Insulin-like growth factor I and II regulate the life cycle of trophoblast in the developing human placenta

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Forbes K, Westwood M, Baker PN, Aplin JD. Insulin-like growth factor I and II regulate the life cycle of trophoblast in the developing human placenta. Am J Physiol Cell Physiol 294: C1313–C1322, 2008. First published April 9, 2008; doi:10.1152/ajpcell.00035.2008.—The main disorders of human pregnancy are rooted in defective placenta- tion. Normal placental development depends on proliferation, differen- tiation, and fusion of cytotrophoblasts to form and maintain an overlying syncytiotrophoblast. There is indirect evidence that the insulin-like growth factors (IGFs), which are aberrant in pregnancy disorders, are involved in regulating trophoblast turnover, but the processes that control human placental growth are poorly understood. Using an explant model of human first-trimester placental villus in which the spatial and ontological relationships between cell populations are maintained, we demonstrate that cytotrophoblast proliferation is enhanced by IGF-I/IGF-II and that both factors can rescue cytotrophoblast from apoptosis. Baseline cytotrophoblast proliferation ceases in the absence of syncytiotrophoblast, although denuded cytotrophoblasts can proliferate when exposed to IGF and the rate of cytotrophoblast differentiation/fusion and, consequently, syncytial regeneration, increases. Use of signaling inhibitors suggests that IGFs mediate their effect on cytotrophoblast proliferation/syncytial formation through the MAPK pathway, whereas effects on survival are regulated by the phosphoinositide 3-kinase pathway. These results show that directional contact between cytotrophoblast and syncytiotrophoblast is important in regulating the relative amounts of the two cell populations. However, IGFs can exert an exogenous regulatory influence on placental growth/development, suggesting that manipulation of the placental IGF axis may offer a potential therapeutic route to the correction of inadequate placental growth.

proliferation; apoptosis; signaling

The maternal environment has profound effects on placental and hence fetal development and pregnancy outcome, as demonstrated convincingly by gene ablation experiments in mice. The effects range from implantation failure [as in leukemia inhibitory factor-null mothers bearing wild-type embryos (45)] or early postimplantation demise [the IL-11 receptor-null mouse (38)] to reduced litter size and fetal growth restriction [granulocyte-macrophage colony-stimulating factor-null mothers (39)]. In humans, the three main disorders of pregnancy—miscarriage, preeclampsia, and growth restriction—are rooted in defective placenta- tion caused, at least in part, by maternal factors (4). In placental development, a cytotrophoblast progenitor population expands and differentiates into one of two lineages: extravillous cytotrophoblast, which invades maternal tissues and vessels (8); and villous syncytiotrophoblast, the transporting epithelium responsible for nutrient and gas exchange between mother and fetus (8).

During pregnancy, there is a constant process of trophoblast turnover and renewal. The villous syncytiotrophoblast layer, which contains nuclei that are exclusively postmitotic, continuously sheds terminally differentiated and apoptotic elements into maternal circulation (19, 31). The syncytiotrophoblast is maintained by the underlying cytotrophoblasts, which are thought to divide asymmetrically to produce one daughter cell that differentiates to fuse with the syncytiotum, and another that remains in the progenitor cell pool. Cytotrophoblast proliferation is of obvious importance for placental growth, so that not all divisions may be asymmetric, but the process must be tightly regulated so that the bilayer structure of the epithelium is maintained, at least during the first trimester, while the tissue grows rapidly (19, 31). Increased or decreased rates of turnover have been associated with different pathological conditions (2, 28, 44).

IGFs are potent stimulators of tissue growth, regulating metabolic status, mitogenesis, differentiation, and survival (21, 46). Human fetal tissues including the placenta express IGF-I and IGF-II from early gestation. A partial deletion in the coding region of the human IGF-I gene results in severe intrauterine growth restriction (48), and IGF levels are correlated with birth weight (47). In mice, ablation of either the IGF-I or -II gene reduces birth weight to 60% that of normal littersmates (7, 29). Moreover, IGF-II-null mice have small placentas, indicating that the IGFs may influence fetal growth by promoting placental growth; this is supported by the demonstration that reduced fetal growth in mice lacking only the placental-specific transcript of IGF-II (9) is associated with reduced placental volume (42). IGF-I and IGF-II mediate their effects primarily by binding to the type 1 IGF receptor (IGF1R) to induce activation of the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. However, it is not known whether these signaling cascades are involved in mediating the trophoblast response to IGF.

