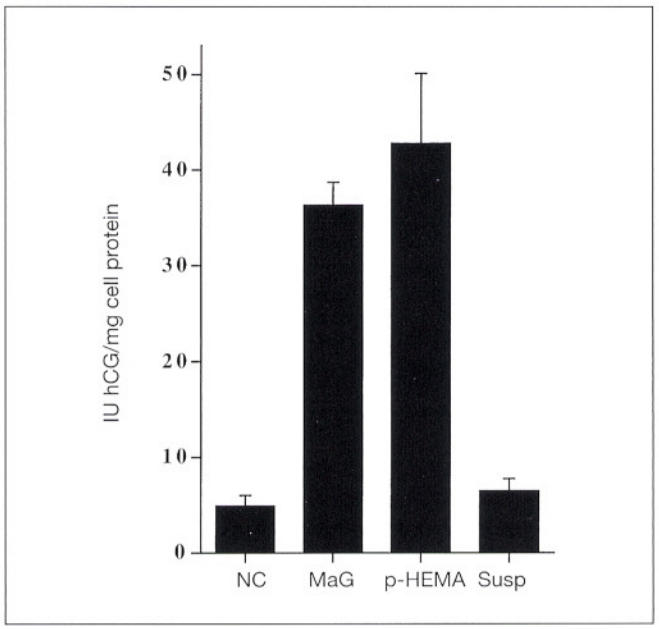

Fig. 5. Production of hCG by BeWo cells grown as monolayers on plastic (NC), on gels of Matrigel (MaG), as aggregates either on plastic coated with 0.1 μg/cm^2 poly-HEMA (p-HEMA) or in suspension culture (Susp.) 15 000 cells per cm^2 were seeded into wells and grown for three days before the media were exchanged. Alternatively, spheroids were generated as described in Materials and methods and transferred to fresh medium after three days. Then accumulation of hCG in the media over an additional 24 h was determined on day 4 and referred to total cell protein.

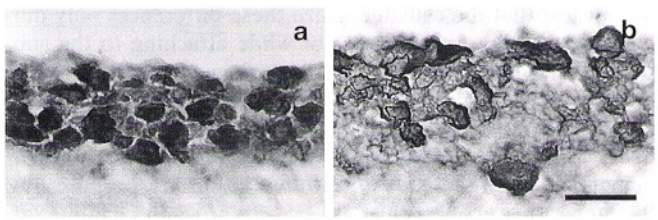

Fig. 6. Immunoperoxidase staining of paraffin sections of BeWo cells (grown on gels of Matrigel for 4 days) with polyclonal antibodies directed against hCG (**a**) or against P1AP (**b**). While hCG is located in the cytoplasm, P1AP is located on the cell membrane. In both cases, cells with varying staining intensity are distributed over all layers of the colonies. – Bar 25 μm.

Tab. I. Detection of matrix molecules and of integrin subunits in BeWo aggregates on gels of Matrigel vs. suspended aggregates (spheroids).

	EDM molecules				Integrin subunits				
	FN	LN	CI	CIV	α1	α5	α6	β1	β4
Aggregates on Matrigel	+	+	+	./.	+	+	+	+	+
Aggregates in suspension	+	+	+	./.	+	+	+	+	+

CI Collagen type I. – CIV Collagen type IV. – FN Fibronectin. – Ln Laminin. – ./.: No staining.

some cell types like mammary epithelial cells shift from flat monolayers to simple cuboidal or columnar epithelia [9, 44, 61, 64] and others like normal and malignant trophoblast cells even pile up to multilayered aggregates on ECM gels [29, 31, 32]. From this correlation, it appears possible that differentiation of cells on matrix gels is directed by increased cell-cell interactions. However, so far only indirect evidence exists for the role of cell-cell interactions in differentiation (see below), and no data have been published on the relevance of cell-cell contacts and cell-cell communication for the behavior of cells on matrix gels. Therefore, we have studied the correlation of the expression of E-cadherin and of gap junctions with the degree of differentiation of BeWo choriocarcinoma cells (with a differentiation behavior on ECM comparable to normal trophoblast [29, 31]) growing as aggregates on ECM gels, on artificial substrates or non-attached in suspension culture.

