Regulation of human villous trophoblast by insulin-like growth factors and insulin-like growth factor-binding protein-1

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Abstract

Many studies have implicated the insulin-like growth factors (IGFs) and insulin-like growth factor-binding protein-1 (IGFBP-1) in the control of the feto-maternal interface of human pregnancy, but many of the data are from cell lines derived from primary trophoblast or from extravillous trophoblast. We have obtained highly enriched villous cytotrophoblast (VCT) from first trimester and term human placentae, and investigated the effects of IGF-I, IGF-II and phosphoisoforms of IGFBP-1. First trimester villous trophoblast cells were regulated by all these factors. IGF-II increased cell numbers 3.5-fold after 96 h in culture, and IGF-I had less effect (1.5-fold increase) (both $P < 0.05$). IGF-II also had a greater effect on the levels of matrix metalloproteinase (MMP)-2 and MMP-9. Phosphorylated and non-phosphorylated isoforms of IGFBP-1 added alone increased cell numbers

and MMP levels ($P < 0.05$). IGFBP-1 did not modify the effects of IGF-II on cell numbers or on MMP production. Term VCT numbers and MMP production *in vitro* were unaffected by IGFs ($P > 0.05$). Cell numbers were increased only by 100 nM IGFBP-1 isoforms ($P < 0.05$), whereas MMP levels released from term cells were optimally increased by 1–10 nM IGFBP-1. Overall, our data show that IGFs regulate only first trimester, but not term, VCT. IGFBP-1 regulates VCT from both gestations, but the effects are concentration and end-point specific. In particular, first trimester cell numbers are more affected by low levels of IGFBP-1, whereas high levels of IGFBP-1 are needed to increase MMP and the converse applies to term VCT; low levels of IGFBP-1 have more effect on MMP levels.

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Introduction

Proper development of the human placenta is essential if normal pregnancy is to be maintained. This includes growth of the placenta, differentiation of the trophoblast and vascular systems, invasion of extravillous cytotrophoblast (EVCT) to form anchoring villi and remodelling of the uterine maternal vasculature to provide adequate nutrition for the rapid fetal growth in the second half of pregnancy. Defects in these processes, which lead to insufficient trophoblast growth and invasion, are associated with some of the major pregnancy complications, including fetal growth restriction, pre-eclampsia and early pregnancy loss (Brosens *et al.* 1972, Robertson *et al.* 1975, Teasdale 1989, Hustin *et al.* 1990). Furthermore, fetal growth restriction *in utero* is associated with an increased risk of cardiovascular disease in adult life (Barker *et al.* 1993). Conversely, excessive trophoblast growth characterises molar pregnancies and choriocarcinomas, and complications of pregnancy such as placenta increta, placenta accreta and placenta percreta are linked to increased

invasion of trophoblast into the myometrium during pregnancy (Morison 1978, Paradinas & Fisher 1995).

The control of human trophoblast functions is not well understood, despite the importance of these cells to pregnancy. Very many of the known growth factors and cytokines are expressed at the feto-maternal interface in fetal placenta, or in maternal decidua (Rice & Chard 1998); this also applies to the receptors which mediate the effects of these potential regulators (Sharkey *et al.* 1995, Sanchez-Cuenca *et al.* 1999). There seem to be few pathways of regulation unique to pregnancy, but rather a complex series of factors which may be involved. A general paradigm has been derived from murine pregnancy, that fetal (paternally derived) factors are concerned primarily with increasing the size and development of the placenta and fetus, whereas maternal factors act in the opposite sense to restrain growth (Barton *et al.* 1985, Surani *et al.* 1987). This is supported by *in vitro* data from human pregnancy tissues, which show that proliferating placentally derived cells support their own growth and that this was inhibited by decidual products (Lewis *et al.* 1993).