Progress in understanding of cell dynamics in placenta has been hampered by the failure of in vitro models to recapitulate the epithelial-stromal organization or regenerate the trophoblast bilayer found in the chorionic villus and by the loss of normal proliferative capacity observed in primary trophoblast cultures. Choriocarcinoma cell lines have been used as an alternative, but these show unrestricted proliferation and limited differentiation. In the present study, we have overcome these difficulties by using an explant model of first-trimester human placenta in which the normal spatial and ontological relationships between the various cell populations are maintained, to examine the ability of IGFs to influence cell turnover.
(cytotrophoblast proliferation, differentiation, and apoptosis), and the mechanisms by which these effects are exerted. We show that cytotrophoblasts retain the capacity to proliferate ex vivo and that a subpopulation of these cells is able to undergo lateral fusion to generate syncytiotrophoblast. IGF-I and -II significantly enhance cytotrophoblast proliferation and syncytial formation and can rescue trophoblasts from apoptosis. Furthermore, we suggest that IGFs act via IGF1R-mediated activation of p42/44 MAPK to enhance proliferation and differentiation, whereas their ability to rescue cytotrophoblasts from apoptosis depends on IGF1R activation of the PI3K pathway.

MATERIALS AND METHODS

Placental tissue culture. Late first-trimester (8–12 wk) placentas were obtained by elective surgical or medical termination of pregnancy. All tissue was collected with informed consent in accordance with Local Ethics Committee approval.

Placental villous tissue was dissected under sterile conditions in a 1:1 mixture of serum-free Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F12 (F12) containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Because of the small size of first-trimester placenta, it was not possible to use a grid to randomly select areas of tissue for sampling, and, therefore, half of the available villous tissue was dissected into 5-mm pieces and random pieces were transferred to fresh medium in 1% agarose-coated 24-well tissue culture plates, while the other half was used for syncytiotrophoblast denudation (see below). Cultures were routinely maintained in 20% O2 at 37°C before being processed for histology or transmission electron microscopy because initial experiments (n = 4) performed in both 6% and 20% O2 concentrations demonstrated that neither basal nor IGF-induced cytotrophoblast proliferation was affected by O2 concentration.

Syncytial denudation. In preliminary experiments, dissected villous tissue was gently agitated in serum-free DMEM-F12 containing various concentrations (0.05–1%) of trypsin for up to 30 min at 37°C and was then transferred to DMEM-F12 containing 10% fetal bovine serum to neutralize the trypsin. Histological analysis revealed that light trypsinization (0.125% trypsin for 15 min) was optimal for removal of syncytiotrophoblast without damage to the underlying cytotrophoblasts (data not shown), and therefore all subsequent experiments were performed using this protocol. After trypsin neutralization, tissue was washed twice in serum-free DMEM-F12 and further dissected into 5-mm-diameter pieces before random selection of pieces for transfer to 1% agarose-coated 24-well tissue culture plates containing fresh serum-free DMEM-F12 for culture as described above.

Histology/transmission electron microscopy. Samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, for 3 h, washed in buffer containing 3 mM calcium chloride, and stored at 4°C until further processing. Seminiferous tubules and electron micrographs were obtained as previously described (43). Briefly, specimens were postfixed in 1% osmium tetroxide in 0.05 M sodium cacodylate buffer with pH 7.3 for 1 h at room temperature. Tissue was dehydrated, cleared in propylene oxide, embedded in resin (Taab Laboratories Equipment, Aldermaston, UK), and then polymerized in gelatin capsules at 60°C for 72 h. For semithin analysis, 0.5–μm sections were cut, mounted on glass slides, and stained with 1% toluidine blue 0 in 1% borax. Suitable areas were selected for examination with a Philips EM 301 electron microscope.

