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The Mitotic Manipulation of Cytotrophoblast Differentiation *In Vitro*

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Abstract

The placental syncytiotrophoblast is of paramount importance in optimising feto-maternal interactions. Syncytiotrophoblast is generated by the differentiation and fusion of underlying cytotrophoblasts. This process is aberrant in complicated pregnancies. We hypothesized that cell cycle withdrawal determines the phenotypic decision-making of cytotrophoblasts. We therefore investigated the effects of broad-spectrum mitotic inhibitors on cytotrophoblast differentiation.

Villous tissue was dissected from term placentae of normal pregnancies and cultured on Netwell supports. Over 48 h, the original syncytiotrophoblast was detached and underlying cytotrophoblasts exposed. The resulting villi were treated with mitotic blockers (Ara-C, colcemid, cyclohexamide, doxorubicin hydrochloride, hydroxyurea, L-Mimosine, purvalanol A). The media was recovered and analysed for lactacte dehydrogenase (LDH) and human chorionic gondadotrophin (hCG), markers of tissue viability and cytotrophoblast differentiation, respectively. The resulting tissue was processed for proliferative activity thorough Ki-67 immunorecognition.

Colcemid, cyclohexamide, hydroxyurea, and purvalanol A showed significant cytotoxicity over 48 h incubation. Villous tissue exposed to 0.01 mM and 0.1 mM Ara-C, doxorubicin hydrochloride and L-Mimosine showed no increase in liberated LDH. hCG production increased exponentially with cytotrophoblast differentiation. Higher concentrations of Ara-C and L-Mimosine significantly encouraged hCG production. In addition, total cell and cytotrophoblast proliferation were reduced with Ara-C and L-Mimosine treatment.

The inhibition of DNA synthesis and replication with Ara-C and L-Mimosine suppressed active proliferation of villus components and exaggerated the biochemical differentiation of cytotrophoblasts. Cell cycle disruption is therefore a basic trigger for cytotrophoblast differentiation. This approach provides a mechanism for encouraging syncytiotrophoblast formation and may hold benefits for conditions where syncytiotrophoblast cover is attenuated.

Keywords: Cytotrophoblast; Syncytiotrophoblast; Explants; Mitosis; Cell cycle

1. Introduction

The anatomy of the human placenta is in large part due to the high rates of cellular proliferation and differentiation of epithelial cytotrophoblasts. Throughout gestation, a proportion of cytotrophoblasts retain their undifferentiated phenotype, whilst the remainder generate two major trophoblast populations; the syncytiotrophoblast, the exchange barrier of the placenta, and the invasive extravillous trophoblasts, which penetrate the maternal decidualised endometrium, modifying the uterine spiral arteries to optimise utero-placental blood flow [1].

The terminally differentiated, multinucleated syncytiotrophoblast has no regenerative potential and is therefore maintained by continuous fusion of cytotrophoblast cells [2]. To counter this recruitment, aged cellular material is packed into membrane-sealed syncytial knots and extruded into the maternal circulation. Apoptosis within the syncytiotrophoblast

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is implicated in this process; whilst differentiation of cytotrophoblasts, a prerequisite for syncytial fusion, also shows signs of early but reversible apoptotic activity [3].

The deregulation of syncytiotrophoblast is a feature of pre-eclampsia and intrauterine growth restriction (IUGR). In pre-eclampsia, enhanced trophoblast apoptosis, combined with elevated cytotrophoblast proliferation, indicates exaggerated trophoblast trafficking, as an adaptive response to direct villus damage or utero-placental underperfusion [4,5]. Evidence supports the idea that syncytiotrophoblast-derived material stimulates the maternal inflammatory response and encourages vascular anomalies which characterise the pre-eclamptic condition [6,7]. In support, plasma from affected women show an excess of circulating syncytial debris [8], whilst placental morphology indicates a marked reduction in syncytiotrophoblast cover; a feature directly associated with a reduction in neonatal birthweight [9].

The necessity for appropriate cell turnover of trophoblasts in healthy pregnancy is clear, however, questions remain regarding the regulation of the syncytiotrophoblast *in vivo*, and the potential of pharmacological agents to influence the cytotrophoblast cell life cycle. Traditionally, proliferation and differentiation are considered mutually exclusive, with cell cycle withdrawal considered an acknowledged prerequisite for terminal differentiation. Here we investigate this concept in syncytiotrophoblast formation *in vitro*, using a series of highly characterised but mechanistically different mitotic blocking agents.

