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Experimental Modulation of Cell-Cell Adhesion, Invasiveness and Differentiation in Trophoblast Cells

Hans-Peter Hohn Hans-Werner Denker

Institut für Anatomie, Lehrstuhl für Anatomie und Entwicklungsbiologie, Universitätsklinikum Essen, Germany

Key Words

Differentiation · Cell-cell adhesion · Invasion · Trophoblast · Choriocarcinoma · Placenta · Embryo implantation

Abstract

The establishment of pregnancy in the human decisively depends on the competence of the early trophoblast to interact during implantation with (1) the uterine epithelium and subsequently (2) with the endometrial stroma and blood vessels. In the interaction with uterine epithe-

Abbreviations used in this paper	
dbcAMP	dibutyryl-cAMP
ECM	extracellular matrix
EMT	epitheliomesenchymal transition
hCG	chorionic gonadotrophin
MMP	matrix metalloproteinase
MTX	methotrexate
PMA	phorbol-(12-myristoyl-13-acetyl)-diester
RA	all-trans-retinoic acid
STS	steryl sulfatase
TIMP	tissue inhibitors of metalloproteinase

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Fax + 41 61 306 12 34 E-Mail karger@karger.ch www.karger.com © 2002 S. Karger AG, Basel 1422–6405/02/1723–0218\$18.50/0 Accessible online at: www.karger.com/cto lium cell-to-cell adhesion appears to be a critical element, involving initially (and astonishingly) apical cell poles of both epithelia. The subsequent invasion of the stroma includes both adhesive interactions with and degradation of extracellular matrix. How these different processes are regulated in detail remains largely unknown. While the invasiveness of the trophoblast is known to be regulated in local and temporal terms it has remained unclear so far whether trophoblast adhesiveness to cells and/or matrix is subject to a coupled regulation or whether both properties involve different, maybe sequentially effective, control mechanisms. It is also not known how the regulation of these activities is related to the differentiation pathways leading to the formation of noninvasive villous trophoblast serving endocrine as well as nutritive functions. This communication reviews experiments using normal cytotrophoblast cells isolated from first trimester or term placentae as well as malignant trophoblast (choriocarcinoma) cells treated with a panel of compounds known to modulate cell differentiation [retinoic acid, methotrexate, dibutyryl-cAMP, phorbol-(12-myristoyl-13-acetyl)-diester]. Parameters indicative of trophoblast differentiation [in particular chorionic gonadotrophin (hCG) secretion] as well as adhesion to uterine epithelial cells and invasion into extracellular matrix in vitro were monitored. While expression of dif-

H.-P. Hohn

Universitätsklinikum Essen, Institut für Anatomie Hufelandstrasse 55, D-45122 Essen (Germany) Tel. +49 201 723 4380, Fax +49 201 723 5916 E-Mail hohn@uni-essen.de ferentiation parameters was increased by all drug treatments, adhesion to uterine epithelial cells in vitro was reduced. Modulation of invasiveness, however, followed a different pattern: while it was reduced in normal trophoblast cells it was even increased in choriocarcinoma cells with various substances. The response of cells with respect to production of extracellular matrix proteins or matrix-degrading proteinases showed a complex pattern that again lacked a stringent correlation with hCG production and adhesion, and in addition also with invasive behavior. These results suggest that adhesiveness of trophoblast to uterine epithelial cells and invasiveness into the uterine stroma (extracellular matrix) are subject to different control mechanisms. They support the view that trophoblast-endometrium interactions involve a cascade of various adhesion and migration processes whose cellular and molecular basis is complex but accessible to experimental investigation using a variety of available in vitro systems.

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Introduction

The survival of the human conceptus and, specifically, the establishment and continuation of pregnancy are dependent on a cell type of the blastocyst that does not contribute to the embryo proper, i.e. the trophoblast. After apposition of the blastocyst to the uterine mucosa the trophoblast has, in a cascade of events, to attach to the uterine epithelium, to penetrate it and its basement membrane and to arrode maternal blood vessels. The first direct cell-to-cell contact that can be recognized with the electron microscope involves the apical plasma membranes of trophoblast and uterine epithelium (perhaps initially that part of the latter that is located next to the junctional belt [Enders and Mead, 1996]). Such an adhesion between the apical poles is absolutely unusual for epithelia and can, therefore, be regarded as a cell biological paradox, because epithelial cells do normally not allow adhesion of any other cells to their apical surface [Denker, 1986, 1990, 1993]. There are exceptions from this rule, however, e.g. the sticking of leukocytes to endothelium (rolling, attachment, extravasation) as occurring under certain conditions.

Interestingly, leukocytes as well as endothelial cells are not generally adhesive to each other but adhesion competence followed by actual membrane contacts has to be induced by complex signaling cascades [Ley, 1996; Campbell and Butcher, 2000; Aurrand-Lions et al., 2002]. It appears to be interesting to ask to what extent similarities might exist when compared with the trophoblast-endometrium system. In the case of the uterine epithelium the adhesion-competent state seems to be transient and of short duration and it forms, according to our concepts, an important aspect of the so-called 'receptive state' of the endometrium, the latter being defined by embryo transfer experiments and shown to be under the control of steroid hormones, i.e. progesterone and estrogens [Psychoyos and Casimiri, 1980]. Likewise for the adhesive partner, the trophoblast, experimental evidence suggests that adhesion competence is not expressed constitutively throughout all developmental stages but is gained temporarily during a specific phase and is dependent on inside-out and outside-in signaling processes, about which a number of interesting details have been uncovered recently [Wang and Armant, 2002].

