Are human placental bed giant cells merely aggregates of small mononuclear trophoblast cells? An ultrastructural and immunocytochemical study

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The ultrastructure of placental bed giant cells in early human pregnancies of 7–12 weeks gestational age is described. Their nature and function was further characterized by confocal immunofluorescence microscopy of paraffin sections labelled for cytokeratin, gap junction connexins (CX) 32 or 43, and placentual hormones, α-human chorionic gonadotrophin (α-HCG) and human placental lactogen (HPL). Placental bed giant cells were observed with two phenotypes; as single large trophoblast cells containing one or more nuclear profiles in a voluminous cytoplasm, and as cell aggregates comprising mononuclear trophoblast cells in close apposition separated by narrow intercellular spaces. Cells within the aggregates are attached to one another by desmosomes, and also possess gap junctions as shown by immunolabelling for CX32 and CX43. By contrast, gap junctions were absent in the true multinucleated giant cells. Organelles present within the cytoplasm of the giant cells and their immunoreactivity for HPL and α-HCG suggest protein synthesis.

Key words: cell aggregate/human/immunocytochemistry/placental bed giant cell/ultrastructure

Introduction

Placental bed giant cells are a sub-population of the extravillous trophoblast cells present at the implantation site. They have been reported to be most numerous during mid gestation, but many are said to be present near term, and they have even been demonstrated up to the fifth day post-partum (Boyd and Hamilton, 1970; Pijnenborg et al., 1981). Based on their morphological appearances at the light microscope level, placental bed giant cells are described as larger than the adjacent trophoblastic cells, and containing two or more nuclei enclosed in a voluminous cytoplasm.

The origin of placental bed giant cells has been much disputed (Boyd and Hamilton, 1970), and has still to be established conclusively. Various investigators have described them as multilobular and multinuclear decidual cells rather than trophoblast cells (Wynn, 1967; Boyd and Hamilton, 1970). Robertson and Warner (1974) suggested that they are derived from the syncytiotrophoblast, while other investigators are of the opinion that they are formed by mitosis without cytokinesis of the interstitial trophoblast (Avery and Hunt, 1969; Pijnenborg et al., 1981; Graham et al., 1992), and others believe that they may be formed as a result of homograft reaction at the placental bed (Park, 1965). It has also been suggested that such cells are a result of endoreduplication, that is, replication of the nuclear material without subsequent mitosis and cell division (Zybina, 1961). However, most embryologists agree that the multinucleated condition is established as a result of cell fusion (Boyd and Hamilton, 1970; Hunt and Avery, 1971; Kliman et al., 1986), although direct evidence to support this theory is lacking.

The most obvious evidence of cell fusion during trophoblast formation in other primates is the presence within the cytoplasm of segments of parallel cell membranes, the standard intercellular distance apart but fused at their margins, thus isolating within the cytoplasm a small portion of former intercellular ‘space’ (Schlatke and Enders, 1975). Other features of cell fusion include gap junctions and remnants of desmosomes (Cavicchia, 1971). Gap junctions are specialized cell membrane domains consisting of clusters of channels called connexons (CX). Ultrastructural studies have shown gap junctions to be pentalaminar structures with a 2 nm gap, found between cells of the trophoblastic layers of the haemochorial placentae of rabbits, rats and mice (Metz et al., 1976). They have been implicated in the process of giant cell formation by cell fusion in the guinea pig (Firth et al., 1980), and have been observed between syncytiotrophoblast and cytotrophoblast cells in the human villous placenta (De Virgiliis et al., 1982). Gap junctions link the cytoplasmic compartments of neighbouring cells, forming a pathway for direct exchange of ions and small molecules (Yeh and Kurman, 1989). At least 12 different mammalian CX have been identified, of which two, CX32 and CX43, have been shown to be expressed in the rat endometrium (Yeh and Kurman, 1989), and CX26 and CX43 in the rat decidua during trophoblast invasion (Winterhager et al., 1993).

In-vitro studies have demonstrated that the permeability, conductance and other properties of gap junctional channels depend on the make-up of their component connexins (Veenstra et al., 1995). The possibility therefore exists that the expression of CX32 and CX43 on trophoblast cells may play an important role in their functional differentiation in different zones of the human endometrium, and in the formation of giant cells.

Desmosomes have also been linked to the formation of placental bed giant cells (Cronier et al., 1994). Staining for desmosomes has been reported at intercellular boundaries in aggregates of cytrophoblast cells isolated from normal term placentas, between cytrophoblastic cells and syncytiohoblasts, and between contiguous areas of the syncytiohoblast (Cronier et al., 1994).