Candidate trophoblast growth factors include the insulin-like growth factors (IGFs) (Han *et al.* 1996), and decidual inhibitors may include insulin-like growth factor-binding protein-1 (IGFBP-1) (Rutanen *et al.* 1985, Pekonen *et al.* 1988) as well as transforming growth factor- β (TGF- β) (Graham & Lala 1992, Graham *et al.* 1992). A series of studies have not only confirmed that changes in the IGF-IGFBP-1 balance are linked to complications of pregnancy such as fetal growth restriction and pre-eclampsia (Howell *et al.* 1989, Giudice *et al.* 1995, Anim-Nyame *et al.* 2000), but have also shown that in a variety of model systems IGFs and IGFBP-1 have direct effects on trophoblast-derived cell proliferation and invasion (Maruo *et al.* 1995, Hamilton *et al.* 1998, McKinnon *et al.* 2001, Chakraborty *et al.* 2002).

IGF-I and IGF-II are single chain polypeptides, each 7.5 kDa, which promote growth and differentiation in a variety of cell types (Jones & Clemmons 1995). Their effects are mediated through specific receptors (insulin-like growth factor receptors (IGFRs)-I and -II), but our understanding of the roles of the receptors is incomplete. IGF-II binds most avidly to IGFR-II (Nielsen 1992), but this receptor has a short intracellular domain which has limited coupling to signal transduction systems; it may interact with inhibitory G-proteins (Okamoto & Nishimoto 1991), but it is not clear whether this mediates all the effects of IGF-II. However, this growth factor can also bind to and activate IGFR-I, although with a lower affinity than IGF-I (Steele-Perkins *et al.* 1988). A further complication in the understanding of the IGF-IGFBP system is that while the binding proteins generally limit the effects of IGFs (Hills & Chard 1995), they may synergise with them (Jones *et al.* 1993a). Furthermore, IGFBP-1 can bind to the Arg-Gly-Asp sequence of the fibronectin receptor ($\alpha_5\beta_1$) and directly modulate cell function independently of IGFs (Jones *et al.* 1993b, Gleeson *et al.* 2001). Both the direct and IGF-related effects of IGFBP-1 may be affected by the degree of phosphorylation (Jones *et al.* 1991), which affects the affinity of the binding protein for its targets.

The primary aim of our study was to investigate the impact of the IGF-IGFBP-1 axis on the biology of human villous cytotrophoblast (VCT). It should be pointed out that many of the previous investigations have used EVCT (Bischof *et al.* 1995, Choy & Manyonda 1998, Irwin & Giudice 1998), cell lines derived from primary EVCT (Graham *et al.* 1993, Li & Zhuang 1997, Hamilton *et al.* 1998, McKinnon *et al.* 2001, Chakraborty *et al.* 2002), immortalised cells (Lewis *et al.* 1996, Lala *et al.* 2002), or choriocarcinomas (Ritvos *et al.* 1988, Librach *et al.* 1991). In the studies presented here we have: (a) developed the methods needed to isolate highly enriched primary first trimester and term VCT; (b) determined the effects of IGF-I, IGF-II and IGFBP-1 phosphoisoforms on VCT numbers *in vitro*; (c) investigated the effects of these same factors on the production of matrix metalloproteinase

enzymes (MMPs). These are commonly used as surrogate markers for trophoblast invasiveness – the gelatinases (MMP-2 and MMP-9) have particularly been implicated in trophoblast invasion, and have been investigated thoroughly in human EVCT (Librach *et al.* 1991, Bischof *et al.* 1995).

Materials and Methods

Tissue samples

Samples of normal first trimester placental tissues were obtained after termination of pregnancy between 8 and 12 weeks gestation. Term placental tissues were obtained after delivery by elective Caesarean section prior to labour. Consent for the use of the tissues was obtained from the patients in accordance with the approval of Hammersmith Hospitals NHS Trust Ethics Committee. Twenty-five tissues of each type were used for the experiments described, as not all studies could be repeated on each cell preparation.

