Implantation, trophoblast differentiation and haemochorial placentation: mechanistic evidence *in vivo* and *in vitro*

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Introduction

The process of implantation and placentation in women involves a series of intercellular interactions, the eventual elucidation of which will result in extensions or even shifts of cell biological paradigms. These include the formation of motile and non-motile mononuclear and multinuclear trophoblast, alterations between faster and slower states of proliferation, changes in adhesive properties and further differentiation of the various trophoblast populations. In addition there is progressive local tissue remodelling by both mononuclear and multinuclear trophoblast as it interacts with the maternal environment (Aplin, 1989; Glasser et al. 1991) including decidua, smooth muscle, endothelium and blood.

Initial interaction with maternal stroma

Following attachment to the endometrial surface, trophoblast penetrates the epithelium and its basement membrane (Fig. 1A; Schlafke and Enders, 1975; Lindenberg et al. 1990; Weitlauf, 1988; Denker, 1990). Contact is thus established with the underlying stroma, which also differentiates under the influence of hormones ('decidualisation'). In humans, this process is not evident until some 5 days after the embryo makes contact with the stroma (cf. Fig. 1A and B; and Hertig et al. 1956; Boyd and Hamilton, 1970). In contrast, in rats and mice, where trophoblastic penetration of the stroma is less extensive, decidualisation is evident in the earliest phase of implantation (Finn and Porter, 1975), a difference that may be significant in placental morphogenesis (see below). Decidual tissue is secretory and a paracrine relationship is established between the mother and her embryo (Bell, 1988).

Motile behaviour in the early syncytiotrophoblast

In the earliest human implantation sites that have been observed, the outer trophoblast layer is largely multinuclear (Fig. 1A). The cells are invasive, colonising glands, degrading local extracellular matrix (ecm) and endocytosing glandular secretory contents (Boyd and Hamilton, 1970). This unusual behaviour appears to have something in common with the osteoclast (Sato and Rodan, 1989). As maternal capillary spaces are invaded, and even before implantation is complete, another process occurs in the trophoblast that depends on cell motility and resembles aspects of the behaviour of sprouting endothelial cells in neovascularisation (Ingber and Folkman, 1989; Grant et al. 1989). Membrane-bounded spaces appear within the cytoplasm and become organised into an intercellular channel system lined by trophoblast plasma membranes (Enders, 1989). This appears to be the surface at which the earliest haemochorial contact occurs (Boyd and Hamilton, 1970), though there is evidence that the maternoplacental vascular circuit only becomes continuous at a later stage (Hustin and Schaaps, 1987). Throughout placentation, the lining of the new channels through which maternal blood passes comprises (non-haemostatic) trophoblast.

Extravillous cytotrophoblast

The events of the next month in women have been little studied. However, it appears that beneath the syncytiotrophoblast from the earliest stages there is an inner stem cell layer of primitive mononuclear cytotrophoblast, dividing and fusing with the overlying syncytiotrophoblast, dividing and fusing with the underlying syncytiotrophoblast to allow expansion of the surface area of the developing placenta (Fig. 1A; and Boyd and Hamilton, 1970; Benirschke and Kaufman, 1990). During the 3rd week after ovulation, villous core mesenchyme develops beneath the cytotrophoblast, and within it there appear foetal blood vessels (Fig. 1B). At this stage the essential components of the haemochorial interface are in place. The major route of nutrient supply to the growing foetus becomes the villous interface, with solute transfer taking place across the syncytiotum between the maternal and foetal blood.

During the 3rd post-ovulatory week, proliferating cytotrophoblast at the tips of the villi penetrates the syncytiotum to make contact with the underlying maternal tissue (Fig. 1B). The mechanism of this process is unknown, but it may involve local degeneration of the syncytiotum.
Fig. 1. (A) Human implantation site at approximately 9 days after conception. The outer layer of the embryo consists of syncytiotrophoblast (STR) in which spaces (lacunae) are already visible. These expand to form the intervillous space. Adjacent to the outer syncytial surface at its interface with the maternal uterine stroma (US) is a zone of tissue degradation. UE, uterine epithelium; CTR, cytotrophoblast layer beneath the syncytium. (B) Materno-foetal interface approximately 4 weeks after fertilisation showing the tip of a placental anchoring villus and the adjacent placental bed including the terminal portion of a maternal spiral artery (a) at its exit into the intervillous space (ivs). By this stage the maternal stromal cells have differentiated into decidual cells (D; heavily outlined). Cytotrophoblast proliferates to form a column at the tip of the anchoring villus and migrates into the decidua interstitium and into arteries. The extracellular matrix is partly collagenous, partly fibrinoid. The villous trophoblast is surrounded by the intervillous space through which maternal blood will flow, nutrients being transferred to foetal blood carried in vessels within the villous stroma.

syncytium at sites of decidual contact (Fisher et al. 1985; Vićovac, Sattar, Jones and Aplin, unpublished data). Cytotrophoblast proceeds to colonise the maternal decidua surface to form a 'shell'. Nothing is currently known of the mechanism whereby villous stem cytotrophoblast is diverted from the villous syncytial differentiation pathway into this extravillous (or intermediate; Kurman et al. 1984) lineage. However, its onset coincides with decidual differentiation in the maternal stroma, and so it is possible that a short-range decidual signal may be responsible (Ran and Braustein, 1991; and Fig. 2).