In spite of this recent progress, we are still far from having a complete picture of all the cellular and molecular properties that define the adhesive/invasive state of the trophoblast, nor is it clear how the two properties of adhesiveness and invasiveness may be related to each other. Indeed, the competence of the trophoblast for adhesion to the uterine epithelium and the ability to penetrate it are usually thought to be coupled and related to the invasive properties of trophoblast cells. However, this assumption has not been substantiated so far. Theoretically, these trophoblast activities could also be expected to be each under different control mechanisms, since epithelial adhesion and stromal invasion must differ with respect to at least some of the cellular processes and the molecules involved. The former represents a cell-to-cell interaction that is coordinated by control mechanisms affecting cell polarity and that includes expression and interaction of proper cell adhesion molecules and their interconnection with an active cytoskeleton providing inside-out as well as outside-in signaling [Denker, 1986, 1990, 1993; Glasser and Mulholland, 1993; Thie et al., 1996b, 1997; Murphy, 1998]. In contrast, invasion of trophoblast into endometrial stroma – which is comparable to invasion of tumor cells into host tissue [Murray and Lessey, 1999] - is based on interactions with the extracellular matrix (ECM) including adhesion to matrix molecules via matrix receptors like integrins (and outside-in signaling via these), enzymatic alteration of ECM, e.g. by matrix metalloproteinases (MMP; modulated by respective inhibitors) and migration in the matrix. Knowledge on the biochemistry of these interactions during trophoblast invasion into matrix has accumulated over the years [Lala and Graham, 1990; Aplin, 1991; Librach et al., 1991; Cross et al., 1994;

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Damsky et al., 1994; Aplin et al., 2000; Bischof et al., 2000b]. In contrast, comparably little is known about the mechanisms involved in the initial event of trophoblast attachment to the uterine epithelium though this has recently been changing and even some possible key player molecules may have been identified [see Glasser et al., 1988; Lindenberg et al., 1989; Denker, 1993; Glasser and Mulholland, 1993; Kimber et al., 1993; Rohde and Carson, 1993; Thie et al., 1995, 1996b, 1997, 1998; Suzuki et al., 1999; Burghardt et al., 2002; Wang and Armant, 2002].

With regard to regulation of trophoblast invasiveness some of the external signals and also some of the regulatory genes apparently involved have been described [Bass et al., 1994; Cross et al., 1994; Damsky et al., 1994; Librach et al., 1994; Conrad and Benyo, 1997; Hamilton et al., 1998a, b; Zygmunt et al., 1998; Caniggia et al., 1999; Janatpour et al., 1999; Bischof and Campana, 2000; Bischof et al., 2000a, b; Janatpour et al., 2000; Knofler et al., 2000; Lala, 2000]. In contrast, comparable information about regulation of the initial event, i.e. trophoblast adhesion to the uterine epithelium, is scant [see Burghardt et al., 2002; Wang and Armant, 2002]. We review here results from a series of experiments focusing on the question of whether and to what extent interconnections may exist between the regulation of differentiation, cell adhesion and invasiveness of trophoblastic cells, focusing on in vitro behavior of normal and malignant trophoblast cells that were treated with substances affecting cell differentiation.

Experimental Models

The dynamic processes of trophoblast adhesion and invasion are rather inaccessible in vivo. Therefore, correlations between cell differentiation and matrix invasion on one hand and between differentiation and the competence for adhesive interactions with uterine epithelial type cells on the other were investigated using in vitro models. Protocols for the isolation of villous cytotrophoblast cells are well established for the first trimester and term placenta [Kliman et al., 1986a; Fisher et al., 1989, 1990]. In the studies reviewed here, normal trophoblast cells from both stages were used whenever possible, first trimester trophoblast representing the highly invasive, term trophoblast the more sessile phenotype. In addition, malignant trophoblast (i.e. choriocarcinoma) cell lines were also employed in our experiments, specifically BeWo, JAr and Jeg-3 choriocarcinoma cells that have served as models for invasive trophoblast in numerous previous investigations [for literature, see Grümmer et al., 1990; Hohn et al., 1992; John et al., 1993a, b; Grümmer et al., 1994; Thie et al., 1996a, 1997, 1998; Hohn et al., 1998]. Normal as well as malignant trophoblast cells were treated with dibutyryl-cAMP (dbcAMP), methotrexate (MTX), phorbol-(12myristoyl-13-acetyl)-diester (PMA) or *all-trans*-retinoic acid (RA), substances that are generally known to affect cell differentiation. These substances were chosen because of their differing modes of action, particularly because it could not be predicted which of the signaling pathways was to be interfered with in order to modulate differentiation versus cell adhesion or invasiveness: RA influences gene transcription by interaction with specific nuclear receptors [Gudas, 1990; Wolf, 1990; Jones et al., 1998; Rohwedel et al., 1999]. dbcAMP affects the cAMPdependent protein kinase present in trophoblast cells [Strauss et al., 1992] while the phorbol ester PMA activates protein kinase C bypassing the inositol triphosphate pathway [Kikkawa et al., 1983; Ron and Kazanietz, 1999]. MTX was used because it has been claimed to induce differentiation in choriocarcinoma cells [Friedman and Skehan, 1979; Friedman et al., 1984] although as an antimetabolite for dihydrofolate dehydrogenase it may just force cells into some type of postmitotic state [Borsa and Whitemore, 1969a, b; Takimoto, 1997].

Cell differentiation was assessed by quantitating the secretion of chorionic gonadotrophin (hCG) and the activity of the cellular steryl sulfatase (STS). Cellular morphology and proliferation rates were also recorded [see Hohn et al., 1992, 1998].

