Trophoblast toxicity assay (TTA): a gestational toxicity test utilizing human placental trophoblasts

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Citation:
Kliman HJ. Trophoblast toxicity assay (TTA): A gestational toxicity test using human placental trophoblasts. In: Alternative toxicological methods. Salem H, Katz SA (eds), CRC Press, Boca Raton, FL, 2003, pgs 567-580

ABSTRACT

Many people in the field of mammalian reproduction have concluded that there is a severe lack of information regarding the effects of the majority of drugs and environmental toxins—and the potential detrimental effects of these agents—during pregnancy. The problem has been attributed to the lack of an assay system which can assess the potential effects of a chemical on the human fetus and placenta. Since the health of the fetus is critically dependent on the quality of placental function during gestation, we have developed an *in vitro* Trophoblast Toxicity Assay (TTA) system to evaluate the potential adverse effects that drugs may have on the placenta (and hence fetus) during human gestation. The system utilizes an *in vitro* trophoblast culture system that we have developed and studied over the last fourteen years.

Trophoblasts are unique cells derived from the cytotrophoblasts of the outer cell layer of the blastocyst which mediate implantation and placentation. Depending on their subsequent function *in vivo*, undifferentiated cytotrophoblasts can develop into 1) absorptive and hormonally active villous syncytiotrophoblasts, 2) extravillous anchoring trophoblastic cell columns, or 3) invasive intermediate trophoblasts. Interestingly, within the villi of the human placenta—at all gestational ages—there exists a population of cytotrophoblasts which remain undifferentiated. My colleagues and I have shown that by studying the differentiation of these villous cytotrophoblasts in culture, it is now possible to correlate *in vitro* trophoblast behavior with *in vivo* biology of the human trophoblast. Therefore, the basis of the Trophoblast Toxicity Assay system is that drugs and chemicals that alter the differentiated functions of trophoblasts in culture are expected to alter the function of trophoblasts *in vivo*. Conversely, agents which do not affect a wide array of differentiated trophoblast functions *in vitro* should not have a deleterious impact on placental function during pregnancy.

The Trophoblast Toxicity Assay focuses on the three major differentiated forms of trophoblasts described above. To perform this assay, we have chosen characteristic markers for each of these trophoblasts: amino acid uptake, and secretion of ß-hCG and progesterone for villous syncytiotrophoblasts; trophouteronectin secretion for anchoring trophoblasts; and PAI-1 secretion for invasive intermediate trophoblasts. My colleagues and I have studied all of these products and have utilized standard assay methods to measure these trophoblast markers. The goal of the Trophoblast Toxicity Assay will be to evaluate the effects of chemicals on the ability of cultured human trophoblasts to take up amino acids and secrete these markers. Dose-response curves will be generated for agent as a function of trophoblast differentiation in culture over a 72 to 96 h period. Statistical methods will be employed to accurately evaluate the differences seen in the treated cultures compared to controls. In concert with the biochemical aspects of the Trophoblast Toxicity Assay, the cultures will be evaluated cytologically to assess for gross effects of these chemicals, and immunocytochemically to assess for cell to cell variability for each of the markers examined.

As well as becoming a practical and useful assay to assess drugs and environmental toxins, the further development of the Trophoblast Toxicity Assay may have significant impact on the evaluation of compounds that have exposure potential during pregnancy and could possibly become a standard safety assay for gestational therapeutics.

HUMAN TROPHOBLASTS *IN VIVO*: THREE DIFFERENTIATION PATHWAYS

Trophoblasts are unique cells derived from the outer cell layer of the blastocyst which mediate implantation and placentation. Depending on their external environment, undifferentiated cytotrophoblasts can develop into **1) hormonally active villous syncytiotrophoblasts, 2) extravillous anchoring trophoblastic cell columns, or 3) invasive intermediate trophoblasts** (Kliman and Feinberg, 1992) (Fig 1). Studies utilizing cultured cytotrophoblasts are beginning to elucidate the specific factors that mediate these pathways of trophoblast differentiation. This chapter will review the differentiation pathways of the cytotrophoblast, what is known about the factors that regulate trophoblast differentiation, the model systems used to study trophoblast biology, and the various hormones that have been shown to be made by these trophoblasts, both *in vitro* and *in vivo*.