Cell proliferation assays. Cell proliferation was assessed by monitoring bromodeoxyuridine (BrdU) incorporation. Twenty-four hours after the start of culture, the medium was replaced with fresh media containing 100 μM BrdU. In experiments involving pathway inhibitors, explants were incubated for 30 min with the IGF1R inhibitor picropodophyllin (PPP; 5 μM); one of two PI3K-specific inhibitors, wortmannin (100 nM) or LY-294002 (LY; 200 nM), or the MAPK-specific inhibitor PD-98059 (PD; 50 μM) (Calbiochem, Merck Biosciences, Nottingham, UK); and then IGF-I (10 nM), IGF-II (10 nM), or vehicle was added to the cultures, which were further maintained in 20% O2 at 37°C before being processed for immunohistochemistry.

The dose of IGF used in these experiments was based on preliminary experiments that investigated a range (1 nM−1 μM) of concentrations. Similarly, preliminary experiments were performed to establish a concentration of each of the pathway inhibitors that did not affect basal cell turnover in placental explants.

Immunohistochemistry. Fresh placental tissue samples or cultured explants were fixed in 4% paraformaldehyde overnight and were then transferred to phosphate-buffered saline. Samples were embedded in paraffin, sectioned (5 μm), and mounted on glass slides. Sections were dewaxed in xylene and rehydrated, and antigen retrieval was then performed by boiling the sections in 0.1 M sodium citrate buffer, pH 6.3. Mouse anti-human chorionic gonadotropin-α (hCGα; 1:750; Abcam) antibody was used to identify trophoblast in the process of differentiation; mouse anti-BrdU (1:500; Sigma) and mouse anti-Ki67 (MIB-1 clone; 1:100; DakoCytomation, Ely, UK) antibodies were used to assess cytrophoblast proliferation (expressed as percentage of positive cytrophoblasts cells) and polyclonal antisera specific for the phosphoisoforms of the IGF1R (pY1162/1163, 1:1,000; Biosource International, Camarillo, CA), Akt (Ser473, 1:1,000; Cell Signaling Technologies, Beverly, MA), and p42/44 MAPK (Thr202/Tyr204, 1:1,000; Cell Signaling Technologies) were used to investigate activation of signaling pathways. Primary antibodies were applied for 2 h at room temperature.

Biotinylated goat anti-mouse IgG (1:200) and biotinylated swine anti-rabbit IgG (1:200) were from DakoCytomation. Immunoreactivity was visualized using the avidin-peroxidase technique as previously described (40), and sections were counterstained with hematoxylin.

Control experiments included omission of both antibodies, omission of primary antibody, or substitution of primary antibody with mouse IgG or rabbit IgG, and in these sections, no nonspecific staining was observed.

Apoptosis assays. Fresh placental tissue samples or explants cultured with and without inhibitors (PPP, 5 μM; wortmannin, 100 nM; LY, 200 nM; and PD, 50 μM) and/or IGF-I/-II (10 nM) were fixed, embedded, and sectioned as described above. Following dewaxing and antigen retrieval (0.1 M sodium citrate buffer, pH 6.3), apoptotic cells were identified using the terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) method (In Situ cell detection kit-POD) in accordance with the manufacturer’s instructions (Roche, Lewes, UK). Sections were then exposed to 0.05% diaminobenzidine in Tris-buffered saline, pH 7.6, for 3–5 min and counterstained with hematoxylin. Three random optical fields were selected from each section and were used to determine the percentage of TUNEL-positive nuclei (150–200 cytrophoblasts per section were counted). Positive control sections were incubated with 1,000 U/ml DNase I in 50 mM Tris-HCl, pH 7.5, and 1 mg/ml BSA before incubation with the TUNEL mixture; negative control sections were incubated with only TUNEL label mix. Data obtained were confirmed by immunohistochemical analysis (using the method described above) of the epithelial cell death marker, M30 (1:250; Roche).

Syncytial regeneration. After trypsinization, explants were immediately fixed or cultured with or without inhibitors (PPP, 5 μM; wortmannin, 100 nM; LY, 200 nM; and PD, 50 μM) and/or IGF-I/-II (10 nM) as described above for up to 7 days. Syncytial regeneration was monitored qualitatively by transmission electron microscopy and quantitatively by assessment of histological [hematoxylin and eosin (H&E) stained] sections. At least three images were chosen randomly from each H&E section; the numbers of both syncytial and cytrophoblast nuclei were counted (at least 200 cytrophoblast nuclei/section) using ImagePro Plus software, and data were expressed as the ratio of syncytial nuclei relative to cytrophoblast nuclei. The viability of explants and also their ability to regenerate syncytium following trypsinization were confirmed by...
analyzing the level of hCG (DRG Diagnostics) secreted into medium over 4 days in culture (data not shown).