Extravillous trophoblast now ceases cell division (Bulmer et al. 1988) but continues to penetrate and invade the maternal interstitium where it can conveniently be detected in sections by use of antibodies to cytokeratins (Khong et al. 1986; and Fig. 4, below). The distribution of these cells is significant: they occur more frequently in the peri-arterial stroma (Pijnenborg, 1990). Cytotrophoblast also migrates from the shell along the inner walls of the maternal spiral arteries that feed the intervillous space (Fig. 1B). This invasion seems to occur in two waves, one in first trimester and the second one in second trimester up to about 18 weeks (Pijnenborg et al. 1980). By this time, the intravascular trophoblast has penetrated the inner myometrial segments of the spiral arteries. Meanwhile, the interstitially invading trophoblast has reached the endometrial—myometrial interface with some cells penetrating the myometrium itself. The purpose of this invasion of maternal tissue by trophoblast is probably the remodelling of arterial walls with loss of smooth muscle and associated elastic and collagenous ECM, these being replaced by a unique fibrin-based polymeric deposit ('fibrinoid'). In this way resistance to the flow of arterial blood into the intervillous spaces is reduced, the arteries are allowed to expand and the supply of nutrients to the growing foetus is both enhanced and made independent of the influence of maternal vasoconstrictors. The foetus—placenta becomes a privileged site.

Non-Invasive cytotrophoblast

At first the post-implantation embryo is surrounded completely by developing villous tissue. However, during the 3rd month of pregnancy, in parts of the placenta superficial to the embryo (chorion laeve), the villi regress, thus favouring the most direct route for maternal blood to the placenta (Benirschke and Kaufmann, 1990). The outer layer of the placenta in the non-villous areas also comprises cytotrophoblast, as a multilayered epithelium abutting directly on the maternal stroma. There is homogeneity in the cell population (Bulmer and Johnson, 1986; Yeh et al. 1989) with some proliferation continuing up to term (Bulmer et al. 1988). For unknown reasons, but possibly having to do with the absence of chemotactic signals originating in the maternal vascular system (Fig. 2), these cells do not invade the adjacent decidua significantly.

Molecular Indices of trophoblast differentiation

Presence of the polypeptide hormone chorionic gonadotrophin (CG) in the maternal circulation provides the earliest evidence of a pregnancy. CG is a dimer of \( \alpha \) and \( \beta \) subunits synthesised and secreted by trophoblast in increasing amounts during the first trimester of pregnancy. Later, dimer secretion declines, though synthesis and secretion of the \( \alpha \) subunit continue to increase to term.
CGα mRNA is detectable predominantly in villous syncytiotrophoblast but also occurs in a subpopulation of villous cytotrophoblast (Hoshina et al. 1982), confirming ultrastructural evidence that the first steps in differentiation are made prior to fusion with the overlying syncytiotrophoblast (Terzakis, 1963; Jones and Fox, 1991; Fig. 2). Immunohistochemical studies confirm that CG is produced mainly in the villous syncytiotrophoblast (Gosseye and Fox, 1984; Kurman et al. 1984).

Placental lactogen (PL) is produced by the syncytiotrophoblast in steadily increasing amounts throughout pregnancy, but is also detected by immunohistochemistry in extravillous populations in the placental bed in endovascular and interstitial locations (Gosseye and Fox, 1984; Kurman et al. 1984).

Numerous other glycoproteins detected in pregnancy plasma originate largely in the secretory syncytiotrophoblast. These include pregnancy-specific β1 glycoprotein (SP-1; a member of the CEA family and immunoglobulin superfamily; Gosseye and Fox, 1984; Takayama et al. 1989; Zheng et al. 1990; Watanabe and Chou, 1988), plasminogen activator inhibitor type 2 (PAI-2; Astedt et al. 1986; Feinberg et al. 1989; see below), and others. Placental-type alkaline phosphatase (Webb et al. 1987) is present on some extravillous cells at term, but is again a product of the terminally differentiated syncytiotrophoblast. Maternal-to-foetal ion- and nutrient-transport functions must also be acquired by the syncytiotrophoblast as it differentiates.