Preparation of primary villous cytotrophoblast (PVCT) cultures

The procedure to isolate VCT was essentially the same for first trimester and term tissues. Villous tissue was carefully dissected and incubated with Hank's balanced salt solution (Life Technologies, Paisley, UK) without calcium or magnesium, but also containing 0.025 M HEPES and 0.125% (w/v) trypsin 250 (Difco, Detroit, MI, USA) at 37 °C for 40 min with regular vigorous shaking. The supernatant, containing isolated cells, was removed and further trypsin activity was stopped by addition of 5 ml heat-inactivated, mycoplasma-screened fetal bovine serum (Helena Biosciences, Sunderland, UK). The remaining trophoblast tissue then underwent two further digestions under the same conditions. Supernatants were then pooled and passed through a 70 μ m sterile cell strainer (Becton Dickinson) to remove any non-digested tissue. The cell suspension was centrifuged at 1600 *g* for 20 min through a 40% percoll solution. Trophoblast cells were collected from the percoll/medium interface and cell debris and erythrocytes that had migrated through the percoll were discarded.

VCT were separated from other cell types by negative immunoselection with a monoclonal antibody W6/32 (Dako, High Wycombe, UK) that recognises epitopes common to human leucocyte antigen (HLA) class I antigens (Lata *et al.* 1992). Initially, cells were incubated with 20% (v/v) rat serum in a pH 7.4 buffer comprising 50 mM PBS, 0.5% (w/v) BSA and 2 mM EDTA for 20 min at 4 °C with constant shaking. Cells were then washed in buffer and incubated for 1 h at room temperature with mouse anti-HLA class I antigen (clone W6/32) at a concentration of approximately 1 μ g per 10⁶ cells in a

total volume of 0.5 ml buffer. After further washing the cells were incubated with 10% (v/v) rat anti-mouse IgG magnetic microbeads (Miltenyi Biotec, Bisley, UK) for 30 min at room temperature. The cell suspension was then passed through a magnetic separation column and cells not expressing HLA class 1 antigens were eluted with 15 ml buffer. After further washing cells were plated out into 96-well plates (approximately 20 000 cells/well) in RPMI containing 2 mM L-glutamine, penicillin (100 IU/ml) and streptomycin (100 µg/ml) supplemented with 10% FCS in an atmosphere containing 5% CO₂.

Immunostaining

The purity of the primary VCT was assessed by immunostaining. Cells in suspension were centrifuged onto glass slides at 500 r.p.m. for 3.5 min at room temperature in a Cytospin centrifuge and then stained for the presence of a specific trophoblast cell cytoskeletal marker cytokeratin 7 (Haigh *et al.* 1999) and for vimentin, a marker of stromal cells, using an avidin–biotin complex (ABC) detection system (Vector Laboratories, Burlingame, CA, USA). Cells were fixed in acetone:methanol (1:1) for 5 min and incubated for 30 min with a 10% hydrogen peroxide solution (in methanol) for 30 min to quench any endogenous peroxidase activity. After washing with PBS cells were incubated for 20 min with normal goat serum (1 in 67 dilution). Cells were washed twice with PBS and then incubated with a monoclonal antibody (50 µl, 1 in 25 dilution) to cytokeratin 7 or vimentin (Dako) for 1 h. Cytokeratin 7 or vimentin positive cells were visualised by further incubations with biotinylated goat anti-mouse IgG, HRP-labelled ABC and diaminobenzidine according to manufacturers' instructions. Cells were then lightly counterstained with Ehrlich's Haematoxylin (Merck, Poole, UK).