Villous syncytiotrophoblast

The hormones secreted by the villous syncytiotrophoblast are critical for maintaining pregnancy (Conley and Mason, 1990; Petraglia et al., 1990). Early in gestation, human chorionic gonadotropin (hCG) is essential to maintain corpus luteum progesterone production. Near the end of the first trimester, the mass of villous syncytiotrophoblast is large enough to make sufficient progesterone and estrogen to maintain the pregnancy. During the third trimester, large quantities of placental lactogen are produced, a hormone purported to have a role as a regulator of lipid and carbohydrate metabolism in the mother. Other syncytiotrophoblast products, to name a few, include pregnancy specific $ß_1$-glycoprotein (Kliman et al., 1986), plasminogen activator inhibitor type 2 (Feinberg et al., 1989), growth hormone (Jara et al., 1989), collagenases (Moll and Lane, 1990), thrombomodulin (Maruyama et al., 1985; Ohtani et al., 1989), and growth factor receptors (Kawagoe et al., 1990; Posner, 1974; Uzumaki et al., 1989). The factors responsible for the regulated synthesis of these compounds has been the subject of a great deal of investigations, some of which will be reviewed below.

In vitro experiments have identified several compounds which are capable of differentiating cultured cytotrophoblasts towards an endocrine phenotype. These include cAMP (Feinman et al., 1986; Ringler et al., 1989b; Ulloa-Aguirre et al., 1987), EGF (Maruo et al., 1987) and hCG itself (Shi et al., 1993). Cyclic AMP has been shown to upregulate hCG and progesterone secretion. In the case of hCG, the mechanism appears to be a direct upregulation hCG gene transcription via a cAMP regulatory region of the genome. For progesterone, increased synthesis appears to be due to a concerted upregulation of a number of enzymes responsible for progesterone biosynthesis, including the side chain cleavage enzyme and adrenodoxin complex—the first steps in the

conversion of cholesterol to progesterone. Not only do these compounds upregulate hormone secretion, they also appear to down-regulate the synthesis of markers of the other pathways of trophoblast differentiation. For example, in the presence of 8-bromo-cAMP, cultured trophoblasts are induced to secrete large quantities of hCG (Feinman et al., 1986). At the same time, their synthesis and secretion of the trophoblast form of fibronectin, trophouteronectin (Feinberg et al., 1991)—a marker of junctional trophoblasts (see Fig. 1)—is turned off (Ulloa-Aguirre et al., 1987). This result suggests that mutually exclusive differentiation pathways result from stimulation by appropriate factors.

Trophoblasts seem to make more than one hormone at the same time—a difficult task for a cell. Once stimulated to become hormonally active, the trophoblast seems capable of producing at least two glycoproteins simultaneously (Kliman et al., 1987), although electron microscopic immunochemistry has demonstrated that these products are located in different secretory vacuoles within the same cell (Hamasaki et al., 1988). This synchronous hormone production may help to explain why the syncytiotrophoblast is multinucleated: multiple copies of the genome may be necessary to allow this complex cell to make numerous products simultaneously while it continues to perform its other functions of absorption and waste excretion.