Studies of the extravillous lineage require unique markers for isolation and characterisation of the cells. These are currently scarce; many epitopes identified on the villous syncytiotrophoblast are also present on the extravillous cells (Bulmer and Johnson, 1985; El-Nasir et al. 1987; Loke et al. 1990), consistent with their common origin. Plasma membrane glycoproteins of the HLA family (major histocompatibility antigens) have received a great deal of attention because of the necessity for the foetal allograft to avoid rejection. The major antigens are absent from all trophoblast subpopulations, but a non-polymorphic member of the family, HLA G, is expressed on extravillous trophoblast (Sunderland et al. 1981; Ellis, 1990).

Certain glycans have been identified as unique to the extravillous cells: for example, after the 10th week of gestation, a terminal non-reducing α-galactosyl epitope is present, but prior to this stage the structure is also expressed on villous cyto- and syncytiotrophoblast (Foidart et al. 1990). The blood group-related antigen sialyl-Leα is present on the interstitial and endovascular populations but absent from villous trophoblast and the cytotrophoblast columns and shell (King and Loke, 1988).

**Syncytiotrophoblast**

What light can cultured trophoblast throw on the above complex series of interactions? Improved techniques for
isolating and culturing villosus cytotrophoblast from normal placentas of various gestational ages have given access to the syncytial differentiation pathway in vitro. In addition, choriocarcinoma cell lines (principally BeWo, JAr and JEG-3, a subline of BeWo), which consist of a mixture of proliferating cytotrophoblast with a small proportion of multinucleate cells, have been used extensively to study hormone production.

At term, the cytotrophoblast population underlying the villous syncytiotrophoblast has become sparse (Jones and Fox, 1991). Nonetheless, mononuclear cytotrophoblast can be isolated (Kliman et al. 1986; Morrish et al. 1987; Kato and Braustein, 1989). Within a few hours of plating out, term cytotrophoblasts form into colonies within which, during the next few days, fusion can occur giving rise to multinuclear cells. There is little or no proliferation. CG and PL are synthesized and secreted but this declines after approximately the 5th day in vitro (Morrish et al. 1987; Kato and Braustein, 1989). Cytotrophoblast isolated from first trimester placental villi shows more proliferative potential, but also gives rise to colonies containing syncytiotrophoblast and producing CG (Stromberg et al. 1978; Cotte et al. 1980).

Villosus synctiotrophoblast is the main site of CG and PL production in vivo. Since multinucleate cells form spontaneously in culture, one can ask whether the expression of polypeptide hormone subunits depends on cell fusion. The central difficulties in the analysis have been in relating hormone production at the single cell level to its concentration in the culture medium, and in determining the extent of cell fusion within mass cultures where intercellular boundaries are not easily discernible in the light microscope. The occurrence of true syncytiotrophoblast in primary cultures a few days old has been demonstrated convincingly by various methods: electron microscopy, microinjection of fluorescent dye and immunostaining for CG or PL in which contiguous areas of cytoplasm share the same staining intensity, while adjacent mononuclear cells in the colony frequently appear unstained (Cotte et al. 1980; Kliman et al. 1986; Daniels-McQueen et al. 1987; Lobo et al. 1987; Morrish et al. 1987). However, numerous authors have recognised the difficulty of distinguishing colonies of flattened cytotrophoblast from syncytia and resorting to terms such as 'apparent' or 'syncytial-like'. Recently, new methods have been suggested that may aid discrimination between the two: Wice et al. (1990) used mild trypsinisation to cause BeWo cells to retraction from one another sufficiently to observe the intercellular boundaries in phase contrast. We (J. D. Aplin and A. Sattar) have observed that BeWo cells have prominent microfilament bundles adjacent to intercellular (but not free) boundaries (Fig. 3). Desmosomes, which occur in intertrophoblastic borders, are found within colonies of primary term trophoblast after several days in serum-containing medium, and the observations indicate the persistence of a population of mononuclear cells. In contrast, in a defined (keratinocyte) growth medium, syncytial differentiation occurs with the loss of desmosomal plaques and the elevation of CG production (Douglas and King, 1990). Thus more stringent criteria will in future be necessary in studies of cytotrophoblast to syncytial transformation.

Notwithstanding the above difficulties, it is probable that in primary trophoblast cultures, the highest production of CG and PL is from multinuclear cells. However, there is evidence that production of CG (and possibly β) does occur in mononuclear cells. First, a proportion of mononuclear cells in the BeWo and JAr cell lines (Martell and Ruddon, 1990; Sibley et al. 1991) as well as in early primary cultures (Kliman et al. 1986) can be immunostained for CG. Second, when term cytotrophoblast is cultured in serum-free medium, cell spreading and fusion do not occur; nevertheless, viability is retained and CG is secreted into the medium (Kao et al. 1988). Third, CGβ (but not the CGβ subunit) can be immunoprecipitated from the culture medium of first trimester cytotrophoblast within 4 h of isolation (Daniels-McQueen et al. 1987). During the first 24–48 h in vitro, CGβ production begins, giving rise to holo-CG in the culture medium. This coincides with the appearance of multinuclear cells in the cultures. During the following 3 weeks production of CGβ stops but CGα production continues. Thus the cells undergo a differentiation process in vitro that resembles their programme in vivo.