Effect of IGFs and IGFBP-1

Primary VCT were plated out at a concentration of approximately 20 000 cells/well in 96-well plates in RPMI containing 2 mM L-glutamine, penicillin (100 IU/ml) and streptomycin (100 µg/ml) supplemented with 10% FCS in an atmosphere containing 5% CO₂. After overnight incubation the medium was changed to serum-free and was supplemented with IGF-I or IGF-II (Peninsular Laboratories, St Helens, UK: 0, 1, 10 and 100 nM) in the presence or absence of 1 nM phosphorylated or dephosphorylated IGFBP-1 (Sigma, Poole, UK), or with phosphoisoforms of IGFBP-1 only (0.1, 1, 10, 100 nM), and incubated for a further 96 h with a change of medium after 48 h incubation. The IGFBP-1 preparations were ≥95% pure with the remaining ≥5% comprising other IGFBP-1 isoforms. Triplicate wells were run for each treatment and experiments were repeated at least three times.

Cell numbers

At the end of the experiments the medium was removed from the cells, which were stored at –20 °C until assay for cell numbers. This method relies on the reduction of a synthetic tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt (MTS; Promega UK Ltd) by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. Medium was removed from cells at the end of each experiment and replaced with 100 µl fresh medium and 20 µl MTS reagent. After 2.5 h incubation the A₄₅₀ was determined. Cell number was determined by comparing absorbance from each well with that from wells containing a known number of cells.

Detection of MMP-2 and MMP-9

MMP-2 and MMP-9 activities were detected by gelatinase zymography as previously described (Graham *et al.* 1993). Media collected from IGF and IGFBP-1 stimulation experiments were mixed in a 4:1 ratio with SDS-PAGE loading buffer comprising 300 mmol/l Tris (pH 6.8), 50% (v/v) glycerol, 10% (w/v) SDS and 0.1% bromophenol blue. Proteins were separated by discontinuous SDS-PAGE using a 12% resolving gel containing 1 mg/ml gelatin under non-reducing conditions. Loading volumes were adjusted to take into account differences in cell number following incubation with different concentrations of IGFs and IGFBP-1.

Gels were washed twice in 2.5% (v/v) Triton X-100 and then incubated for 18 h at 37 °C in zymography digestion buffer (5 mmol/l Tris, 20 mmol/l NaCl and 0.5 mmol/l CaCl₂). Gels were immersed in staining solution comprising 0.5% Coomassie blue-G in 30% methanol and 10% acetic acid for 2 h at room temperature and then destained in staining solution with the Coomassie blue omitted. MMP activity was seen as clear areas within the gel where the gelatin substrate had been hydrolysed by gelatinase activity.

Quantification of MMP activity

This was accomplished using image analysis software inherent in Adobe Photoshop. The process is described briefly below. For each lane in a gel the area containing all MMP activity was selected and the mean pixel brightness calculated automatically. Data were expressed as a percentage of the control (unstimulated) value. For MMP-2 and MMP-9 this process was repeated, except that only the area of gel representative of each individual MMP was used for the mean brightness calculations. Again the data were expressed as a percentage of the unstimulated value. This process is necessary because levels of the MMPs in the samples are not equivalent, and this separation allows the detection of changes in any MMP present at low levels that may be lost in the total assessment of activity.

Statistical methods

All experiments were repeated at least three times. The data are expressed as mean $\pm$ S.E.M. for replicate experiments. Because of the variability in baseline cell numbers, in experiments where the effects of IGFs and IGFBP-1 were studied the cell number was expressed as the percentage of the control level from each experiment. Changes in cell numbers were compared by one-way analysis of variance (ANOVA) and the Bonferroni/Dunn test was used to determine variation within each group.

Results

Immunocytochemistry of primary villous cytotrophoblast

First trimester and term placentae were digested to produce mixed single-cell suspensions which contained cells positive for cytokeratin 7 ($78.1 \pm 2.3\%$, trophoblast), HLA ($32.6 \pm 2.4\%$) and vimentin ($19.1 \pm 2.9\%$). These preparations were enriched for VCT by removal of all cells which express HLA class 1 antigens, as described in Materials and Methods. After this procedure, $98.9 \pm 0.3\%$ ($n=4$) of the cells were cytokeratin 7 positive and therefore of trophoblast origin. These preparations contained few HLA+ ($1.3 \pm 0.4\%$) and vimentin+ ($1.1 \pm 0.7\%$) cells. Similar data were obtained from first trimester and from term tissues.