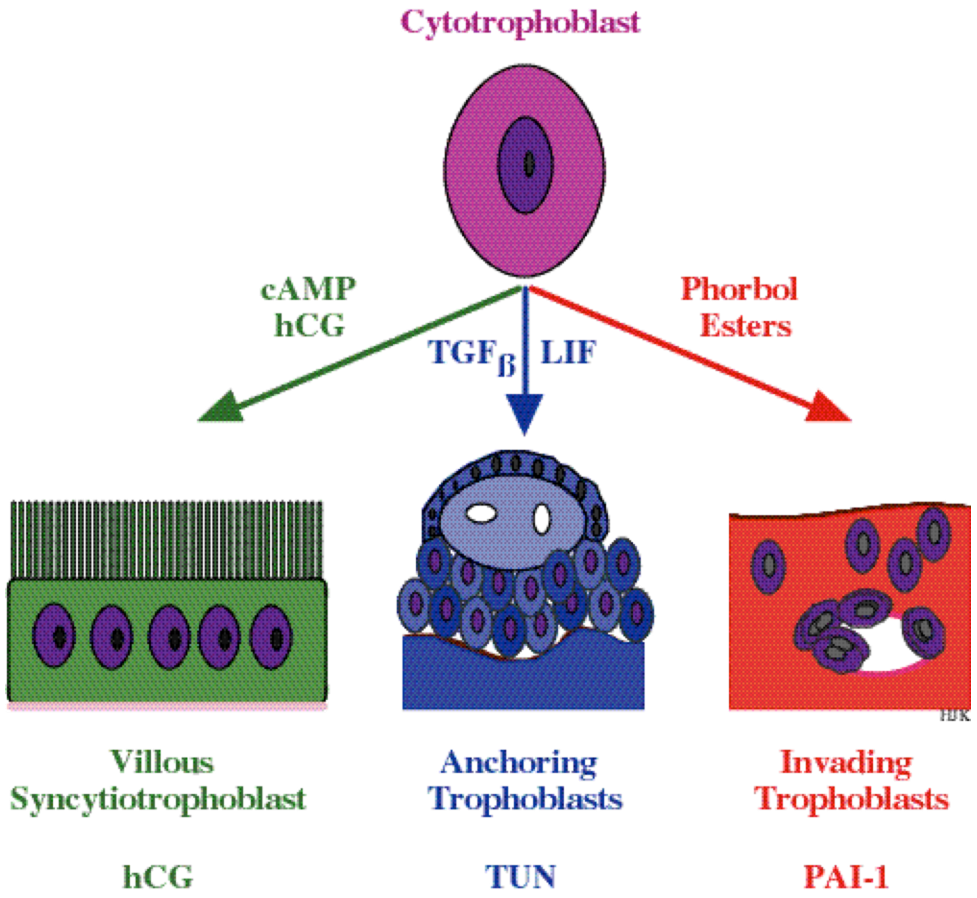

Figure 1. Pathways of trophoblast differentiation . Just as the basal layer of the skin gives rise to keratinocytes, the cytotrophoblast—the stem cell of the placenta—gives rise to the differentiated forms of trophoblasts. **Left)** Within the chorionic villi, cytotrophoblasts fuse to form the overlying syncytiotrophoblast. The villous syncytiotrophoblast makes the majority of the placental hormones, the most studied being hCG. cAMP, EGF, and even hCG itself have been implicated as stimulators of this differentiation pathway. In addition to upregulating hCG secretion, cAMP has also been shown to down-regulate trophouteronectin (TUN) synthesis. **Center)** At the point where chorionic villi make contact with external extracellular matrix (decidual stromal ECM in the case of intrauterine pregnancies), a population of trophoblasts proliferates from the cytotrophoblast layer to form the second type of trophoblast—the junctional trophoblast. These cells form the anchoring cell columns that can be seen at the junction of the placenta and endometrium throughout gestation. Similar trophoblasts can be seen at the junction of the chorion layer of the external membranes and the decidua. The junctional trophoblasts make a unique fibronectin—trophouteronectin—that appears to mediate the attachment of the placenta to the uterus. TGFß and LIF have been shown to induce cultured trophoblasts to secrete increased levels of trophouteronectin, while down-regulating hCG secretion. **Right)** Finally, a third type of trophoblast differentiates towards an invasive phenotype and leaves the placenta entirely—the invasive intermediate trophoblast. In addition to making human placental lactogen, these cells also make urokinase and plasminogen activator inhibitor-1 (PAI-1). Phorbol esters have been shown to increase trophoblast invasiveness in *in vitro* model systems and to upregulate PAI-1 in cultured

trophoblasts. The general theme that comes from these observations is that specific factors are capable of shifting the differentiation pathway of the cytotrophoblast towards one of the above directions, while turning off differentiation towards the other pathways. See text for details.

Anchoring trophoblasts

It has been generally accepted that some form of cell-extracellular matrix interaction takes place at the attachment interface between the anchoring trophoblasts and the uterus. Recently, a specific type of fibronectin—*trophouteronectin (TUN)*—has been implicated as the protein responsible for the attachment of anchoring, extravillous trophoblasts to the uterus throughout gestation (Feinberg et al., 1991; Feinberg and Kliman, 1993). This specialized form of fibronectin appears to be made wherever trophoblasts contact extracellular matrix proteins. The factors that may be responsible for activating trophoblast TUN production include TGFß (Feinberg et al., 1994) and leukemia inhibitory factor (LIF) (Nachtigall et al., 1993). TGFß has been identified in the region of the utero-placental junction, possibly made by both decidual cells in that area and by the trophoblasts themselves (Lysiak et al., 1992). LIF has been identified in human endometrium (Stewart, 1994), but has not been shown to be made by trophoblasts. Interestingly, both TGFß and LIF have been shown to upregulate TUN secretion from cultured trophoblasts while down-regulating hCG secretion (Fig. 1) (Feinberg et al., 1994; Nachtigall et al., 1993).