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Ultrastructural/immunocytochemical study of placental bed giant cells

Figure 1. A semi-thin (1 µm) resin section of placental bed stained with methylene blue. (a) Survey micrograph showing few large cells with two or more nuclear profiles. (b) A cell aggregate in a different area of the same section. Scale bars = 20 µm.

Figure 2. Electron micrograph of placental bed section showing a cell aggregate with its outer membrane interdigitating neatly into the membrane of an apposing cell (arrowhead). m, mitochondria; n, nucleus. Scale bar = 2 µm.

in early pregnancy are still lacking due to the difficulties in isolating the implantation site and in identifying the giant cells. In the present study, we have attempted to: (i) describe the fine structure of placental bed giant cells in the early human placental bed using transmission electron microscopy of resin-embedded sections; and (ii) examine the mode of formation and function of giant cells using confocal microscopy of formaldehyde-fixed, paraffin-embedded sections immunostained with antibodies to cytokeratin, CX32 or CX43 and placental proteins.

Materials and methods
First-trimester human placental tissue of 7–12 weeks gestational age was obtained from 10 individuals after elective termination of uncomplicated pregnancies at Addenbrooke’s Hospital, Cambridge, with the permission of the local Ethical Committee. After collection, the tissue was dissected under culture media to identify the placental bed. It was then fixed by immersion in 4% formaldehyde in 0.1 M PIPES buffer pH 7.6 for 1 h at 4°C for light microscopy, or in 4% glutaraldehyde in 0.1 M PIPES buffer pH 7.6 for 1.5 h at 4°C for ultrastructural studies. Tissue selected for light microscopy was either encapsulated in OCT (Tissue-Tek, Bayer Diagnostic, Glamorgan, UK) and frozen, or embedded in paraffin wax.

Cryostat sections (5 µm thickness) from each batch of tissue were stained with a monoclonal antibody against cytokeratin to confirm the presence of trophoblast cells. On this basis, the decidua was classified as decidua basalis if staining for cytokeratin was present, or decidua parietalis if it was absent.
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Figure 5. A few irregular, densely osmophilic, lipid inclusions (O) are seen in the cytoplasm of some large trophoblast cells with multinuclear profiles. Also present are stacks of Golgi apparatus (G), mitochondria (m), glycogen particles (g) and a dilated rough endoplasmic reticulum (er). Scale bar = 1 µm.

Light microscopy

Single immunostaining for cytokeratin

All immunostaining was carried out in a humid atmosphere, at room temperature. Paraffin sections of placental bed were de-waxed in xylene, rehydrated in an ascending series of ethanol solutions and washed thoroughly in running tap water, then in deionized water. Sections were then incubated with 50 µg/ml Proteinase-K (Boehringer Mannheim, Lewes, East Sussex, UK) for 10 min and washed thoroughly with Tris buffer pH 7.5. They were then incubated with 0.5% bovine serum albumin (BSA; Sigma Chemicals Ltd, Poole, Dorset, UK) for 10 min. Excess fluid was removed and sections were incubated for 30 min with rabbit polyclonal anti-cytokeratin antibody diluted 1:100 in 0.5% BSA in Tris buffered saline (TBS). After thoroughly rinsing in Tris buffer, sections were incubated with anti-rabbit fluoro-isothiocyanate (FITC; Dakopatts Ltd, High Wycombe, Bucks, UK) diluted 1:150 in 0.5% BSA for 30 min, or with a biotinylated anti-rabbit antibody diluted 1:100 in 0.5% BSA for 30 min, and then with a solution of Vectastain Elite ABC avidin–biotin–peroxidase (Vector Laboratories Ltd, Bretton, Peterborough, UK). After thoroughly rinsing in Tris buffer and double-distilled water, the sections for fluorescent microscopy were mounted in Citifluor mounting medium and examined immediately. The sections labelled with peroxidase were incubated with 1 mg/ml 3,3-diaminobenzidine (DAB; Dako Limited, Ely, Cambs, UK) plus 0.03% (v/v) H2O2, dehydrated in ethanol, cleared in xylene and mounted in DPX.