Effect of IGFs and IGFBP-1 on cell numbers

Preliminary studies showed small positive effects of IGFs on numbers of primary first trimester VCT after 48 h of culture and greater effects after 96 h (data not shown). The longer incubation was therefore used in all remaining experiments. These studies also showed that there was a variable decrease in cell numbers (of 10%–40%) associated with the first change of medium (after 24 h of culture), but no change in cell numbers thereafter under basal culture conditions.

IGF-I increased first trimester VCT numbers after 96 h of culture (Fig. 1a); the greatest effect was a 1.5-fold increase with 100 nM IGF-I. IGF-II also had dose-dependent effects on first trimester VCT numbers, but the optimal dose was lower (10 nM), and the mean effect greater (3.5-fold) (Fig. 1a). Under the same conditions, numbers of term VCT were unaffected by both IGFs (Fig. 1b).

The addition of phosphorylated IGFBP-1 (pIGFBP-1) or unphosphorylated IGFBP-1 (nIGFBP-1) also increased first trimester VCT numbers after 96 h of culture (Fig. 2a), and the greatest effect was with 1 nM binding proteins. Only 100 nM of both IGFBP-1s significantly increased numbers of term VCT *in vitro* (Fig. 2b). Co-incubation of first trimester VCT with IGF-II and IGFBP-1 phospho-

Figure 1 Numbers of enriched VCT from (a) first trimester or (b) term placenta following incubation with IGF-I (□) or IGF-II (hatched). Data are means $\pm$ S.E.M. ($n=5$). * $P<0.05$ vs control (medium alone).

isoforms showed that neither form of IGFBP-1 inhibited the effects of IGF-II (Fig. 2c) on cell numbers.

Effect of IGFs and IGFBP-1 on MMP-2 and MMP-9 secretion

Both MMP-2 and MMP-9 could be detected in the culture medium from first trimester and term VCT and IGF-II seemed to increase levels of both MMPs, visualised as larger clear areas in the zymography gels (Figs 3a and b). We determined total levels of MMP-2 activity plus MMP-9 activity, as well as the activity of individual MMPs, as described in the Materials and Methods section. The levels of MMP-2 and MMP-9 activity varied between different cell preparations, so data are expressed as a ratio to the corresponding controls.

In first trimester VCT, all the factors studied (IGF-I, IGF-II, nIGFBP-1 and pIGFBP-1) increased total MMP levels (Fig. 4a) and generally the greatest effects were observed at 100 nM peptide. MMP-2 was increased by IGF-II and pIGFBP-1 (Fig. 4b), while IGF-I and nIGFBP-1 had numerically similar effects that did not

Figure 2 Numbers of enriched VCT from (a) first trimester or (b) term placenta following incubation with phosphorylated (□) or non-phosphorylated (hatched) IGFBP-1. * $P < 0.05$ vs control (medium alone). In (c) first trimester VCT cells were co-incubated with IGF-II (concentrations as shown) (□), IGF-II+piIGFBP-1 (1 nM) (hatched) or IGF-II+niIGFBP-1 (1 nM) (■). All data are means $\pm$ S.E.M. ($n = 4$). In (c) all additions of IGF-II and IGFBP-1 (alone or in combination) increased cell numbers ($P < 0.05$ vs. no addition), but there was no additional effect of co-incubation compared with IGF-II alone.

reach statistical significance. MMP-9 was increased by the highest dose of all four peptides, although piIGFBP-1 had the greatest effect (Fig. 4c).