Invading trophoblasts

As human gestation progresses, invasive populations of extravillous trophoblasts attach to and interdigitate through the extracellular spaces of the endo- and myometrium. The endpoint for this invasive behavior is penetration of maternal spiral arteries within the uterus (Pijnenborg, 1990). Histologically, trophoblast invasion of maternal blood vessels results in disruption of extracellular matrix components and development of dilated capacitance vessels within the uteroplacental vasculature. Biologically, trophoblast-mediated vascular remodeling within the placental bed allows for marked distensibility of the uteroplacental vessels, thus accommodating the increased blood flow needed during gestation. Abnormalities in this invasive process have been correlated with early and mid-trimester pregnancy loss, preeclampsia and eclampsia, and intrauterine growth retardation (Robertson et al., 1986).

As would be anticipated when considering invasive cells, these trophoblasts produce a variety of proteases (Fisher et al., 1989; Milwidsky et al., 1993; Queenan et al., 1987) and protease inhibitors (Feinberg et al., 1989) which are utilized to regulate the invasive process. In addition to the protease systems, invasive trophoblasts also make protein hormones, most notably human placental lactogen (Kurman et al., 1984).

IN VITRO MODEL SYSTEMS TO STUDY TROPHOBLAST DIFFERENTIATION

The most commonly used approaches for examining the regulation of hormone production by trophoblasts have come from *in vitro* studies. Model systems developed to study placental and trophoblast function have included placental organ and explant culture, trophoblast culture, chorion laeve culture, choriocarcinoma cell line culture, and placental perfusion studies (Kliman and Feinberg, 1992). Recently, most investigators have turned to trophoblast cell culture since it eliminates the complications of more heterogeneous cell systems (Fig. 2). Since the cytotrophoblast is the precursor of all other trophoblasts, a variety of methods have been proposed to purify this cell type from the human placenta (Bax et al., 1989; Belisle et al., 1986; Branchaud et al., 1990; Dodeur et al., 1990; Fisher et al., 1990; Kliman et al., 1986; Loke, 1990; Loke et al., 1989; Shorter et al., 1990; Truman et al., 1989; Yagel et al., 1989a).

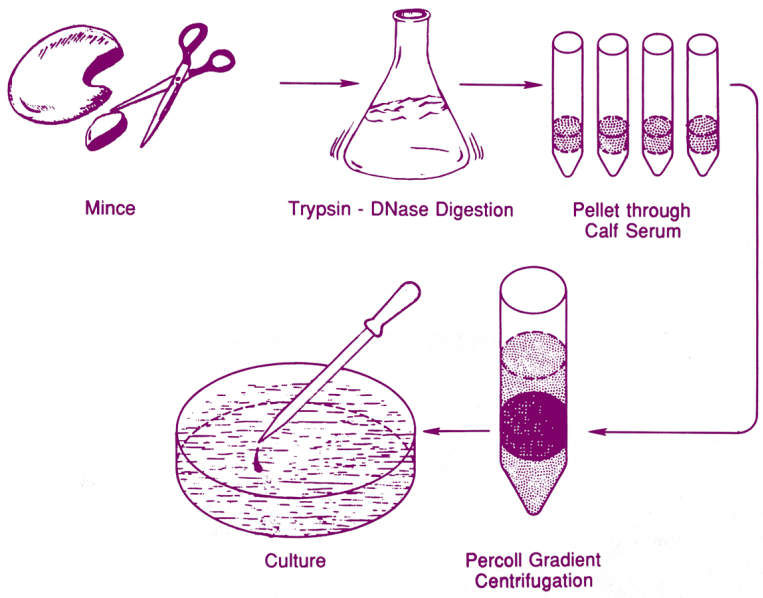

Figure 2. Purification of cytotrophoblasts from term placenta. A term placenta is minced and digested with trypsin and DNAse. The supernatant is passed through calf serum to inactivate the digestive enzymes, then these pellets are pooled and placed on a Percoll gradient to separate out the cytotrophoblasts (Kliman et al., 1986).