Adhesion Assay

Rates of trophoblast adhesion to uterine epithelial cells were determined in an established attachment assay [John et al., 1993a, b] in which multicellular choriocarcinoma cell spheroids were brought in contact with monolayers of endometrial cells grown on coverslips. After incubation for routinely 30 min nonadhering cell spheroids were removed under mild centrifugal forces (see fig. 1). These experiments were only performed with BeWo, Jeg-3 and JAr choriocarcinoma cells. According to previous investigations these cell lines can form mechanically robust multicellular spheroids under proper suspension culture conditions [Grümmer et al., 1990]. Normal trophoblast cells did not reliably form stable multicellular spheroids as needed for this assay and thus could not be included here. Multicellular spheroids were preferred to cell suspensions [as used by Rohde and Carson, 1993] because when cell suspensions are used it cannot be dis-



Fig. 1. a Experimental design for the attachment assay quantifying adhesion of choriocarcinoma cell spheroids to endometrial cell types [John et al., 1993a]. **b** Cross-sectional sketch of the confrontation arrangement. **c** JAr cell spheroids adhering to RL95-2 cell monolayer after 30 min of incubation and following centrifugation (bar represents $100 \,\mu$ m).

criminated whether the interaction is via the basolateral or the apical (as physiological for implantation initiation) plasma membrane. Also in single cell suspensions epithelial cells tend to lose their apicobasal polarity. In contrast, in multicellular choriocarcinoma spheroids the outer layer cells indeed display elements of trophoblast-like polarity [Grümmer et al., 1990], thus representing the situation found in an implanting blastocyst more closely. The endometrial cells chosen for confrontation culture differed in their adhesiveness for trophoblastic cells: (1) RL95-2 endometrial adenocarcinoma cells [Way et al., 1983] do not display epithelial polarity but nevertheless basic epithelial characteristics such as primitive junctions and cytokeratins [Thie et al., 1995, 1996a]. These RL95-2

Experimental Modulation of Trophoblast Behavior cells do allow attachment of choriocarcinoma cell spheroids to their apical surface [John et al., 1993a, b] and were, therefore, used as a model for the receptive uterine epithelium. (2) AN3CA cells, in contrast, originating from lymph node metastasis of an endometrial adenocarcinoma [Dawe et al., 1964] are dedifferentiated and retain barely any epithelial characteristics. These cells, remarkably, when grown as a monolayer, did not support adhesion of choriocarcinoma cell spheroids [John et al., 1993a]. Hence, AN3CA cell monolayers were used as a nonadhesive control.

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Invasion Assay

The invasion assay used (see fig. 2) had originally been developed for investigating tumor cell invasiveness in cell-free ECM, in particular invasion through a basement membrane-type ECM (Matrigel [Albini et al., 1987; Repesh, 1989]). The test system employs a Boyden chamber that is modified by a matrix coating of the porous membrane. Boyden chamber systems without any matrix barrier blocking the pores had primarily been developed for assessing cell motility due to chemotaxis and/or chemokinesis [Boyden, 1962; Zigmond and Lauffenburger, 1986]. For the invasion assay the porous membrane (pore size = 8 µm) is covered with a thin gel of Matrigel blocking the pores. For assaying invasiveness, cells are suspended and transferred onto the surface of the matrix. Invasive cells are then able to penetrate the gel and move through the pores to the lower surface of the filter. After different time periods (see below), cells and gel on the upper surface are removed before the cells on the lower surface are stained and counted. This model has repeatedly been used to analyze invasion of trophoblast cells [Librach et al., 1991; Hohn et al., 1998].

Modulation of Differentiation

Modulation of differentiation by treatment with RA, MTX, dbcAMP or PMA (see above) was assessed in normal trophoblast and in choriocarcinoma cells grown as monolayer cultures using the secretion of hCG as a reliable parameter [Hohn et al., 1998, 2000]. In all cell types the secretion of the hormone was more or less clearly increased with all treatments. In normal trophoblast cells isolated from first trimester and term placenta (fig. 3), dbcAMP and PMA each induced a highly significant boost in hCG production while RA and MTX raised hCG secretion only slightly [Hohn et al., 1998]. A similar overall trend (although data differing in detail between groups) was seen with all three choriocarcinoma cell lines when grown as multicellular spheroids (as used in studies of cell adhesion) (fig. 4) [Hohn et al., 2000].

The cytoplasmic enzyme STS was investigated as an additional differentiation marker since this enzyme also (like hCG) appears to be associated with more mature stages of trophoblast (trophoblast cells isolated from term placenta displaying much higher activities of the enzyme than cells from first trimester, i.e. about 300 vs. 5 pmol/min \times mg protein [see Hohn et al., 1998]. This may well be due to the fact that, once transferred into cell culture, cytotrophoblast cells from term placenta undergo mor-



Fig. 2. In the Boyden chamber-type invasion assay cells are seeded onto a layer of basement membrane-like ECM gel (Matrigel) reconstituted on a porous filter membrane. After certain periods of time the cells that have penetrated gel and filter are stained and counted.

phological differentiation very rapidly resulting in the formation of symplasm by fusion of individual cells [Kliman et al., 1986b; Coutifaris et al., 1991; Hohn et al., 1993]. Correspondingly, the highest levels of the enzyme are detected in syncytiotrophoblast rather than in cytotrophoblast [Dibbelt et al., 1989; Ugele et al., 1992]. Modulators of differentiation had similar effects in both, first trimester or term trophoblast [Hohn et al., 1998]: slight but significant reduction with RA or dbcAMP but no change with MTX or PMA. In choriocarcinoma cells, in contrast, STS showed a tendency to be increased in BeWo and Jeg-3 cells, with a significant increase obtained in BeWo cells with dbcAMP or PMA and in Jeg-3 cells with MTX or PMA. The enzyme was not detected in JAr cells [see also Dibbelt et al., 1994].







Fig. 4. Modulation of hCG secretion in choriocarcinoma cell spheroids. The experimental design as described in figure 3 for normal trophoblast cells. $p \le 0.1$; $p \le 0.05$; $p \le 0.01$ as compared to the respective control (control = regular medium for MTX and dbcAMP; EtOH = control medium containing 0.1% ethanol for RA and PMA) [reproduced with permission from Hohn et al., 2000].

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Fig. 5. Modulation of the capacity of choriocarcinoma cell spheroids to attach to uterine epithelial monolayers. BeWo, JAr and Jeg-3 choriocarcinoma cells were treated with the same series of differentiation modulators as in figures 3 and 4, and their adhesive capacity was probed with the attachment assay (fig. 1) using 30 min of confrontation culture with RL95-2 cells. Adhesion rates were calculated from the total number of adhering spheroids referred to the total number transferred onto the endometrial monolayer (numbers added up from 3-6 experiments with 3-6 parallel coverslips carrying about 20 spheroids). Bars on the columns represent confidence limits (95% level) determined for the total number of spheroids with the respective treatment [reproduced with permission from Hohn et al., 2000].