In term VCT, IGF-II had a modest effect on total levels of MMP activity (Fig. 5a) at a concentration of 100 nM. Both isoforms of IGFBP-1 had more potent effects (Fig. 5a) at lower concentrations (1–10 nM), and IGF-I was without significant effect. Changes in MMP-2 activity in

Figure 3 Zymograph gel showing MMP-2 and MMP-9 released from enriched VCT from (a) first trimester or (b) term placenta after 96 h. The volume of medium added was corrected for the cell numbers present. Cells were incubated with IGF-II as shown. MMP standards were present in lane 1.

term VCT (Fig. 5b) generally reflected the overall changes in MMP activity, in that IGF-I and IGF-II had modest stimulatory effects at 100 nM, whereas niIGFBP-1 (10 nM) and piIGFBP-1 (1 nM) had more effect at lower levels. The highest concentration of piIGFBP-1 inhibited MMP-2 activity (Fig. 5b). Only niIGFBP-1 significantly increased MMP-9 activity in term VCT (Fig. 5c). The IGFs had modest stimulatory effects, but these did not reach statistical significance and piIGFBP-1 had little effect at any concentration (Fig. 5c).

Combinations of IGFs with IGFBP-1 had no overall effect on gelatinase production compared with the IGFs alone (results not shown). This applies to both first trimester and term VCT.

Discussion

For these studies we were able to generate a highly enriched population of VCT ($\geq 95\%$ cytokeratin 7 positive) from first trimester and term placentae. Numbers of contaminating cells remained low during the culture

Figure 4 Effects of IGF-I (□), IGF-II (light hatched), nIGFBP-1 (heavy hatched) or pIGFBP-1 (■) on gelatinase activity released from enriched first trimester VCT after 96 h of culture. Total (MMP-2+MMP-9) (a), MMP-2 (b) and MMP-9 (c) activity are shown. All data are means $\pm$ S.E.M. ($n=4-6$). * $P<0.05$ vs control (medium alone).

Figure 5 Effects of IGF-I (□), IGF-II (light hatched), nIGFBP-1 (heavy hatched) or pIGFBP-1 (■) on gelatinase activity released from enriched term VCT after 96 h of culture. Total (MMP-2+MMP-9) (a), MMP-2 (b) and MMP-9 (c) activity are shown. All data are means $\pm$ S.E.M. ($n=4-6$). * $P<0.05$ vs control (medium alone).

Table 1 Summary of the effects of IGF family proteins on human VCT *in vitro*

	IGF-I	IGF-II	nIGFBP-1	pIGFBP-1	Trimester
Cell numbers	+ (H)	++ (H)	++ (L)	++ (L)	1st trimester
	0	0	+ (H)	+ (H)	3rd trimester
MMPs	+ (H)	+ (H)	+ (H)	+ (H)	1st trimester
	0	0	+ (L)	+ (L)	3rd trimester

0: no effect; +: moderate positive effect; ++: strong positive effect. (H): high dose optimal. (L) low dose optimal.

period, thus bypassing the problems of contamination which can complicate such long-term experiments (Choy & Manyonda 1998). The VCT remained viable for at least 96 h of culture and the small-scale methods used to estimate cell numbers produced reliable data. The purity of the preparation and culture characteristics are comparable with those of Guilbert *et al.* (2002) who also obtained highly enriched villous cells. Others have used similar negative selection methods to generate and to characterise villous cells from term placentae (Douglas & King 1989, Morrish *et al.* 1991, Yui *et al.* 1994, Aboagye-Mathiesen *et al.* 1996), but there are few studies on the functional regulation of these VCT.

Our findings are summarised in Table 1. This demonstrates clearly that the IGFs, most notably IGF-II, regulate only first trimester VCT and that the highest concentrations used (100 nM) are most effective. This applies to cell numbers and to MMP production. While the former observation is not surprising, as VCT cells are known to proliferate, the latter finding was unexpected as villous cells are not thought to be invasive. It must be noted that MMP-1 (interstitial collagenase) (Moll & Lane 1990) and MMP-9 (Isaka *et al.* 2002) have been detected in VCT of intact villi, and the latter is consistent with high levels of MMP-9 detected in our VCT (Fig. 3). The functional significance of the MMPs in VCT requires further investigation. It is well established that the placental expression of IGF-II declines with pregnancy (Han *et al.* 1996). This decline could be linked to the altered *in vitro* responses that we have found, but further studies are needed to identify the mechanism.