**Data analysis.** Data were arc sine (proliferation and apoptosis) or log_{10} (regeneration) transformed before analysis using SPSS 13.0 for Windows. Comparisons were made using one-way ANOVA followed by planned contrasts. Data were considered significant at \( P < 0.05 \).

**RESULTS**

Proliferation continues in villous cytotrophoblast explants as evidenced by immunohistochemical analysis of BrdU incorporation (Fig. 1A). Oxygen concentration had no effect on cytotrophoblast proliferation because the number of BrdU-posi-

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**Fig. 1.** Characterization of basal trophoblast proliferation and regeneration in a first-trimester villous explant model. A and B: first-trimester (8–12 wk) placenta was cultured in serum-free medium for 2 days, with bromodeoxyuridine (BrdU) included for the final 24 h. Removal of syncytium was effected by light trypsinization before culture (B). Immunopositive cytotrophoblast nuclei are evident in the control tissue (A), but where syncytium is absent (B), none of the cells in the cytotrophoblast layer have incorporated the marker. C–F: transmission electron microscopy. Dotted lines in the images track the trophoblast basement membrane. C: control tissue shows the characteristic bilayer with cytotrophoblast (CT) beneath syncytium (ST). D: after trypsin treatment, the syncytium is absent. E: after 2 days in culture, newly fused cytotrophoblasts are shown that lack lateral intercellular membranes. Note that desmosomes (arrows) are present between adjacent cytotrophoblasts in the basal layer and between the basal and superficial cell layers. F: following 4 days in culture, a newly formed syncytiotrophoblast layer is apparent. G: following trypsinization, the ratio of syncytial to cytotrophoblast nuclei decreases before being restored to the control value after 4 days (means ± SE of 4 independent experiments). NS, nonsignificant. H: immunohistochemistry using anti-human chorionic gonadotropin-alpha (hCGα; day 0 (D0)) showing largely immunonegative cytotrophoblasts (arrows) beneath an immunopositive (detaching) syncytium. I: at day 2 (d2) after syncytial removal, cytotrophoblasts (arrows) show increased hCGα reactivity, indicating differentiation in culture. J: in some experiments, mouse IgG was used as primary antibody.
First trimester cytotrophoblast proliferation is not affected by oxygen concentration

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<th>O2 Concentration, %</th>
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Values are means ± SE. Villous explants (n=4) were cultured in 6% or 20% oxygen for 24 h. Bromodeoxyuridine (BrdU; 100 μM) was added, and cultures were continued for a further 48 h. Explants were then fixed, wax-embedded, and sectioned to assess cytotrophoblast proliferation via immunohistochemical analysis of BrdU-positive nuclei. One-way analysis of variance with planned contrasts was used to assess significant (P<0.05) differences between the groups: *significantly different from control (no treatment). Paired analysis of data from individual placentas (basal versus IGF-treated) revealed that IGF-I-stimulated cytotrophoblast proliferation at 6% oxygen was not significantly different from that observed at 20% oxygen.

Figure 2. IGF receptor type 1 (IGF1R) signaling pathways can be activated in placental explants. Villous tissue was cultured for 24 h and then fixed (A, D, and G) or incubated with IGF1R inhibitor picropodophyllin (PPP; C), phosphoinositide 3-kinase (PI3K) inhibitor LY-294002 (LY; F), or MAPKK inhibitor PD-98059 (PD; J) for 30 min, before exposure to IGF-I for 24 h (B, E, F, H, and I). Activation of signaling pathways was determined by immunohistochemical analysis using antibodies to phosphorylated (p)IGF1R, pAkt, and p42/44 MAPK as indicated. Staining in both the trophoblast and stromal layers is increased after IGF treatment, and all three inhibitors reduce the level of staining, indicating reduced phosphorylation. Results are representative of at least 3 independent experiments.