Conversely, in mass cultures of BeWo or JAr cells, morphological differentiation can occur in the absence of detectable CG production as monitored by immunofluorescence (Martell and Ruddon, 1990). Thus it would appear that multinucleation and the production of CG are independent events, correlated under normal conditions of placental development. The cell culture observations also lend support to the idea (Hoshina et al. 1982) that a transitional (CGα-producing) form of mononuclear cytotrophoblast exists in the villous differentiation pathway.

**The control of villous differentiation**

What controls syncytial differentiation? Considerable efforts have been devoted to a search for humoral factors that might influence this process. It has been known since the late 1970s that elevation of intracellular cyclic AMP levels causes increased production of CG by trophoblast
(reviewed by Pattillo et al. 1983), though no physiological first messenger has been identified. A CRE (cyclic AMP-responsive element) has been demonstrated in the 5'-promoter region of the CGa gene (Darnell and Boime, 1985). Non-trophoblastic cell lines, after transfection with a construct containing the 5'-CRE region of CGa, failed to respond to cyclic AMP, suggesting that other controls also influence trophoblast differentiation. The CGa gene appears to be controlled by a different mechanism (Otani et al. 1988).

While term trophoblast in culture is stimulated by 8-Br-cyclic AMP to produce elevated levels of CG, SP-1 production is suppressed, and these responses are independent of the formation of syncytiotrophoblast (Feinman et al. 1986; Ullao-Aguirre et al. 1987; Kato and Braunstein, 1989). Placental lactogen (PL) production is unaffected, and BeWo cells respond to 8-Br-cyclic AMP or forskolin (which activates adenylyl cyclase) by forming syncytiotrophoblast, secreting hCG, and increasing alkaline phosphatase levels (Wice et al. 1990). Since the last three substances are products of late pregnancy, in contrast to CG, which is produced from the peri-implantation phase onwards, it has been suggested that the cyclic AMP-stimulated BeWo cells resemble an earlier (i.e. first trimester) type of syncytiotrophoblast (Wice et al. 1990). The BeWo cell observations are important in suggesting that extracellular signals could alter not only the kinetics of transition from cytotrophoblast to syncytiotrophoblast, but also the decision to differentiate along the villous pathway. There are also indications that normal cytotrophoblasts, when stimulated in culture by cyclic AMP, cease the production of components characteristic of the extravillous phenotype (see below). Conversely, cultured cytotrophoblasts can be inhibited from CG production by a small protein released by cultured decidual cells (Kao et al. 1990) and this substance also blocks cyclic AMP-mediated stimulation; these results may indicate that decidua plays a role in controlling the secretory phenotype of extravillous trophoblast (Fig. 2).

It has been shown that unphosphorylated adenosine derivatives stimulate CG production both in JAR cells and normal term trophoblast in culture (Martell and Ruddon, 1990; Otani et al. 1989; Sibley et al. 1991). Elevation of both the alpha and beta subunits occurs, and since the latter does not respond to cyclic AMP, an independent mechanism is likely to be involved. JAR cells are also inhibited by adenosine derivatives from dividing.

Evidence for intercellular interactions in the control of CG production has recently been adduced: significantly increased amounts of CG (and PL) are produced by term trophoblast in coculture with JAR cells and normal term trophoblast in culture (Martell and Ruddon, 1990; Otani et al. 1989; Sibley et al. 1991). Elevation of both the alpha and beta subunits occurs, and since the latter does not respond to cyclic AMP, an independent mechanism is likely to be involved. JAR cells are also inhibited by adenosine derivatives from dividing.

Studies of the effects of growth factors and cytokines on trophoblast function are at an early stage. Morrish et al. (1987) and Barnes et al. (1990) have shown that epidermal growth factor (EGF) treatment gives rise to enhanced syncytiotrophoblast formation and increased production of CG and PL in culture. In first trimester cultures CG production also increases but without increased cell fusion (Barnes et al. 1990). This is consistent with the presence of EGF receptors in the villous trophoblast; however, receptors are also found in the extravillous populations (Bulmer et al. 1989). The same receptors may be occupied by TGF-β, which is a product of decidua in rat (Han et al. 1987). TGF-β1 is also a decidual product in mouse (Tamada et al. 1990). IL-1α and β and TNF-α are produced by human decidua (Romero et al. 1989) and there is evidence that IL-1α can stimulate CG production by trophoblast (Yagel et al. 1989; Silen et al. 1989), as can IL-6 (Nishino et al. 1990). In mouse, CSF-1 is produced by decidua and its receptors are present in both decidua and adjacent trophoblast (Arceci et al. 1989; Regenstreif and Rossant, 1989). There is evidence that retinoic acid may stimulate placental hormone secretion by trophoblast (Kato and Braunstein, 1991). Polymamines (Moore et al. 1988), inhibin (Petraglia et al. 1989, 1990) and interferons (Sekiya et al. 1986) may also have a role. More information is required about growth factor/second messenger systems in trophoblast differentiation.