Modulation of Adhesiveness

Multicellular spheroids of BeWo, Jeg-3 or JAr choriocarcinoma cells have previously been employed in an organ culture model system designed to mimic initial steps of embryo implantation. In confrontation cultures, spheroids were brought in contact with the epithelium of human endometrial explants [Grümmer et al., 1994].

Interestingly, in this model, the three cell lines displayed differences in their ability to adhere to the uterine epithelium and to invade it, in spite of the fact that all three cell lines were equally invasive in general invasion assays (using other types of host tissue or ECM). This suggests that the interaction of trophoblast type cells with the uterine epithelium shows a degree of selectivity not found in other host tissues or ECM models. In the adhesion assay used in the experiments discussed now, this system was simplified for better standardization and scaling up, i.e. the endometrium was replaced by a monolayer of RL95-2 endometrial carcinoma cells [John et al., 1993a]. This assay system, although without any doubt quite artificial when compared to the in vivo situation at embryo implantation, shows, interestingly, again a marked degree of cell type specificity, in this case studied with respect to the host (uterine) tissue cells. The choriocarcinoma spheroids attach easily to RL95-2 monolayers but not to AN3-CA endometrial carcinoma cells or to fibroblasts and display only low levels of adhesion to HEC1 endometrial car-

cinoma cells [John et al., 1993a, b]. A marked degree of specificity of this interaction is also suggested by the observation that adhesion of JAr cell spheroids to RL95-2 cell monolayers is inhibited by proteins extracted from either JAr cells or RL95-2 [Hohn et al., 2000]. Attachment of JAr cells to RL95-2 appears to depend on integrins, calcium and Rho signaling and on a reorganization of the actin cytoskeleton [Thie et al., 1997, 1998; Tinel et al., 2000; Thie and Denker, 2002]. A possible role of components of the ECM will be discussed below.

In our experiments on the modulation of the ability of trophoblastic cells to undergo this interaction, adhesion competence of choriocarcinoma spheroids tended to be reduced by all of the modulator drugs in all three cell lines (fig. 5). Major reductions were obtained with dbcAMP and with PMA in all cell types and with MTX in JAr and in Jeg-3. RA treatment resulted in only a slight reduction in all three cell lines, as did MTX in BeWo cells. The same experiments could not be performed with normal trophoblast cells isolated from either placental state because these (and in particular trophoblast from term placenta) did not form stable multicellular spheroids at reliable rates.

Modulation of Invasiveness

The invasive potential of normal trophoblast cells from either first trimester or term placentae or of malignant trophoblast (choriocarcinoma) cells and its experimental modulation were studied in a Boyden chamber system with a gel of Matrigel blocking the pores as an invasion barrier as described above. The different types of normal and malignant trophoblast cells differed considerably with respect to their invasive capacity in this test system. Choriocarcinoma cells reached the lower surface of the filter membrane, after penetrating the Matrigel-covered filters, after about 15 h of incubation, whereas penetration took about 2 days for first trimester trophoblast and about 4 days for term trophoblast. On the other hand, after first cells had penetrated the barrier it always took considerable additional time periods (differing between the different cell types) until larger numbers of cells reached the lower surface. Therefore, Jeg-2 cells were quantitated after 2 days and BeWo, JAr and first trimester trophoblast cells after 3 days. In contrast, trophoblast cells from term placentae showed a noninvasive behavior in this test, because even after 7 days only low numbers of invasive cells were found on the bottom face of the membrane [Hohn et al., 1998].



Fig. 6. Invasion assay: Typical pattern of uneven penetration of Matrigel by JAr cells. Cells that had reached the lower surface of the chamber (see fig. 2) were stained 3 days after start of the assay (50,000 cells per well) and can be seen to be accumulated here in concentric rings. An individual experiment is illustrated in which an exceptionally high number of cells have reached the lower surface so that they can be visualized even at low magnification. **a** Control. **b** MTX. **c** PMA. Diameters of filters = 6.5 mm [reproduced with permission from Hohn et al., 1998].

A difficulty with this test is that all cell types penetrate matrix and filter membrane inhomogeneously (fig. 6). This may be caused by the fact that the gel of Matrigel cast on the filter has an inhomogeneous thickness resulting from the formation of concentric menisci in the periphery of the filters in contact with the plastic walls of the chamber. This higher thickness in the periphery and lower thickness in the center may result in differences in the mechanical properties, which in turn have been shown to influence the behavior of choriocarcinoma cells [Hohn et al., 1992, 1993, 1996; Hohn and Denker, 1994]. In the case of trophoblast cells isolated from the first trimester as well as in Jeg-3 cells the thickness of Matrigel influenced cell morphology and most interestingly the proteolytic activity of the cells [Kliman and Feinberg, 1990].