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blast nuclei were readily apparent in untreated explants (Fig. 3A), and proliferation was significantly enhanced by treatment with either IGF-I or -II (Fig. 3, C and E, respectively). Data obtained using the cell cycle marker Ki67 (not shown) supported these findings. IGF-stimulated cytotrophoblast proliferation was not affected by oxygen concentration (Table 1). Thus IGF applied to the syncytial surface, which mimics the in vivo exposure of syncytium to IGF present in maternal blood in the intervillous space, can influence the proliferation of the underlying cytotrophoblast layer. Similar experiments were performed in the presence of signaling molecule inhibitors to dissect the pathways involved in mediation of the cellular response to IGF. Both PPP (Fig. 3G) and PD (Fig. 3, D and G) reduced the percentage of IGF-I-induced BrdU-positive cytotrophoblasts ($P < 0.001$). Similarly, both PPP (Fig. 3G) and PD (Fig. 3, F and G) reduced IGF-II-induced cytotrophoblast proliferation. This suggests that IGF-I and -II both act through the IGF1R to activate the MAPK and promote cytotrophoblast proliferation.

**IGF promotes proliferation in the absence of syncytiun.** Because basal cytotrophoblast proliferation does not occur in the absence of a syncytiun (Fig. 1B), we were interested to determine whether application of IGF-I or IGF-II to exposed cytotrophoblast cells could alter the proportion of cytotrophoblast passing through S phase. IGF-stimulated proliferation of denuded villous explants was monitored by immunohistological assessment of BrdU incorporation (Fig. 4). Both hormones significantly enhanced cytotrophoblast proliferation because labeled cells that lacked an overlying syncytiun could be readily observed at day 2.

**Fig. 3.** Cytotrophoblast proliferation is mediated by the MAPK pathway. A–F: villous explants were cultured for 24 h and BrdU was then added. Explants were then incubated with the MAPKK inhibitor PD for 30 min (B, D, and F) and then exposed to vehicle (A and B), IGF-I (C and D) or IGF-II (E and F) for 48 h. Sections were stained using anti-BrdU antibody and counterstained with hematoxylin. BrdU-positive cytotrophoblast nuclei are seen in control tissue and following exposure to IGF-I or IGF-II. Fewer nuclei are labeled in IGF-treated tissue after exposure to PD. G: explants preincubated with PD as above were compared with those treated with the IGF1R inhibitor PPP or the PI3K inhibitor wortmannin before IGF-I or IGF-II. Three random areas from each placenta were counted, and the number of positive cells is expressed as a percentage of the total number of cytotrophoblasts (means ± SE of at least 4 independent experiments). PPP and PD but not wortmannin inhibited BrdU incorporation relative to IGF-treated controls. One-way analysis of variance with planned contrasts was used to assess significant ($P < 0.05$) differences between the groups: *significantly different from control (no treatment); $b$significantly different from IGF-I alone; $c$significantly different from IGF-II alone.
IGF-I and -II influence the rate of syncytial regeneration.

We next examined if IGFs can affect cytotrophoblast fusion by monitoring the regeneration of syncytium following treatment of tissue with trypsin (Fig. 5). In the presence of either IGF-I (Fig. 5, B and E) or IGF-II (Fig. 5E), the ratio of syncytial to cytotrophoblast nuclei was significantly enhanced by day 2. We suspected that IGFs enhance syncytial regeneration by increasing the number of cytotrophoblasts (through proliferation) that are available for fusion. Nevertheless, numerous in vitro studies have demonstrated that, in addition to promoting mitogenesis, IGFs are potent stimulators of differentiation (21), and thus the effect of IGF on cytotrophoblast differentiation was considered an alternative/additional mechanism. However, neither IGF-I nor IGF-II appeared to have a significant effect on the number of cytotrophoblasts expressing the differentiation marker hCG (not shown).

Therefore we examined whether IGF-induced syncytial regeneration was mediated by the same signaling pathway as IGF-induced proliferation. Trypsinized explants were exposed to IGF-I (10 nM) or IGF-II (10 nM) following pretreatment with PD (50 μM), wortmannin (100 nM), LY (200 nM), or PPP (5 μM). After 2 days in culture, the ratio of syncytial to cytotrophoblast nuclei was examined. As with IGF-induced proliferation, IGF-induced syncytial regeneration was unaffected by preincubation with wortmannin (or LY; data not shown); however, PPP significantly reduced the ability of both IGF-I (Fig. 5, C and E) and -II (Fig. 5E) to promote syncytial regeneration (P < 0.001). Similarly, PD reduced the effect of IGF-I (Fig. 5, D and E) and -II (Fig. 5E) on syncytial regeneration.