Both phosphoisoforms of IGFBP-1 affected VCT from first and third trimester placentae and the effects varied with gestational age. Low concentrations (~1 nM) of IGFBP-1 had the greatest effect on first trimester cell numbers (Table 1, Fig. 1a), whereas high concentrations affected MMP production (Table 1, Fig. 4). The exact opposite of this applied to term VCT, in that high concentrations of IGFBP-1 were needed to increase cell numbers and lower concentrations affected MMP levels (Table 1, Figs 1b and 5). Neither of the IGFBP-1 isoforms inhibited the effects of IGF-II (Fig. 2c) on first trimester cells, indicating that the effects of the binding protein are not mediated through inhibition of the effects of IGFs.

In other systems, IGFBP-1 is known to bind to the Arg-Gly-Asp (RGD) sequence of $\alpha_5\beta_1$ integrin, and thereby regulate cell functions (Gleeson *et al.* 2001). It is not clear if this applies to VCT as these cells are generally α_5 negative (Bischof *et al.* 1993). Also, the differential effects of different concentrations of IGFBP-1 on cell numbers and on MMPs indicate that two different signalling pathways are involved. Finally, the effects of IGFBP-1 on VCT change with gestational age (Table 1). A simplistic interpretation would suggest that decidual IGFBP-1 would support VCT proliferation and invasive potential (MMPs) at all gestational ages. However, it could be argued that the levels of IGFBP-1 that would reach the VCT would be relatively low, given that the cells are physically distant from the main source of IGFBP-1 (the decidua) and the syncytium interposes between the maternal blood and the VCT. Under such conditions, IGFBP-1 would mainly support the proliferation of VCT in the first trimester, and maintain invasive potential in the third trimester.

While some studies have implicated IGFBP-1 in the inhibition of placental or fetal development (Howell *et al.* 1989, Giudice *et al.* 1995), other data show that IGFBP-1 has positive effects. Overexpression of human IGFBP-1 in the decidua of transgenic mice increased placental size (Crossey *et al.* 2002), suggesting that in murine pregnancy IGFBP-1 supports placental development. Gratton *et al.* (2002) have reported that IGFBP-1 expression in human decidua was decreased in women suffering from pre-eclampsia, a disorder characterised by defective placentation. Again, this indicates that IGFBP-1 supports placental development rather than decreasing it, which is consistent with our findings. In a culture system of term human trophoblast, pIGFBP-1 inhibited IGF-I-stimulated uptake of an amino acid (Yu *et al.* 1998), whereas nIGFBP-1 potentiated the effect of IGF-I. Establishing the mechanism of action of IGFBP-1 isoforms in human VCT would help in resolving these differences.

Our data cannot discriminate between increased trophoblast proliferation or decreased apoptosis (increased cell survival), so we cannot state that IGFs or IGFBP-1 increased proliferation of these cells. A recent paper (Smith *et al.* 2002) has shown that IGF-I is a survival factor for third trimester trophoblast cells at a concentration of

2.7 nM, which is consistent with our findings of increased cell numbers at similar concentrations (1–10 nM). The impact of IGF-II on first trimester trophoblast survival has yet to be assessed. Future studies should be planned to discriminate between changes in apoptosis and in cell proliferation.

Taken together, our findings expand the previous data on a role for the IGF-IGFBP-1 axis in human pregnancy (Maruo *et al.* 1995, Han *et al.* 1996, Li & Zhuang 1997, Hamilton *et al.* 1998) to include the villous trophoblast cell population. Others have shown that IGF-II is a more potent regulator than IGF-I of placental function (Hamilton *et al.* 1998), but there are few precedents for the positive effects of IGFBP-1, which require further study.

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