With respect to changes in invasiveness elicited by the treatment with RA, MTX, dbcAMP or with PMA the different cell types can be grouped into two categories [Hohn et al., 1998]. In the first category, changes in invasiveness appeared to be correlated inversely with modulation of differentiation, i.e. treatments that increased hCG production significantly decreased invasiveness. This was the case with both types of normal trophoblast (first trimester and term), PMA causing the strongest reduction of invasiveness (fig. 7). BeWo cells might also be grouped into this category because all substances used tended to reduce invasiveness of cells although the decrease after treatment with RA and PMA was not significant (fig. 8). In the second category of cells, i.e. JAr and Jeg-3 cells, reduction of invasiveness was obtained with some substances (dbcAMP in JAr, RA in Jeg-3 and MTX in both cell

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Fig. 7. Invasiveness of normal trophoblast cells treated with differentiation modulators. Invasive cells were counted on the lower surface of the filters (cf. fig. 2 and 6) after 3 days for first trimester trophoblast and after 7 days for trophoblast cells isolated from term placenta. The data were calculated from 4-5 experiments with two parallel wells for each treatment. Invasion represents the portion of cells on the lower surface of the filter membranes referred to the total cell numbers determined at the same time points in parallel cultures on tissue culture plastic (coated with Matrigel at the same density as the filters in the assay) under the same conditions. ${}^{a}p \leq 0.05; {}^{b}p \leq 0.02; {}^{c}p \leq 0.01; {}^{d}p \leq$ 0.001 as compared to the control [reproduced with permission from Hohn et al., 1998].

types), but other substances interestingly increased invasiveness: PMA in JAr and in Jeg-3 cells and also RA in JAr cells (fig. 8). The changes in JAr cells were significant [Hohn et al., 1998]. Since no increase of invasiveness was seen with any of the drugs in normal trophoblast, there appears to be an interesting difference between normal and malignant trophoblast cells, in their response to modulators of differentiation with respect to their resulting invasive behavior.

Modulation of Matrix Interactions as Related to Invasion

The ability to penetrate into groups of other cells and into ECM is a property common to tumor cells, trophoblast cells, certain embryonic cell types, leukocytes and, notwithstanding differences in detail, also to bacteria. Apart from cell-cell interactions discussed above (with able phenomena include cell adhesion through matrix receptors like integrins, inside-out and outside-in signaling via those molecules, motility on ECM, modifications of stromal ECM by the activity of MMPs and modulation by tissue inhibitors of metalloproteinases (TIMPs) or by

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MTX PMA RA dbcAMP respect to the uterine epithelium as a confrontation partner for the invasive cells) this involves extensive interactions with ECM which have attracted considerable interest in various cell systems because they may be more simple to understand than cell-cell interactions, and because they include a fascinating reciprocity: modification of the ECM by the invading cells, but also modulation of cell behavior by the ECM [see Noel et al., 1994; Stracke et al., 1994; Levine et al., 1995; Burrows et al., 1996; Bussemakers and Schalken, 1996; Heino, 1996; Aplin, 1997; Wernert, 1997; Rovensky, 1998; Crowe and Shuler, 1999; Murray and Lessey, 1999; Aplin et al., 2000; Friedl and Brocker, 2000; Lauwaet et al., 2000; Bischof et al., 2001; Liotta and Kohn, 2001; Merviel et al., 2001a, b]. Observ-





Fig. 8. Modulation of choriocarcinoma cell invasion assessed after 2 days for Jeg-3 cells and after 3 days for BeWo and for JAr cells. Invasion was calculated from 3–5 experiments with two or three parallel wells for each treatment and represents the portion of cells on the lower surface of the filter membranes (cf. fig. 2 and 6) relative to the total cell numbers determined in parallel cultures on tissue culture plastic under the same conditions. Invasion defined as in figure 7. ^a p \leq 0.001; ^b p \leq 0.05; ^c p \leq 0.1; ^d p \leq 0.2 as compared to the control [reproduced with permission from Hohn et al., 1998].

the deposition of matrix molecules. In order to obtain information about such factors that may be responsible for changes in invasive behavior or in endometrial cell adhesion in trophoblast cell types we analyzed the effects of RA, MTX, dbcAMP or PMA on the motility of the cells on ECM, on their production of collagenases and of matrix molecules as well as on their adhesion to matrixcoated surfaces. These experiments were carried out using

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JAr cells because these showed the most interesting, i.e. heterogeneous, pattern of stimulated versus decreased invasiveness (see above).

First, we addressed the question of whether modulation of invasive behavior might be a result of alterations of general cell motility. JAr cell motility was assayed in a Boyden chamber assay with filter membranes whose pores (8 μ m in diameter) were not blocked by ECM (as in

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the invasion assays, see above) but left open although the membrane had received a monomeric coating of either Matrigel, collagen type I or of fibronectin. In these experiments, no significant changes were seen after treatment with the four differentiation-modulating agents except for a clear but slight increase of motility on fibronectin after treatment with PMA (data not shown).

Further it was asked whether altered MMP production might be instrumental in the observed modulation of invasiveness. The secretion of MMP was analyzed by zymography after separation of secreted proteins by electrophoresis on polyacrylamide gels containing 1 mg/ml gelatin. JAr cells secreted a prominent gelatin-degrading enzyme with an apparent molecular mass of 72 kD.

Some minor bands were detected in addition at 66 and 63 kD (fig. 9a). This is the typical pattern for the presence of matrix metalloproteinase 2 (MMP-2) also known as 72kD-type IV collagenase or gelatinase A [Emonard et al., 1987]. The presence of this enzyme was further verified by Western blot analysis using a specific antibody [Birkedal-Hansen et al., 1988] (fig. 9b). However, in our studies, no evidence was found that the rate of production of this enzyme can be held responsible for the observed differences in invasiveness since MMP-2 displayed higher activity under the influence of MTX or dbcAMP whereas invasiveness was reduced under the same conditions (cf. fig. 9 and fig. 8). Obviously invasion of JAr cells could also be regulated via other proteinases or the appropriate inhibitors. MMP-9 (92-kD type IV collagenase, gelatinase B) which has previously been found to be essential for invasion of normal trophoblast in vitro [Librach et al., 1991] was not detected in our experiments. The plasminogen activator-plasmin system as well as other serine proteinases found in trophoblast cells [Librach et al., 1991; Bischof and Martelli, 1992; Graham and Lala, 1992; Logan et al., 1992; Bischof et al., 1994, 2000b; Graham et al., 1994; Salamonsen, 1999; Bany et al., 2000; O'Sullivan et al., 2001a, b; Whiteside et al., 2001] might well be involved here, but data on regulation of their expression and/or activity by RA, MTX, PMA or dbcAMP are lacking.