2. Materials and methods

Placentae from uncomplicated pregnancies were sampled randomly using a transparent sampling sheet [10]. Within thirty minutes of delivery, chorionic non-anchoring villi were dissected out, cut into pieces of approximately 5 mg and placed, three per well, on Netwell supports in 1.5 ml standard placental explant media (CMRL-1066, 5% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 1 μg/ml insulin, 0.1 μg/ml hydrocortisone, and 0.1 μg/ml retinyl acetate). In 20% oxygen, the syncytiotrophoblast of these explants is extensively detached over 48 h of culture with the subsequent exposure of the villous cytotrophoblasts [11,12]. Further culturing allows cytotrophoblasts to spontaneously differentiate into syncytiotrophoblast like regions. This process can be monitored by human chorionic gondadotrophin (hCG) release [12].

Over a subsequent 48 h period, cultured explants were incubated in 6% oxygen and exposed to individual mitotic blockers; Ara-C (1-beta-D-arabinofuranosylcytosine), colcemid, cyclohexamide, doxorubicin hydrochloride, hydroxyurea, L-Mimosine and purvalanol A at either 0.01 mM or 0.1 mM final concentrations. At the end of these additional incubations, the media were recovered and analysed for hCG and lactate dehydrogenase (LDH); markers of differentiation and tissue viability, respectively. hCG was measured by quantitative immunoradiometric assay (ICN Pharmaceuticals, Basingstoke, UK) and LDH by the catalyzed reduction of pyruvate as previously described [12]. Both measurements were related to total explant protein per well, following solubilisation at the end of culture in 0.3 M NaOH. Proliferative activity was defined by immunostaining of paraffin-embedded tissue sections, using a mouse monoclonal antibody to human Ki-67 antigen (Clone MIB-1, Dako UK Ltd. Ely, UK) [12]. Positive nuclei in the stroma, endothelium and cytotrophoblast were related to total non-syncytiotrophoblast nuclei as determined by image analysis and point counting; again as previously described [13]. Unless otherwise stated, reagents were purchased from Sigma Chemical Co., Poole, UK.

3. Results

The modes of action of all mitotic blockers are given in Table 1. Of these agents, colcemid, cyclohexamide, hydroxyurea, and purvalanol A at 0.1 mM and 0.01 mM demonstrated significant cytotoxicity over a 2-day culture period (Fig. 1). Alternatively, tissue exposed to 0.01 mM and 0.1 mM Ara-C, doxorubicin hydrochloride and L-Mimosine showed no significant elevation in LDH liberation (Fig. 1). From day 2 to 4 of culture, corrected hCG levels increased exponentially in the absence of mitotic blockers, as exposed cytotrophoblasts underwent biochemical differentiation (data not shown). At higher concentrations (0.1 mM), Ara-C and L-Mimosine significantly encouraged hCG production over vehicle controls (Fig. 2); doxorubicin hydrochloride had no significant effect (2.21 ± 0.04 (0.01 mM) and 2.31 ± 0.08 (0.1 mM) vs. 2.47 ± 0.13 (control), $p > 0.05$). Ara-C and L-Mimosine actively reduced cytotrophoblast mitosis and inhibited proliferation in all mitotically active components of the villus (i.e. all cells excluding the syncytiotrophoblast) (Fig. 3).

4. Discussion

The inhibition of DNA synthesis and replication *in vitro* using Ara-C and L-Mimosine, suppressed the proliferation of villos components and exaggerated the biochemical differentiation of cytotrophoblast cells. This indicates that cell cycle disruption and withdrawal are direct triggers for villous cytotrophoblast differentiation and a mechanism for syncytiotrophoblast formation; a fundamental requirement of placental development and a critical determinate of pregnancy outcome. The molecular mechanisms governing cytotrophoblast differentiation are increasingly recognised, with the definition of upstream transcriptional regulators, such as Mash-2 and Hand-1, and downstream effectors, including adhesion molecules, fusion proteins and proteinases (for review see refs. [14,15]). In comparison, little is known about the orchestration of events which determine cytotrophoblast proliferation, differentiation and syncytiotrophoblast formation.

Our observations, conducted in primary tissue culture, are in line with the immunostaining of placental bed biopsies from first trimester pregnancies which indicate that active

Table 1
Mitotic blockers and modes of action

Blocking agent	Mode of action
Ara-C	Selective inhibitor of DNA synthesis
Doxorubicin hydrochloride	Intercalates in DNA and inhibits reverse transcriptase and RNA polymerase
Colcemid	Mitotic spindle inhibitor – arrests cells in metaphase
Cyclohexamide	Inhibits peptidyl transferase and protein synthesis
Hydroxyurea	Inactivates ribonucleoside reductase, inhibiting DNA synthesis
L-Mimosine	Iron chelator – inhibits DNA replication
Purvalanol A	Cell-permeable cyclin-dependent kinase (Cdk) inhibitor