Combined labelling for gap junction proteins (CX32 and CX43) or human placental lactogen (HPL) or human chorionic gonadotrophin (α-HCG)

Following immunostaining for cytokeratin, some sections were incubated with 0.5% BSA in Tris buffer for 10 min at room temperature. Excess fluid was removed and the sections were incubated with mouse monoclonal anti-CX32 (Chemicon International Ltd, Harrow, UK) or CX43 (Chemicon International Ltd) diluted 1:1000 in 0.5% BSA overnight at 4°C or mouse monoclonal HPL (Sigma) or mouse monoclonal α-HCG (Sigma) diluted 1:100 in 0.5% BSA for 30 min at room temperature. After thoroughly rinsing in Tris buffer, the sections were incubated with anti-mouse Texas red (Sigma) diluted 1:150 in 0.5% BSA for 30 min at room temperature. They were then rinsed in Tris buffer, thoroughly washed in double-distilled water, and mounted in Citifluor (Agar Scientific Ltd, Stansted, Essex, UK) before viewing in a Leica TCS-NT confocal microscope (Leica Microsystems Ltd, Knowlhill, Milton Keynes, UK). Negative controls included omission of primary antibody and positive controls of human myocardium were stained for CX43 and rat liver for CX32. Adjacent sections stained with haematoxylin and eosin were examined for comparative histology.

Ultrastructural study

Tissues were fixed in 2% glutaraldehyde in 0.1 M PIPES buffer pH 7.4 for 1 h at 4°C. They were washed in three changes of 0.1 M PIPES buffer pH 7.3, post-fixed in 1% osmium tetroxide in 0.1 M PIPES at pH 7.4 containing 1.5% potassium ferricyanide and 2 mmol/l calcium chloride for 1 h at room temperature. Tissues were then washed in 0.1 M PIPES buffer pH 7.4 for 2 min followed by 0.05 M sodium maleate buffer, pH 5.2 for 10 min at 20°C, and then immersed in 2% uranyl acetate in 0.05 M sodium hydrogen maleate.
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Figure 7. A large trophoblast cell with long cell processes (p) is seen adjacent and in physical contact with a neighbouring cell of different ultrastructure. The cytoplasm of both the cells contains large electron-lucent globules (GL) of proteinaceous texture. Part of the trophoblast nucleus (n) is seen. Scale bar = 2 µm.

buffer for 1 h. After rinsing in deionized water, tissues were dehydrated in an ascending series of ethanol solutions, treated with propylene oxide (two changes of 15 min each), and transferred to a 50:50 mixture of propylene oxide and Spurr’s resin (TAAB Laboratories Ltd, Aldermaston, Berks, UK). After an overnight agitation, tissues were treated for 8 h in 25:75 propylene oxide and Spurr’s resin, and four changes of 100% Spurr’s resin over 36 h. They were next transferred to latex moulds filled with resin and thermally cured at 60°C for 48 h. Sections (0.5–1 µm thickness) were cut using a Reichart Ultracut S and stained with methylene blue for light microscopy. Areas of interest were identified and areas of necrosis selectively avoided. Ultra-thin sections of about 40–60 nm (showing silver to silver-grey interference colours) were cut and mounted on copper grids. These were double stained with uranyl acetate and lead citrate for 30 s each and examined in a Philips CM100 electron microscope at an accelerating voltage of 80 kV.

Results
Semi-thin (1 µm) resin-embedded sections from first-trimester human placental bed were stained with methylene blue and viewed under light microscopy (Laborlux II; Leitz, Wetzlar, Germany) before electron microscopic examination. The sections showed cells of various size and shape, confirming a heterogeneous cell population. Among the decidual cells were numerous leukocytes and a significant number of large cells containing two or more nuclear profiles in a voluminous cytoplasm (Figure 1a and b).

Ultrastructural observations
Electron microscopic examination of selected areas showed essentially two populations of the large cells. The first population appeared as groups of small mononuclear cells in close apposition with each other, with their outer membrane interdigitating into neighbouring cells (Figure 2). These were so tightly interposed that their cell membranes indented, producing

Figure 8. Photomicrograph of placental bed section immunostained for cytokeratin and DAB. The reaction product of DAB is seen (a) in the cytoplasm of the trophoblast cell containing a cluster of nuclei (C) and (b) in the large trophoblast cell containing numerous nuclear profiles (X). (c) Immunofluorescent image of an adjacent section labelled with cytokeratin and fluorescein-isothiocyanate (FITC). Two large trophoblasts (X) are seen among a few isolated mononuclear trophoblasts. Scale bars: (a,b) 20 µm; (c) 10 µm.
some large cells appeared to be adjacent and in physical contact with neighbouring cells of a different ultrastructure (Figure 7). The cytoplasm of both the cells contained large electron-lucent globules which were also present in the extracellular space. No mitotic figures or remnants of desmosomes were observed in the cells’ cytoplasm.