In general, it must be said that the existing literature on the relevance of any of the mentioned enzymes for trophoblast invasion is quite contradictory. From experiments mainly using function-perturbing antibodies or inhibitors (TIMPs, plasminogen activator inhibitors) Librach et al. [1991] concluded that MMP-9 was the main player with a minor role for the urokinase-type plasminogen activator but not for interstitial collagenase (equivalent to MMP-1?). However, relevance of MMP-2 for inva-



Fig. 9. Expression of MMP-2 in the presence of differentiationmodulating agents. a Zymography. b Western blot. Gelatinase activity was detected by zymography (a) according to Heussen and Dowdle [1980] and Birkedal-Hansen and Taylor [1982]: JAr cells were grown in the presence of fetal calf serum and the respective differentiation-modulating substance. After they had been grown in fresh media without serum for an additional 24 h secreted proteins were separated on 10% polyacrylamide gels containing 0.2% gelatin under nondenaturing conditions. Applied protein was normalized for cell mass representing 3 µg of cellular DNA in the respective culture. After separation the gels were incubated in PBS containing CaCl₂ and ZnCl₂ (1 µM each) at 37 °C overnight before they were stained. A prominent band of gelatin degradation with variable intensity (i.e. enzyme activity) is detected at 72 kD; minor bands seen at 66 and 63 kD are thought to be isoforms of the 72-kD enzyme [Emonard and Grimaud, 1990; Corcoran et al., 1996]. Med = Fresh media were used as a control; Std. = protein standards were applied in lane 1 of a and lane 7 of b. The presence of MMP-2 was verified by Western blot analysis (b) under the same electrophoretic conditions as for a except for omitting the inclusion of gelatin. The specific antibody against MMP-2 was kindly provided by H. Birkedal-Hansen [Birkedal-Hansen et al., 1988].

sion cannot be excluded from these studies since antibodies specifically perturbing MMP-2 function were not used and the TIMPs used may well have inhibited both MMP-2 and MMP-9. In contrast, Graham et al. [1994] proposed that MMP-2 plays the central role, because in their investigations human first trimester trophoblast in vitro as well as JAr and Jeg-3 cells did not express MMP-9 but only MMP-2 (in a regulated fashion). This would be in



Fig. 10. Modulation of adhesion to ECM components in JAr cells. Cells were grown as monolayers and treated with differentiation-modulating substances from 3 days before the attachment assay was performed as described by Hohn et al. [1992]. Tissue culture plastic (96-well plates) was coated with 25 μ g/ml of individual ECM molecules or a 1:10 dilution of Matrigel in PBS. Nonspecific binding sites were saturated with BSA. 150,000 cells/cm² were transferred onto the coated wells and incubated for 1 h at 37 °C in a moist atmosphere with 5% CO₂ before adhesion rates were assessed. Averages are from 5 experiments with three parallel wells for each condition (substrate and treatment).

agreement with our findings (see above) insofar as the presence of MMP-2 is concerned, but not with respect to any correlation between production of this proteinase and regulation of invasiveness. Differences in the reported findings concerning MMP-9 could be due to cell culture conditions. According to Maquoi et al. [1997] both the MMP-2 and MMP-9 are expressed in choriocarcinoma cells only when cells are cultured on or in ECM substrates [as by Librach et al., 1991] whereas MMP-9 is not produced on tissue culture plastic (culture system used in our investigations on the proteinase production and by Graham et al. [1994]). The MMP-9 gene is known to be downregulated by progesterone and, therefore, reduced invasiveness and reduced expression of MMP-9 are in line with higher levels of progesterone in term placenta [Shimonovitz et al., 1998; Bischof et al., 2001]. These data suggest downregulation of MMP-9 expression with proceeding placental (trophoblast) differentiation. Therefore, it would be interesting to assess MMP-9 expression in normal trophoblast cells under PMA treatment in our system, since phorbol esters have been shown to stimulate MMP-9 expression in normal trophoblast [Bischof et al., 2001]. This appears to be in contrast with our finding of reduced invasiveness under PMA treatment if MMP-9 is assumed to play a crucial role here, although the difference in the cell type studied (choriocarcinoma vs. normal trophoblast) must perhaps be taken into account. Based on these conflicting observations it may appear reasonable to assume that differential expression of any of these proteinases (MMP-2 or MMP-9) may not be the critical factor that regulates differential invasiveness (see above). This leaves room for considering differential expression of TIMPs as a way of how trophoblast invasiveness may be regulated [Graham and Lala, 1991, 1992; Huppertz et al., 1998; Salamonsen, 1999].

Apart from matrix degradation, intermittent adhesion to ECM components is thought to be instrumental in invasion [Liotta and Rao, 1985; Stetler-Stevenson et al., 1993] and could likewise play a role in regulating this process. When the adhesion of JAr cells to substrates coated with components of ECM was assessed the cells displayed very little adhesiveness for monomeric coatings of laminin, collagen type I or collagen type IV (fig. 10). This was markedly different with fibronectin coatings. About 60% of the cells adhered to fibronectin after incubation on the substrates for 1 h. About 40% attached to coatings with Matrigel (a complex mixture of different matrix molecules similar to the composition of basement membranes [Kleinman et al., 1986]). Treatment of the cells with differentiation-modulating agents failed to alter the low adhesiveness to laminin, collagen I or to collagen IV, while attachment to fibronectin or to Matrigel tended to be reduced with all four substances used (although significant changes were only seen with attachment to fibronectin in the presence of MTX, fig. 10). A comparison with modulation of invasiveness (cf. fig. 8, JAr, and fig. 10)

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does not suggest any simple or direct mechanistical link between these two properties of the cells; if any there seems to be an inverse correlation between invasiveness and adhesion (to fibronectin and Matrigel) in case of modulation by RA and PMA. There was, however, no equidirectional response to all the modulating agents. With MTX, invasion as well as adhesion were both reduced.