Cell contacts and communication thought to be involved in cell-specific phenotype regulation may involve adherens junctions as well as gap junctions [4, 6, 63]. Cadherins are a family of Ca^{++}-dependent cell-cell adhesion molecules with characteristic expression patterns in each cell type. A relevance for normal cellular behavior is suggested by the inverse correlation of tumor cell invasiveness with cadherin expression [8, 26, 42]. From such data it cannot be concluded, however, whether or not cell-cell contacts may be important for normal differentiation, in particular in epithelial cells. Indeed, experimental evidence argues for a suppressive rather than a stimulatory role of cell-cell adhesion in differentiation, in some systems. When, for example, during cleavage of the mammalian zygote, blastomeres are completely surrounded by other cells (comparable to cells within aggregates) differentiation to trophoblast is suppressed and the cells remain pluripotent (reviewed in [14]). A more indifferent role of cell contacts with respect to cell differentiation is observed in other systems: Mouse mammary epithelial cells grown on matrix gels usually establish organotypic alveolar morphology, and a proper response to lactogenic hormones is obtained only under these conditions in contrast to traditional (flat) monolayers. Nevertheless, cell communication appears not to be required for lactogenic differentiation in these cells as shown with cells embedded in matrix gels at low density [62]. Spatial isolation of hepatocytes does also not prevent cell shape-dependent differential production of albumin [59].

The cell adhesion molecule E-cadherin is known to be expressed in trophoblast and choriocarcinoma cells [12, 20], and its expression is discussed to be involved in regulation of differentiation in normal trophoblast cells but not in choriocarcinoma cells [12]. However, it appears more probable that the down-regulation of E-cadherin observed in normal trophoblast is rather a consequence of formation of symplasms, a specific feature of trophoblast differentiation, than causing it. Our experiments provide evidence that cell contacts and molecules involved are not the decisive factor for differentiation (in our system) even when cells are in contact. The increase in cell-cell contacts is associated with increased production of hCG only in aggregates attached to a substrate (gels of Matrigel or poly-HEMA-coated plastic) but not in suspended aggregates. In contrast to hCG, E-cadherin mRNA levels were comparable in all cultures.

Cell-cell communication via gap junctions has also been discussed to participate in the control of cell differentiation (reviewed in [6, 25, 67]). Possibly, however, this type of junction may primarily serve the maintenance of homeostasis in

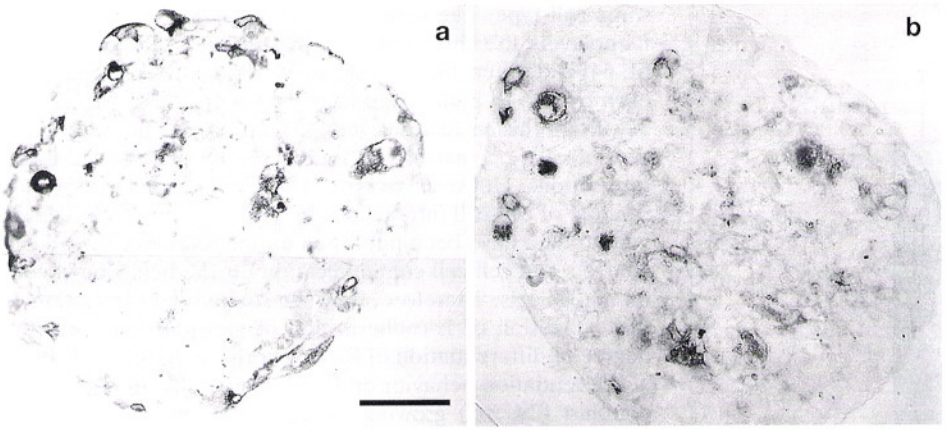

Fig. 7. Detection of cells expressing hCG (**a**) or P1AP (**b**) by immunoperoxidase staining on cryostat sections of cell spheroids grown in suspension for 4 days. As seen in Figure 6 for cells grown on Matrigel, antigen-expressing cells are randomly distributed over the entire section of the spheroid, for hCG as well as for P1AP. – Bar 20 μm.

tissues or the compartmentation in organs rather than controlling basic cell type-specific differentiation patterns. This is supported by indirect evidence as already discussed for mammary epithelial cells and for hepatocytes (see above) as well as by observations of cardiac malformations in mice lacking Cx43 [49]. Consequently, expression of connexins and gap junction formation may rather be a result of differentiation than a cause of it as suggested by data on adipocyte differentiation [65]. In agreement with this, cell-cell communication was not correlated with increased expression of differentiation markers in our experiments.