Kao et al. (1988) have shown that term cytotrophoblast in serum-free medium remain mononuclear on plastic but may be triggered to spread and form multinucleated (possibly syncytial) colonies by plating onto surfaces containing fibronectin, laminin or collagens I, III, IV or V. The activity of fibronectin may be inhibited by peptides containing the RGD cell recognition motif. This complements many other reports that ECM components can be permissive for the expression of a differentiation programme.

Intercellular interactions must obviously be regulated in both the syncytiotrophoblast and extravillous differentiation pathways, but little is yet known about the molecular mechanisms of trophoblast adhesion. Cytotrophoblasts established either in monolayer or suspension culture form into aggregates or colonies in the first few hours, provided that divalent cations are present (Kliman et al. 1986; Babalola et al. 1990). Desmosomes are prominent between cytotrophoblast and syncytiotrophoblast in vivo (Beham et al. 1988) and in vitro (Douglas and King, 1990; Babalola et al. 1990). Another type of calcium-dependent adhesive interaction is mediated by the glycoprotein E-cadherin, found at the borders between villous cytotrophoblast and syncytiotrophoblast (Fisher et al. 1989). Fully differentiated rat trophoblast expresses another member of the cadherin family, P-cadherin (Nose and Takeichi, 1986) as does the adjacent maternal decidua. Integrin α6β4 (Sonnenberg et al. 1990) is present at the interface between villous cytotrophoblast and syncytiotrophoblast as well as at the basal trophoblastic cell surface (Figs 4, 5), but its function is undefined.

The mechanism and control of trophoblast invasion

The invasive phase of trophoblast ontogeny lasts into the 5th month of pregnancy in women, and various hypotheses have been elaborated to explain its control: (1) trophoblast has an intrinsic differentiation programme in which invasive behaviour ceases 4–5 months after implantation with the differentiation of motile extravillous trophoblast into non-motile placental bed giant cells; (2) trophoblast differentiation proceeds from invasive to non-invasive phenotypes under the control of soluble (endocrine, paracrine or autocrine) factors; (3) the transition from invasive to non-invasive phenotype is the result of exposure during the later stages of invasion to the myometrial environment; (4) trophoblast is intrinsically invasive but spread is limited by the maternal decidua functioning as a barrier (Billington, 1971).

In principle these possibilities should be distinguishable by experiments in cell culture. There is also valuable (but not conclusive) evidence available from in vivo obser-
Fig. 4. Immunofluorescence of integrin α6 (monoclonal antibody GoH3; Sonnenberg et al. 1990) and cytokeratin (monoclonal antibody CAM 5.2; Makin et al. 1984) at the materno-foetal (anchoring villus-decidual) interface in first trimester. Left top and bottom, integrin α6 showing staining in the villous trophoblast and more weakly in adjacent decidua (de). Right top and bottom, both villous and extravillous trophoblast populations stain strongly for cytokeratin. Frames at bottom left and right are adjacent sections and indicate that as the trophoblast leaves the placental villus to migrate into the decidual interstitium, α6 integrin expression is diminished. Observations. The evidence, reviewed in the following sections, suggests that mechanism (1) is dominant, though other mechanisms do contribute.

Initial invasion by syncytiotrophoblast gives way to later invasive behaviour on the part of cytotrophoblast; these two processes should be seen as distinct, though interdependent. It is to be expected that the onset of cytotrophoblast invasion is heralded by a panoply of changes in cell–cell and cell–matrix interactions as cells escape from the tips of anchoring villi. Cell–matrix interactions might include matrix remodelling (production or breakdown) by trophoblast as well as changes in cell surface receptors for matrix components, as anchorage to the villous basement membrane is lost and the maternal vascular and decidual matrices encountered. It is also possible that the maternal ECM could in turn influence the trophoblast differentiation state.

Fig. 5. Immunofluorescence (monoclonal antibody 5B5; Sonnenberg et al. 1991) of integrin β4, which forms a heterodimer with the α6 subunit. Top, first trimester villous tissue showing staining in the stem cytotrophoblast (CTR) but not the overlying syncytiotrophoblast; ivs, intervillous space; vs, villous stroma. Bottom, cultured BeWo cells also show a pericellular distribution of the β4 subunit.