**Light and confocal microscopy observations**

Haematoxylin and eosin-stained sections of the placental bed showed a heterogeneous cell population. Among the various cells present were a few large cells which were readily distinguished from other cells by their large size and apparent multinucleate nature, some containing up to six nuclear profiles. Most of the large cells were in close proximity to the invaded maternal spiral arteries. Immunolabelling of adjacent sections with anti-cytokeratin antibody confirmed their trophoblastic origin (Figure 8a–c). In order to distinguish between true giant cells and cell aggregates, evidence of cell junctions was sought. Adjacent sections were double-immunolabelled for cytokeratin and the gap junction proteins CX32 or CX43. CX32 and CX43 were observed on the surface of cells immunolabelled with anti-cytokeratin antibodies, thus confirming their trophoblastic origin. Some of these cells were large and multinucleated, whereas others were small and mononucleated. Some aggregates appeared to be formed by several small mononucleated cells intimately apposed to a larger multinucleated cell (Figures 9 and 10a,b). Reactivity was lower within the isolated mononuclear interstitial trophoblast cells dispersed within the decidual stroma. A strong labelling for CX43 was also observed on various cells in the decidual stroma (Figure 11). CX32, on the other hand, was less abundant in the decidual stroma, but was seen in between the epithelial cells of endometrium glands (Figure 12).

Control sections of human myocardium showed a strong labelling with anti-CX43 (Figure 13), whereas CX32 was undetected and thus served as a negative control. In contrast, a strong labelling for CX32 was observed on rat liver sections (Figure 14), but not with anti-CX43.

To investigate the function of the large trophoblast cells and trophoblast cell aggregates, some placental bed sections were immunostained for α-HCG and HPL. Strong staining for α-HCG (Figure 15a and b) and HPL (Figure 15c and d) was observed in the cytoplasm of the large trophoblast cells. In contrast to HPL, which was also observed on numerous interstitial trophoblast cells in the decidua, α-HCG was distributed mainly on the large trophoblast cells and on the cell aggregates. Strong labelling for both HPL and α-HCG was observed on the syncytiotrophoblast of the cell columns, which served as an internal positive control.

**Discussion**

Immunocytochemical labelling confirmed the trophoblastic origin of placental bed giant cells in keeping with the light microscopy observations of Pijnenborg et al. (1981) and Wells and Bulmer (1988). At the light microscopy level, it is difficult to appreciate whether a structure is an aggregate of cells or a true multinucleated syncytium because of the limitations of...
resolution. This ultrastructural study has for the first time allowed demonstration of the fine morphology of the placental bed giant cells in early pregnancy. While some appeared as large individual cells with several nuclear profiles enclosed in extensive cytoplasm, others appeared as small single cells in close apposition with one another, giving the appearance of a single, large multinucleate cell. It is notable that the intercellular gap between cells within an aggregate was not sufficiently wide to be resolved by the highest power of the light microscope. It is therefore of interest to examine the degree to which the ultrastructural findings coincide with, or differ from, the immunocytochemical observations made at the light microscopy level.

Experimental evidence of a role for cell-to-cell communication via gap junctions in the control of cell proliferation and differentiation is well established (Lo and Gilula, 1979; Loewenstein and Rose, 1992). It has been suggested by Firth et al. (1980) that gap junctions connecting different layers of the trophoblast (cytotrophoblast and syncytiotrophoblast) could play a role in trophoblast development as a starting point for cellular fusion. One of the most important morphological features in this present study is the presence of gap junctions and junctional complexes between the mononuclear cells within cell aggregates, suggesting the potential of cell-to-cell communication. CX43 was also observed in abundance in the decidual stroma. This finding is in keeping with that of Winterhager et al. (1993), who demonstrated expression of CX43 in the rat endometrium during early pregnancy. The gap junctions may modulate cytoplasmic exchange between cells within an aggregate, or mediate the formation of morphogenetic gradients (Lo and Gilula, 1979). The presence of gap junctions on the trophoblast cells within aggregates and their absence on the single large trophoblast cells implies a process of internalization following coalescence of individual cells. Previous studies have reported gap junctions to be internalized
Figure 12. Confocal visualization of placental bed section double-immunolabelled for gap junction CX32 and cytokeratin. (a) The cytokeratin-positive glandular epithelial cells are shown. (b) A positive reaction for CX32 (arrow). Scale bar = 10 µm.