In cell aggregates on matrix gels, not only cell-cell contacts are changed but also cell-matrix contacts. When piling up, only the basalmost layer of cells can be in contact with the gel. In such cultures it had not been clear, in the past, whether all cells or only those in direct contact with the gel display features of stimulated differentiation. Our results demonstrate that neither proliferating cells nor those expressing trophoblastic differentiation markers are preferentially located in certain layers in aggregates on ECM gels or in suspension. The same random distribution has been found for differentiating normal trophoblast cells growing on ECM gels (Boßerhoff et al., unpublished). Therefore, the suggested effects of ECM on cells in upper layers of colonies on matrix gels, i.e. without immediate contact to the ECM, remain enigmatic. There may be several possible mechanisms: (*i*) growth factors and cytokines may have been trapped in matrix gels [56]. (*ii*) Matrix proteases (produced by BeWo cells on ECM gels: Hohn, unpublished; [16]) may generate active peptides from matrix molecules in the gels. Both types of molecules may reach the cells in upper layers by diffusion and elicit specific responses. However, both mechanisms are unlikely because we have obtained comparable results on matrix gels, in spheroids attached to nonspecific poly-HEMA substrates and with artificial synthetic matrix surrogate gels [30]. In addition, matrices have been compared with each other that have the same composition but are either used as rigid supports or as gels, and differentiation was stimulated remarkably only when matrices were provided as gels [31]. (*iii*) The cells themselves may have deposited different types of ECM molecules in different types of aggregates eliciting differential behavior (in all layers). (*iv*) BeWo cells might express different matrix receptors depending on their environment comparable to normal trophoblast [13]. However, in our system immunohistochemical staining demonstrated no differences between aggregates on ECM gels and suspended spheroids in the patterns of ECM molecules and integrins (Tab. I).

A rounded cell shape has been proposed to be important in the control of differentiation [30, 66]. The data reported here demonstrate that in contrast to normal keratinocytes in vitro [66] differentiation in our system requires anchorage of aggregates to a substrate. This is also suggested by previous investigations comparing BeWo cells attached to substrates of glyoxyl-agarose or growing non-attached on non-adhesive regular agarose [30]. A major role in cell differentiation on extracellular matrix gels is ascribed to the physical properties of the substrate in terms of flexibility/malleability allowing for a rounded morphology [3, 9, 31, 44, 61, 64]. Reduced adhesion on poly-HEMA may mimic this situation. Consistent with this, our investigations on normal and malignant trophoblast cells growing on non-flexible vs. flexible ECM substrates [29, 31] suggest that the cells recognize these differences only during long-term interaction but not while attaching to the substrate. Since the cytoskeleton is interconnected to the environment [34, 38, 63] it may be involved in "sensing" the physical properties of the substrate (as also implied by a previous observation [30]). Explanations for how the cytoskeleton participates in the regulation of differentiation are proposed by the "tensegrity" concept [23, 33–35]. In order to facilitate differentiation, the tensile forces of the cytoskeleton obviously have to be in balance with extracellular tensile forces establishing a tensegrity system for the extracellular matrix across the cytoskeleton to the nucleus [33–36]. Altering this cellular mechanical force balance (e.g. by using rigid vs. flexible substrates [29, 31, 44]) results in integrated changes in cell, cytoskeletal and nuclear shape [58]. In the nucleus, binding of DNA and RNA to the nuclear matrix are discussed to participate in the regulation of gene expression [10, 17, 45]. On the other hand, many components of cellular regulation are associated with the cytoskeleton [2, 27, 28, 47, 48, 57]. Variations in cytoskeletal arrangement may also affect the function of the nuclear envelope [19] and of nuclear pores that are linked to intermediate filaments [11, 46]. The involvement of cellular motor proteins in integrating cell shape and cellular functions has also been shown [52]. Alterations of cell shape may affect kinesin-dependent transport of organelles and also of mRNA along the microtubule cytoskeleton. Changes in cell shape are, therefore, likely to cause modulation of gene expression. An interesting model how cytoskeletal elements may regulate cell differentiation has been proposed concern-