Matrix degradation in culture

Trophoblast isolated from first trimester placenta, unlike third trimester isolate, retains considerable proliferative potential in vitro (Stromberg et al. 1978; Cotte et al. 1980; Yagel et al. 1988; Yeger et al. 1989). Although a subpopulation of the cells differentiate down the villous pathway, mononuclear cytotrophoblast persists for several passages. Loke and Burland (1988) have detected in first trimester isolates a proportion of cytokeratin-positive cells carrying the HLA class I framework epitope W6/32. This is present in vivo only on the extravillous population (HLA-G; see above), and so their data support the contention that there are extravillous-type cells present or that they appear during culture.

It has been established by several groups that early trophoblast expresses matrix-degrading proteolytic activity. The human peri-implantation blastocyst secretes a type IV collagen-degrading enzyme (Puistola et al. 1989). Newly hatched mouse blastocysts adhere to, and grow out on, a variety of extracellular matrix substances including fibronectin, laminin and several collagen types (Armant et al. 1986a; Sutherland et al. 1988; Carson et al. 1988) and trophoblast outgrowth leads to local clearance of fibrillar material visible in the scanning electron microscope (Glass et al. 1983). Explant cultures of first trimester human chorionic villi also degrade collagen gels (Vico, Sattar, Jones and Aplin, unpublished data) and this is consistent with the presence of the $52/55 \times 10^3 M_\text{r}$, interstitial collagenase in first trimester villous tropho-
blast (Moll and Lane, 1990). This enzyme is also expressed by the extravillous population (Yagel et al. 1988; Moll and Lane, 1990). Fisher et al. (1989) have identified several distinct metalloproteinase species in human trophoblast including a \(92 \times 10^6\) M, gelatinase that is expressed in first trimester cells, at much lower levels in second trimester and lower still in third trimester. Emonard et al. (1990) have demonstrated that first trimester trophoblast and BeWo cells are stimulated to produce more type IV procollagenase by the addition of soluble laminin; when the same cells are cultured on gels of type I collagen or the basement membrane analogue Matrigel (instead of plastic), interstitial procollagenase production is stimulated in addition to the type IV procollagenase.

Urokinase-type plasminogen activator (uPA) is produced by peri-implantation mouse embryos (Strickland et al. 1976) and also by human cytotrophoblast (Martin and Arias, 1982; Queenan et al. 1987). Given evidence that both uPA and collagenases may contribute to matrix breakdown by trophoblast (Yagel et al. 1988; see below) it is interesting to note that plasmin can activate interstitial procollagenase (He et al. 1989). The presence of different plasminogen activator inhibitors in villous (PAI-2) and extravillous (PAI-1) trophoblast (Feinberg et al. 1989) is probably indicative of tightly regulated cellular micro-environments in which proteases are both produced and inhibited by the same cells, giving rise to highly localized degradation (Glass et al. 1988). PAI-1 is a potent inhibitor of ECM breakdown by invasive tumour cells (Cajot et al. 1990). The tissue inhibitor of metalloproteinases (TIMP) is produced by decidual cells in mouse (Nomura et al. 1989), suggesting that the maternal compartment may also play a role in protease regulation (see below).

Yagel et al. (1988) showed that early passage first trimester trophoblast shows significant invasive behaviour in the denuded amnion assay, in which cells are required to attach to basement membrane and then penetrate it and the underlying dense fibrillar stroma. Up to 4 % of the cells could penetrate into the stroma in a period of 6 days, a value comparable with those obtained from the human choriocarcinoma cell line JAr and the mouse metastatic melanoma cell line B16F10, and roughly three to four times higher than that observed when a non-metastatic cell line was used. Invasion was abrogated by inhibitors of collagenase or plasmin or by treatment of trophoblast isolated from term placenta, when plated onto the amnion surface, forms a confluent cell layer that differentiates into a polarised syncytiotrophoblast (Aplin and Charlton, 1990; Earl et al. 1990). Laminin is also produced by normal trophoblast and choriocarcinoma cells in culture (Peters et al. 1985), while in vivo, it is present in the villous basement membrane as well as in association with extravillous cells. A variety of isoforms are present: the A and merosin heavy chains and the light chains Bl, B2 and Bl (Leivo et al. 1989; Earl et al. 1990; Engvall et al. 1990).

Given the extensive fibrinoid deposit that forms in the walls of the uterine spiral arteries during their transformation in pregnancy, it is possible that, in addition to degrading the arterial musculoelastic matrix and depositing endogenous ECM components, trophoblast may act to nucleate the deposition of fibronectin and other maternal ECM components. Conversion of fibrinogen may be triggered by the production of uPA by trophoblast (Martin and Arias, 1982; Queenan et al. 1987). Complement components are also deposited (Wells et al. 1987) and there is evidence that the trophoblast surface contains molecules including membrane cofactor protein (Purcell et al. 1990) and decay accelerating factor (Holmes et al. 1990), which protect against attack by maternal complement. However, no detailed study has yet addressed the mechanism of vascular transformation. In guinea pigs, where similar arterial widening takes place, the major structural alterations seem to occur in advance of the wave of invading trophoblast (Hees et al. 1987).