Figure 13. Confocal visualization of human myocardium immunolabelled for gap junction CX43. Punctate labelling in an en-face view of intercalated disk prepared from an optical series of transversely sectioned cells. Scale bar = 10 µm.

by endocytosis for lysosomal degradation (Severs et al., 1996) and ubiquitin-mediated proteosomal proteolysis (Laing and Beyer, 1995). In addition, in-vitro studies have reported gap junctions to precede fusion (Cronier et al., 1994), and hence they may play an important role in giant cell formation.

Intercellular fusion is a rapid process, and thus is difficult to visualize on static images. However, one may infer the occurrence of such an event by the presence of intracytoplasmic cell structures such as the persistence of residual cell partitions and remnants of intracytoplasmic desmosomes. The electron microscopy results presented here show no evidence of intercellular fusion equivalent to that which occurs during formation of the syncytium (Boyd and Hamilton, 1960). There was no evidence of remnants of intracytoplasmic desmosomes or mitotic figures in the multinucleated giant cells to suggest either cell fusion or division. Thus, although strongly supportive, our results cannot provide conclusive evidence of cell fusion as the origin of giant cells.

The fine structure of placental bed giant cells suggests that they are not totally effete or passive cells (Robertson, 1987), but are involved in at least some aspects of synthetic activity. The numerous polyribosomes and mitochondria, abundant rough endoplasmic reticulum, extensive Golgi complex and cytoplasmic vesicles are indicative of protein synthesis. Giant cells have long been linked with the production of enzymes and elaboration of hormones (Boyd and Hamilton, 1960), and our findings indicate they may elaborate protein hormones such α-HCG and HPL. This is in good agreement with Kurman et al. (1984), but contradicts the findings of Gosseye and Fox (1984) and Loke and King (1995). This discrepancy may be due to the different fixation methods used, and to the sensitivities of the immunolabelling techniques employed. Immunocytochemical studies cannot demonstrate that the products synthesized are secreted, but it is interesting that Pedersen et al. (1998) have recently reported the presence of HPL in maternal serum during normal pregnancy, and its significant decrease in diabetic mothers.

In-vitro studies of human trophoblast cells have shown them to aggregate and subsequently fuse to form a syncytium in which placenta-specific hormones such as human somatotrophin, HCG and HPL have been detected (Cronier et al., 1994; Richards et al., 1994). HCG has also been reported to promote the expression of cadherin, a cell adhesion molecule that facilitates cellular aggregation (Shi et al., 1993) and gap junctional communication (Cronier et al., 1994). Thus, from our observations here and the descriptions in the literature, we suggest that cell fusion may be stimulated and reinforced by autocrine and paracrine pathways.
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Figure 15. Confocal visualization of double-immunolabelled placental bed section with cytokeratin and α-HCG or HPL placental proteins. (a) A large cytokeratin-reactive trophoblast cell with multinuclear profiles (X), among a few isolated small trophoblast cells showing. (b) α-HCG immunoreactivity is seen only in the cytoplasm of the large trophoblast cell and not in the small mononuclear trophoblasts. (c) A large cytokeratin-reactive trophoblast cell with multinuclear profiles (X) with (d) HPL immunoreaction in the cytoplasm. Scale bars: (a,b) 10 µm; (c,d) 5 µm.

Unlike the villous syncytiotrophoblast, the placental bed giant cells express HLA-G class I major histocompatibility complex antigens (Loke et al., 1997). The HLA-G on giant cells may facilitate interaction with the maternal cells in the placental bed. This interaction may lead to some cells undergoing programmed cell death (apoptosis) (Al-Lamki et al., 1998), while others may undergo terminal differentiation to giant cells. Giant cells have been shown to produce protease and protease inhibitors (Sasagawa et al., 1987), leucine aminopeptidase (Loke and Butterworth, 1987) and have lectin-binding affinity (Earl et al., 1990).

It therefore seems likely that in early placental development, there are at least two possible fates for the interstitial extravillous trophoblast cells migrating into the endometrium; apoptosis and giant cell transformation. While both processes may serve to limit trophoblast invasion, the transformation of trophoblast cells into isolated masses of syncytium may also ensure adequate local production of hormones and other products that are critical to maintaining a normal pregnancy.

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References