IGF-I and -II can rescue cytotrophoblasts from apoptosis by activating the PI3K pathway. Apoptosis in cytotrophoblast progenitor cells is another important aspect of cell turnover in the placenta. The level of apoptosis in trophoblast in explants was investigated using TUNEL and the epithelial cell death marker M30 (cleaved cytokeratin 18; Fig. 6A). Although cell viability is maintained for the short time frame (2 days) needed to perform proliferation assays, prolonged culture (4 days in serum-free medium; Fig. 6A) did lead to an increase in the number of cells undergoing apoptosis. However, cytotrophoblast apoptosis could be reduced by addition of either IGF-I or IGF-II 24 h after the start of the experiment (Fig. 6, B–G). PPP and wortmannin (and also LY; data not shown) reversed IGF-I- and IGF-II-mediated enhancement of cell survival, whereas PD did not affect the ability of either IGF-I or IGF-II to rescue cells from apoptosis (Fig. 6H). Similar data were obtained using the cell death marker M30 (Fig. 6I).

**DISCUSSION**

Placental explants in serum-free culture maintain the spatial and ontological relationships between cells and allow effective study of cytotrophoblast proliferation, differentiation, and apoptosis without the complications of multiple exogenous growth factors, binding proteins, and cytokines (32). Use of a mild enzymatic method to remove syncytiotrophoblast allowed direct access to the subjacent cytotrophoblast progenitor cell population (5, 6, 32). Cytotrophoblasts in explanted tissue remain viable for several days either in the presence or absence...
of syncytium and are capable of undergoing proliferation and differentiation, thus allowing cell kinetics to be examined. When syncytium was absent, no cytotrophoblast proliferation was observed. Importantly, this loss of basal cytotrophoblast proliferation suggests that the syncytium is required for maintenance of normal tissue kinetics. Syncytiotrophoblast is always a postmitotic compartment in vivo (3, 26, 34), and this is reflected in explant culture in which Ki67-positive nuclei are entirely absent from syncytium.

Many reports have demonstrated the presence of cell-cell contacts (gap junctions, cadherin-mediated attachments, and desmosomes) in villous trophoblast, and there is evidence that gap junctions are important for proliferation (5, 20, 30, 35). Our data strongly suggest that contact between the syncytiotrophoblast and underlying cytotrophoblasts is required for proliferation of the latter. This may explain why primary cytotrophoblasts exit from the cell cycle following isolation (33). Alternatively, since we also demonstrate that IGF applied directly to exposed cytotrophoblasts can initiate proliferation and differentiation, it is possible that the basal syncytium delivers soluble factors that, in turn, influence cytotrophoblast behavior. FGF-4 has similarly been shown to stimulate cytotrophoblast proliferation (6).

Removal of the syncytium also allowed mechanisms of syncytial formation to be investigated. Marker studies (hCG1/H925 and glial cell missing homolog-1 the latter not shown) indicate the presence of differentiated cytotrophoblasts in the absence of syncytiotrophoblast. Morphological observations show clearly that regeneration of a continuous syncytium occurs within 4 days of removal of the native syncytium. This is consistent with previous reports of regeneration in term tissue explants after spontaneous loss of syncytium (43). Morphological studies also suggest the possibility that a subset of cytotrophoblasts can fuse laterally in the absence of an existing syncytium. Real-time observations will be required to confirm this phenomenon, which challenges current models of placental development in which cytotrophoblasts divide asymmetrically to produce one daughter cell that differentiates to fuse with the overlying syncytium and another that remains in the progenitor cell pool. Cytotrophoblast-cytotrophoblast fusion

Fig. 5. IGF acts through the MAPK pathway to enhance the rate of syncytial regeneration. Following trypsinization, denuded villous explants were cultured for 24 h and then incubated with PPP, PD, LY (not shown), or wortmannin for 30 min before IGF-I (or IGF-II; data not shown) treatment for a further 72 h. Tissue sections were then stained with hematoxylin and eosin. Note the presence of regenerating syncytium after IGF treatment (arrows; B) but not in PPP-treated (C) or PD-treated (D) tissue. The ratio of syncytiotroplast nuclei was determined by counting 3 random areas of each placenta (E; means ± SE of 3 independent experiments).