The expression of receptors for extracellular matrix

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**Matrix deposition and matrix receptors**

In addition to its involvement in ECM degradation, it is likely that extravillous trophoblast contributes to the (largely maternal) ECM through which it migrates. This contribution could be made in either of two ways: the secretion of endogenous ECM components or the 'seeding' of maternally derived deposits, e.g. from plasma. Production of fibronectin has been demonstrated in cultures of cytotrophoblast and it is interesting that this is diminished in the presence of 8-Br-cyclic AMP, which stimulates syncytiotrophoblast differentiation (Queenan et al. 1987). Thus fibronectin might be a product of the extravillous cell, and this is consistent with immunohistochemistry of the placental bed (Aplin et al. 1988; Aplin and Charlton, 1990; Earl et al. 1990). Laminin is also produced by normal trophoblast and choriocarcinoma cells in culture (Peters et al. 1985), while in vivo, it is present in the villous basement membrane as well as in association with extravillous cells. A variety of isoforms are present: the A and merosin heavy chains and the light chains Bl, B2 and B3 (Leivo et al. 1989; Earl et al. 1990; Engvall et al. 1990).
components including collagens, laminin, fibronectin and vitronectin is probably a prerequisite of syncytiotrophoblast invasion of the maternal stroma (Arman et al. 1986a,b). Of a panel of ECM components tested, BeWo cells adhere most efficiently to fibronectin (Aplin and Charlton, 1990), while JAr cells adhere well to fibronectin, laminin and type IV collagen (Hall et al. 1990). BeWo cells express integrins a2b1, a5b1, avb3 and a6b4 (Aplin, Mould and Sattar, unpublished). (For a recent review of the integrins, see Humphries (1990).) Of these the first three have all been shown to function as fibronectin receptors in different systems, but we have been able to demonstrate receptor activity for only a5b1 in BeWo (Aplin and Sattar, unpublished). JAr cells also use a6b1 as a fibronectin receptor, while a1b1 acts as a collagen type IV receptor and a6b1 as a laminin receptor. BeWo and JAr therefore differ considerably in integrin expression and adhesive phenotype. All these matrix ligands are present in locations in which trophoblast invasion occurs (Aplin et al. 1988) and may well be used by the migrating cells for anchorage. The BeWo cell line is heterogeneous and we have isolated a largely mononuclear subline that migrates up a fibronectin gradient in a modified Boyden chamber assay more efficiently than the parent cells. The subline may be closer to the extravillous phenotype. Its integrin complement is identical to that of the parent line, suggesting that integrin activation can occur at the cell surface without a change in the level of expression (Adams and Watt, 1990; O'Toole et al. 1990).

The extracellular ligand for integrin a6b4 has yet to be identified. This integrin dimer is expressed at the basal plasma membrane in many epithelia, both simple and stratified (Sonnenberg et al. 1990), including villous trophoblast (see above; Figs 4, 5). It disappears progressively from the extravillous cells as they move away from the villi (Aplin, unpublished). This is the first example of modulation of integrin expression in trophoblast differentiation, but its functional significance remains to be assessed.

Taken together these results suggest that trophoblast behaviour can be modulated by the matrix environment, but the intrinsic differentiation programme is the most dominant influence. Current data are consistent with the villous cytotrophoblast of first trimester being a stem cell for both syncytial and extravillous cell populations (Fig. 2). If the mononuclear cells initially isolated for culture retain this multipotentiality, present culture methods evidently promote the syncytial differentiation pathway. Alternatively, this is a default pathway from which cells have to be diverted to enter the extravillous pool. Third trimester cytotrophoblast appears already largely committed to the syncytial lineage. There is only one report (Yagel et al. 1988) that cells with a truly invasive (as opposed to extensive) capacity have been isolated. Syncytium is a terminally differentiated form, and cells in the placental bed remaining after pregnancy regress and disappear. This could be taken as providing further evidence of an intrinsic differentiation ‘clock’, or instead reflect the dependence of trophoblast on the specific maternal environment of pregnancy.

The role of the maternal environment in trophoblast invasion

Decidualisation of the endometrium is extensive in women, occurring throughout the stroma, while in rats, mice and certain non-human primates, it occurs only in the vicinity of the implanted embryo. In addition to its paracrine function (Bell, 1988), decidua has been postulated to act in the control of trophoblast invasion (Billington, 1971). This contention is based on experiments in which mouse blastocysts were transferred from the uterus to other soft tissues including spleen and testis (Kirby, 1965a,b; reviewed by Billington, 1971). Trophoblast masses were reported to invade both stroma and blood vessels and give rise to local tissue destruction in these sites. When transplanted to undecidualised endometrium, the trophoblast was reported to invade more deeply than in decidua of pregnancy (Billington, 1971). These results were interpreted to indicate that decidua plays a barrier role, resisting the invasive behaviour of the trophoblast. A more cautious interpretation might be that the lytic activity of trophoblast is less efficiently regulated in extraterine sites.