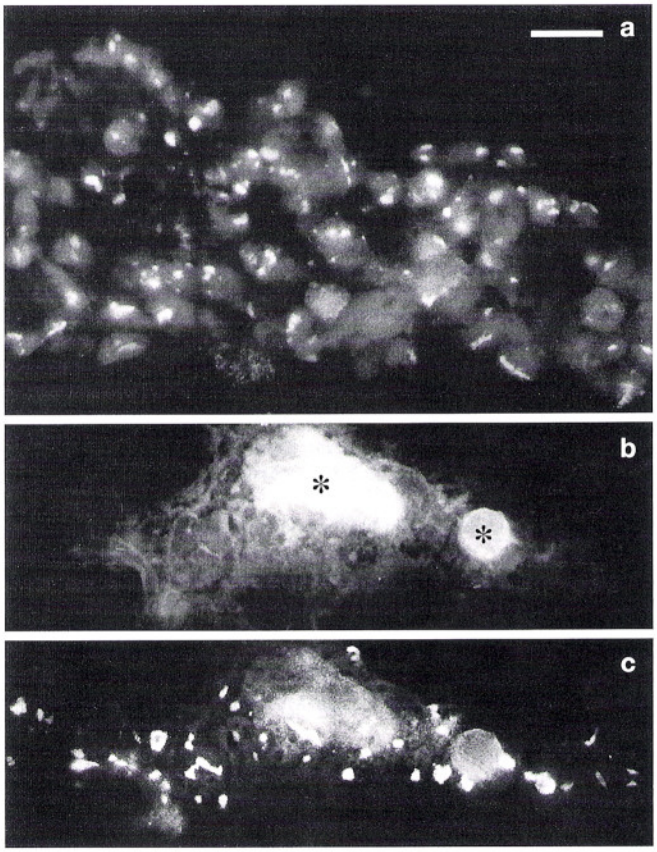

Fig. 8. Distribution of cells expressing the proliferation-associated nuclear antigen Ki-67 during growth of BeWo cells on gels of Matrigel. – **a.** Ki-67-positive cells (note distinct bright spots) are seen randomly distributed in all layers of the colony. – **b, c.** Double immunofluorescence staining for hCG (**b**) and Ki-67 (**c**) in another aggregate of cells grown on gels of Matrigel for 4 days. Cryostat sections were incubated consecutively with rabbit polyclonal antibodies against hCG followed by an FITC-conjugated goat anti-rabbit-IgG antibody and a mouse monoclonal antibody against Ki-67 detected with a rhodamine-conjugated goat-anti-mouse-IgG antibody. Note that cells strongly expressing hCG (*asterisks* in **b**) are free of Ki-67-specific spots in (**c**). The strong FITC fluorescence (cytoplasmic labeling of hCG) is not completely suppressed by the filter combination used in (**c**). – Bar 25 μm.

ing the release of messenger molecules from caveolae into the cytoplasm [1]. Effects of the ECM on differentiation on matrix-dependent transcription factors and ECM-response elements regulating different genes that have been identified [15, 41, 54, 55] may also be transmitted by the cytoskeleton. A specific biomechanical response element has been described in endothelial cells and in platelets where effects of shear forces are transduced [50, 51]. These data do not prove, however, that such elements respond to mechanical forces only.

In conclusion, the data presented here appear to exclude cell-cell contact and communication as well as direct cell-matrix contact as essential environmental elements for the differentiation of BeWo choriocarcinoma cells. These observations support the view that cell shape and a balanced cellular tensegrity are basic regulatory factors for phenotype determination.

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