These observations may appear to be somewhat in conflict with the results of studies on the role of trophoblast-ECM interactions via integrins as related to invasiveness. Indeed it has been suggested by immunohistological and functional investigations as well as by integrin knockout experiments that differential (adhesive?) interaction of normal trophoblast cells with the ECM through differentially expressed integrins may play a major role in the control of trophoblast differentiation and, in particular, of trophoblast invasion [Korhonen et al., 1991; Damsky et al., 1992, 1993, 1994; Fassler and Meyer, 1995; Stephens et al., 1995; Merviel et al., 2001a]. A probable explanation for the complexity of responses that (trophoblast) cells show in their invasive behavior may be seen in the complexity of the composition of the ECM, the existence of microdomains, and the complexity of signaling events elicited by the various integrins which appear to cooperate in ways that are only partly understood [Plopper et al., 1995; Aplin et al., 1998, 1999; Aplin and Juliano, 1999; Crowe and Shuler, 1999; Dedhar, 1999; Berditchevski, 2001; Eliceiri, 2001; Schwartz, 2001; van der Flier and Sonnenberg, 2001; Juliano, 2002].

On the other hand, as mentioned above (Modulation of Adhesiveness), adhesion mediated by ECM molecules may be involved in trophoblast attachment to the uterine epithelium [Lessey, 1998; Bowen and Burghardt, 2000; Kimber, 2000; Lessey, 2000; Burghardt et al., 2002; Wang and Armant, 2002]. Existing models postulate that in the state of adhesion competence certain integrins like $\alpha_5\beta_1$ and, in particular, $\alpha_{v}\beta_{3}$ translocate to or are newly expressed at the apical surface of the trophoblast as well as of the luminal uterine epithelium in a way that now matrix molecules (in particular, molecules like osteopontin, fibronectin, vitronectin, tenascin, thrombospondin or laminin) can serve as bridging molecules establishing contact and adhesion between both cell types. From this point of view the results of our investigations on modulated adhesiveness for molecules normally found in the ECM do correspond well with changes in trophoblast adhesiveness for endometrial cells, i.e. these two properties seem to be modulated in a parallel manner (see above).

Finally, ECM deposition by trophoblast cells must be considered as a factor that may contribute to the regulation of invasion into stroma. It is well known that in general cells modify their environment also by the deposition of matrix molecules and that invasion and metastasis are guided by the ECM [Bosman et al., 1993; Ruoslahti, 1994]. The type of ECM produced as well as the topography of deposition depend on the general cell phenotype [mesenchymal vs. epithelial; Hay, 1995]. Since the matrix molecules in turn signal to the cell interior via integrins, there is reciprocity in these phenomena of matrix production and interaction with the ECM. The type of integrins expressed by the cells depends on their epithelial or mesenchymal phenotype, in the same way as matrix production. Trophoblast cells undergo a partial epitheliomesenchymal transition (EMT) upon acquisition of invasiveness [Denker, 1993; Thie et al., 1996b; Vicovac and Aplin, 1996]. Eperimental data suggest that trophoblast cells may indeed be guided, during their invasion, by interacting via differentially expressed integrins [Damsky et al., 1992, 1993, 1994; Stephens et al., 1995] with matrix molecules resident in the decidua but also with such molecules deposited by the trophoblast cells themselves.

Based on these concepts, modulation of the production of major matrix proteins, i.e. fibronectin, laminin, collagens type I and type IV, was assessed in choriocarcinoma cells. JAr monolayers were treated with the differentiation modulators as described above, and the production of ECM components was studied in cell extracts as well as in supernatant media by dot blot analysis. The results were quite comparable for secreted as well as cell-bound forms of all matrix components tested (fig. 11). While treatment with RA or with PMA caused no or only slight shifts in the concentration as compared to the controls, MTX and dbcAMP clearly stimulated the secretion of all matrix molecules into supernatant media as well as their deposition in cell-bound matrix that could be extracted from the cell monolayer. When these results are compared with the observation of invasiveness (cf. fig. 11 and fig. 8) it becomes obvious that there is no simple correlation between matrix production and invasiveness, e.g. ECM production by JAr cells remains almost stable upon treatment with RA or PMA while invasion is increased. An inverse correlation (increased ECM production concomitant with reduced invasion) is observed after treatment with MTX or with dbcAMP. It could be argued, here, that increased deposition (or production or secretion) of matrix may impede invasiveness. However, the overall picture emerging about the response of JAr cells to the various treatments remains quite heterogeneous, with respect to any

correlations between the modulation of matrix invasion, matrix production, adhesion to matrix and matrix degradation by proteinases. This may reflect the complexity of the process of invasion and the diversity of signal transduction pathways influenced by the drugs that were used for modulation of cell behavior. In addition, it most probably also documents that any concepts about direct links between invasiveness and the state of differentiation of cells are prone to suffer from oversimplification.

Summary and Conclusions

According to the morphological sequence of events constituting the initial phase of embryo implantation, the properties that trophoblast cells must have in order to play their role here may be broken down into two major qualities: (1) the ability to undergo an adhesive interaction with the apical cell pole of uterine epithelial cells (apical cell adhesion competence) and (2) the ability to invade ECM (invasion competence). It must be asked whether this distinction is only of a descriptive value or whether it may reflect indeed different sets of properties which the trophoblast cells must have during implantation initiation, and whether such different sets of properties can be expressed by the cells with a certain degree of independence. For the latter aspect it should be of interest to see whether these properties show different and independent modes of regulation.

The data discussed above suggest indeed that a differential regulation of these two properties of trophoblast cells is observed when differentiation modulator agents are used. Apical cell adhesion competence was downregulated in parallel with an increased differentiation marker expression, while ECM invasiveness did not respond in parallel but showed quite heterogeneous patterns of modulation.