In mouse, trophoblast invasion is much more restricted than in humans, so that it is unwise to attempt too close an analogy between the two species. Randall et al. (1987) have argued against a specific role for uterine tissue in the regulation of human placentation on the basis of observations made in tubal implantation sites. Here villous morphogenesis, cytotrophoblast invasion and consequent vascular remodelling proceed in a manner very similar to that seen in the uterus. Decidualisation of the tubal stroma (a Mullerian duct-derived tissue) occurs to a limited extent. This evidence points towards a trophoblastic differentiation programme, the aim of which is to achieve haemochorial contact with relatively limited dependence on the specific environment.

However, it has been suggested that the condition of placenta accreta in women, in which trophoblast invades beyond its normal scope, penetrating deeply into the myometrium and even (in percreta) the adjacent serosa, may be accounted for by the presence of decidual tissue defective in a proposed barrier function (Robertson et al. 1985; Khong and Robertson, 1987). More simply, it may be that the endometrium is thinner at or after implantation in these women, and that the inflexible timing of the trophoblastic differentiation programme leads to deeper penetration. The occurrence of placenta percreta indicates that the myometrium is not in itself capable of resisting the degradative activity of villous trophoblast; however, there is some evidence that it may restrict the interstitial migration of extravillous cells (Khong and Robertson, 1987; Benirschke and Kaufman, 1990).

Studies of human endometrial extracellular matrix (Aplin et al. 1988; Aplin and Jones, 1989) do not readily support the simple idea of a physical barrier to invasion, since during decidualisation collagen fibril densities decrease, fibril bundles become scarce and collagen type VI, which probably plays a role in linking together fibril bundles of the major collagens, decreases. Thus the ECM becomes less fibrous, and indeed this change may allow the local structural expansion required for successful placentaion.

Given the ability of cultured first trimester human trophoblast to degrade and invade extracellular matrix, it may very well be that decidual tissue has a specialised composition and organisation designed both for self-protection and restricting or, at least, directing this invasive and lytic potential. One postulate is that cell recognition occurring between trophoblast and maternal cells (resident differentiated decidual stromal cells or bone marrow-derived populations such as macrophages and
large granular lymphocytes) leads to the release of cytokines and growth factors, which in turn act to influence trophoblast differentiation or invasion. This model could provide a role for HLA-G on trophoblast (King and Loke, 1990). In this way the two tissues may cooperate in the morphogenesis of the placenta.

When human first trimester villi or choriocarcinoma cells are cultured in direct contact with decidua, no cytotrophoblast infiltration occurs over a period of a few days (Vivoc, Sattar, Jones and Aplin, unpublished data). Similarly, first trimester cytotrophoblast aggregates show rather little invasive activity in coculture with undecidualised endometrium (Kliman et al. 1990). In both cases, however, a ‘contact necrosis’ is observed reminiscent of localised necrotic areas seen in the normal placental bed (Buckley and Fox, 1989). Although reluctance of the cells to invade in vitro may simply reflect experimental deficiencies, the distribution of interstitial extravillous trophoblast in the placental bed (more in the vicinity of the spiral arteries than elsewhere; Pijnenborg et al. 1981; Pijnenborg, 1990) might on the other hand suggest the presence of a chemotactic factor in maternal arterial blood (Fig. 2). This would also account for vascular colonisation and remodelling by extravillous trophoblast in ectopic implantation sites (Randall et al. 1987). More studies are required.

Conclusions

The present balance of evidence suggests strongly that trophoblast has an intrinsic and carefully timed differentiation programme designed to enable placenta to meet the requirements of the developing foetus. The maternal decidual environment is specialised to allow this programme its correct expression. Trophoblast-derived proteases and a motile early syncytium interact in a controlled way with the maternal environment to allow haemochorial contact, and the developing villous syncytium provides the embryo with the means to support the corpus luteum and import nutrient supplies. The least understood component of this complex equation is the extravillous trophoblast, which, in the first and second trimesters of pregnancy, penetrates the decidua and myometrium. These cells, like the villous syncytiotrophoblast, are derived from villous cytotrophoblastic stem cells, but they represent a distinct differentiation pathway that has so far proved intractable to study in vitro.

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References


*Note added in proof*

Librach et al. (J. Cell Biol. 113, 431–449, 1991) have shown that the 92×10^3 M<sub>t</sub> type IV collagenase is essential for the penetration of first trimester trophoblast through a layer of Matrigel. The enzyme is absent from third trimester cells. TIMP inhibits both enzyme activity and cellular penetration.