One-way analysis of variance with planned contrasts was used to assess significant (P < 0.05) differences between the groups: *significantly different from control (no treatment); †significantly different from IGF-I alone; ‡significantly different from IGF-II alone. The effect of wortmannin did not reach significance relative to the respective IGF-treated controls.
probably occurs in primary culture, although this point has been contested (18). Such a process may provide a damage repair mechanism in case of local loss of syncytium from the maternofetal interface.

IGFs are known to regulate proliferation, differentiation, and cell survival in various cell types. However, there are few data on their effects on human placental development. Both IGF-I and IGF-II are produced by placental cells (14), including the syncytiotrophoblast and cells in the villous stroma, and in addition to locally produced IGFs, the liver releases high levels of IGFs into circulation (27). During pregnancy, levels of IGF-I and IGF-II in blood are dramatically increased (to 50–100 ng/ml...
and 150–400 ng/ml, respectively) (13), and there are clinical data demonstrating a correlation between maternal IGF levels and birth weight (47). These observations suggest that maternal IGF can influence fetal growth. The demonstration that IGF-I or IGF-II applied to the maternal side of the placenta enhances cytotrophoblast proliferation supports the hypothesis that maternal IGF may exert this influence at least in part through the regulation of normal placental development. The influence of IGF on the proliferation of placental cell lines is controversial because some studies have shown IGF-I and -II to be stimulatory, whereas others have been unable to detect any effect, although it is possible that these negative findings may be due to the use of inappropriately low IGF concentrations (13), the confounding influence of serum binding proteins, or the fact that isolated primary cytotrophoblasts exit the cell cycle and undergo spontaneous differentiation (24).

We have demonstrated that the ability of IGF to influence trophoblast turnover involves IGF1R-mediated activation of intracellular signaling pathways. These receptors are present on the both the maternal-facing syncytiotrophoblast membrane and on cytotrophoblasts (16, 37). The IGF1R is more abundant in the microvillus membrane than in the fetal-facing basal membrane, suggesting that IGF from maternal circulation may act on receptors on the syncytial surface to influence cytotrophoblast proliferation. However, transcriptional activity within the syncytiotrophoblast is limited, and our data demonstrating that IGF applied directly to cytotrophoblasts influences proliferation suggest that IGF1R present on cytotrophoblasts may regulate maternally derived IGF signals. In intact explants, exogenous IGF delivers a signal across the syncytium to influence cytotrophoblast proliferation. Clearly, the pathways by which this is effected require further investigation.

In other cellular systems, IGF influences cell turnover by inducing autophosphorylation of the IGF1R, which in turn allows activation of PI3K and MAPK pathways (46). Inhibitor studies demonstrate that IGF-I acts via IGF1R-mediated activation of the MAPK pathway in first-trimester placenta to influence proliferation and differentiation and via the PI3K pathway to influence cell survival. Although there are no previous data relating to IGF-induced intracellular pathways in human placental explants, members of the MAPK family have been shown to have a role in regulation of the differentiation of cytotrophoblast into syncytiotrophoblast (10, 22). Targeted gene disruption in mice demonstrates that MAPK is essential for normal placental development (15). Similarly, Akt has been reported to regulate rodent placental development (49), and a reduction in the activation of the PI3K/Akt pathway has been implicated in intrauterine growth restriction (IUGR) (1, 49). Placental expression of the IGF1R is also reduced in pregnancies complicated by IUGR (25), and cytotrophoblast turnover is abnormal (44). A reduction in IGF levels is associated with IUGR (12, 23, 36), and experimental reduction in either IGF-I or -II results in decreased proliferation and survival of placental fibroblasts, whereas overexpression results in increased cell survival, proliferation, and migration (32). Exogenous IGF has been shown to increase placental growth in guinea pigs (41). Manipulation of the IGF axis in the placenta may offer a potential therapeutic route to the correction of growth restriction during pregnancy.

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