An interpretation of these phenomena observed must be sought with caution. Differentiation is not a clearly defined term, nor is invasiveness and neither is endometrial receptivity, a topic which is omitted from the present discussion but which is dealt with in another communication [Thie and Denker, 2002]. The meaning of 'differentiation' largely depends on the criteria used in order to describe this state (morphology, biochemistry/molecular biology, cell physiology/behavior). As far as the trophoblast is concerned, a varying number of different trophoblast cell types, more or less differentiated, have been described which can be found in vivo in the various stages of pregnancy, depending on the sets of criteria used





Fig. 11. Influence of modulated differentiation on production of ECM molecules. Cells were grown on poly-*L*-lysine-coated plastic in serum-free media (containing a fibronectin-stripped serum replacement, Ultroser^G, Pharmacia) in the presence of the respective substance. After 3 days media were retrieved for analysis before the cells were extracted with 0.5% sodium dodecyl sulfate in PBS. Aliquots of media and of cell extracts were standardized against cellular protein, transferred to nitrocellulose filters (using a dot/slot blot apparatus), and probed with antibodies against fibronectin (FN), laminin (LN), collagen type I (CI) or collagen type IV (CIV).

[Aplin, 1991, 1996; Vicovac and Aplin, 1996]. Accordingly, it remains difficult to talk about trophoblast 'differentiation' in general terms. However, there is no doubt that biochemical markers like hCG and STS as well as morphology have been found to be reliable and useful markers for at least some differentiated trophoblast phenotypes. Therefore in a rough, first order approximation it is hoped that the latter parameters give some information about the result of treatments that aim at increasing the overall status of differentiation in experiments as done here. The definition of physiological properties of cells like adhesion competence and invasiveness, on the other hand, depends even more on subtle details of the test systems used. The in vitro test systems employed here (the cell-cell adhesion assay and the ECM invasion assay) definitely simplify the in vivo situation and are certainly artificial in many respects. Therefore, any changes in cell behavior that are recorded with these tests cannot easily be extrapolated to other test systems or the in vivo situation.

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However, with these precautions in mind it may be possible to arrive at some conclusions on the basis of the discussed data.

The competence of trophoblast-type cells to establish apical adhesion to uterine epithelial cells appears to be linked directly to their state of differentiation, at least in the choriocarcinoma cell model used here. This may be a consequence of the fact that apical adhesion competence must theoretically be expected to depend on a destabilization of apicobasal polarity as proposed earlier not only for uterine epithelial receptivity but also for trophoblast adhesiveness [Denker, 1986, 1993]. When the differentiated state of an epithelium (trophoblast in this case) is increased (as by the differentiation modulator treatments used in the present series of experiments) the apicobasal polarity, a typical characteristic of highly differentiated simple epithelia, can be expected to be stabilized, meaning that apical adhesion competence will be downregulated. This is indeed seen in our experiments on interaction of trophoblast with RL95-2 monolayers. Interestingly, this view may apply not only to cell-cell but also cellmatrix adhesion competence (of the apical cell pole) of trophoblast cells. The findings of Armant's group [Wang and Armant, 2002] suggest for the mouse system that the competence of the apical pole of blastocyst trophoblast for integrin-mediated ECM adhesion and transmission of calcium signaling is dependent on a downregulation of apicobasal polarity. One would expect that a stable upregulation of polar organization, if this could be achieved by any treatment affecting the state of differentiation as in our experiments, would also counteract trophoblast adhesion competence in that system, although this has not been tested.

ECM invasion competence, on the other hand, appears to be linked to the regulation of the differentiated state in a more complex way, as far as the pattern of responses suggests that was seen in our experiments with differentiation-modulating drugs. As already mentioned, this may mirror the complexity of the elementary processes behind the matrix penetration mechanisms (see above). Invasiveness was affected heterogenously in JAr and Jeg-3 cells. It was reduced in JAr and Jeg-3 cells in the presence of some substances (JAr: MTX or dbcAMP; Jeg-3: RA or MTX) but was (even highly) stimulated with others (JAr: RA or PMA; Jeg-3: PMA) [Hohn et al., 1998]. BeWo cells displayed an inverse correlation between invasiveness and differentiation (which was observed in normal trophoblast cells in a similar fashion).

Such heterogeneity is also mirrored by the interaction of JAr cells with ECM in that no significant changes were

observed for motility on matrix and a tendency of reduced adhesion to matrix components. Slight but also some strong increases are seen with respect to ECM production. The same holds true for the secretion of a matrix-degrading enzyme (MMP-2), which makes it difficult to come up with any simplistic models explaining changes in invasiveness by altered interactions with ECM and which suggests that additional investigations are necessary.

In conclusion, invasiveness into ECM and adhesiveness of trophoblastic cell types to uterine epithelial cells appear to be under different control mechanisms. This supports the view that the two different elementary processes of implantation initiation, i.e. epithelial adhesion/ penetration and stromal invasion, involve somewhat different cellular mechanisms, and that this concept may be of more than a descriptive value. The initial interaction of trophoblast with the uterine epithelium appears to be critically dependent on destabilization/downregulation of apicobasal polarity of these two epithelia, a prerequisite for signaling and adhesion processes at the apical cell poles. Any regulatory processes influencing the expression of epithelial cell polarity must, therefore, be expected to influence trophoblast adhesion competence (and uterine epithelial receptivity). It is not clear whether a de novo expression of adhesion molecules is required for this or only redistribution and activation cascades.

For the acquisition of matrix invasion competence, in contrast, one would expect a complex set of properties to be critical that involves again a downregulation of apicobasal epithelial polarity of trophoblast cells, but in addition also the de novo expression of mesenchymal-type adhesion proteins, e.g. integrins, a switch to mesenchymal-type ECM production and a reorganization of the cytoskeleton, all these being phenomena known from EMT [reviewed by Denker, 1993]. Thus one might expect any signaling involved in regulating EMT events to potentially play a role in trophoblast invasion. As for EMT events in embryology, it is still unclear how exactly the acquisition of invasiveness may be related to apical adhesiveness to other epithelia (see above). It may be hoped that studies on the interconnections between these processes will help to shed light on regulations behind the events that are critical for embryo implantation initiation.

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