We have demonstrated by time-lapse cinematography that when these mononuclear cytotrophoblasts are placed in Dulbecco's Modified Eagles' Medium (DMEM) containing 20% (v/v) heat-inactivated fetal calf serum (FCS), they flatten onto the culture surface within 3-12 h, migrate towards each other to form aggregates within the first 24 h, and over the next 24 h of culture, form syncytiotrophoblasts (Fig. 3) (Kliman et al., 1986). Concomitant with these morphologic changes, these trophoblasts synthesize and secrete a number of cell products, including protein hormones, peptide hormones, steroid hormones, growth factors, and cytokines. We and others have used these cells to elucidate the products of trophoblast differentiation and to explore the mechanisms by which their synthesis and secretion is regulated.

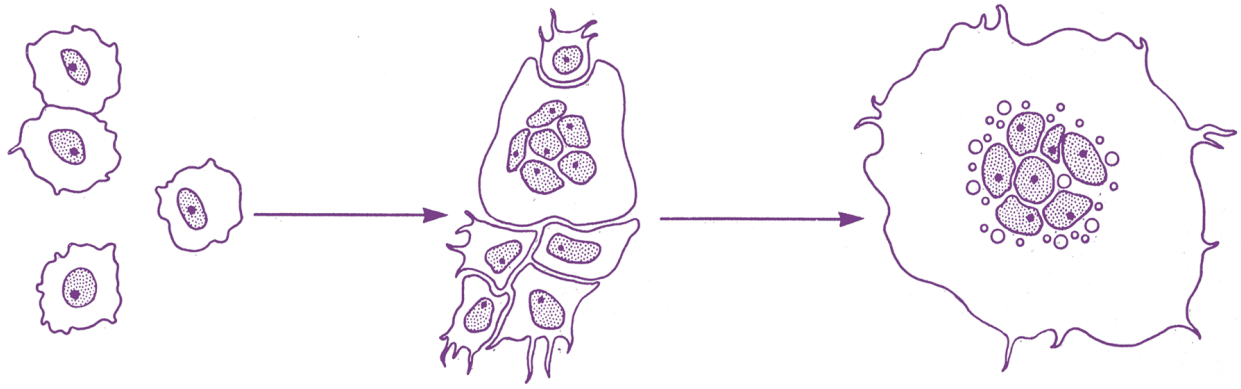

Figure 3. In vitro morphologic differentiation of cytotrophoblasts. After purification the cytotrophoblasts are dispersed individual cells (left). When plated in culture media containing serum these cells flatten out and begin to move towards each other. After 24 h in culture, aggregate begin to appear, with some evidence of cell fusion (center). After 72 h in culture most of the trophoblasts have fused and formed large, multinucleated syncytiotrophoblasts (Kliman et al., 1986).

TROPHOBLASTS AS ENDOCRINE CELLS

Trophoblasts synthesize and secrete a vast array of endocrine products (for reviews see references (Blay and Hollenberg, 1989; Conley and Mason, 1990; Cunningham et al., 1997; Jones, 1989; Petraglia et al., 1990; Ringler and Strauss, 1990; Sirinathsinghji and Heavens, 1989)). Collectively, these hormones function to regulate trophoblast growth and differentiation, affect fetal growth and homeostasis, modulate maternal immunologic, cardiovascular and nutritional status, protect the fetus from infection, and prepare the uterus and mother for parturition.

PROTEIN HORMONES

Chorionic gonadotropin

The most widely studied trophoblast hormone product is chorionic gonadotropin. This glycoprotein is critical to pregnancy since it rescues the corpus luteum from involution, thus maintaining progesterone secretion by the ovarian granulosa cells. Its usefulness as a diagnostic marker of pregnancy stems from the fact that it may be one of the earliest secreted products of the conceptus. Ohlsson et al. (Ohlsson et al., 1989) have demonstrated by *in situ* hybridization that ß-hCG transcripts are present in human blastocyst trophoblasts prior to implantation. Placental production of hCG peaks during the eighth to the tenth week of gestation, and tends to plateau at a lower level for the remainder of pregnancy. This difference in the rate of hCG secretion may be mimicked to some extent by trophoblasts cultured from first versus third trimester placentae. Kato and Braunstein (Kato and Braunstein, 1990) have demonstrated that trophoblasts from first trimester placentae secrete greater amounts of hCG than trophoblasts purified from term placentae, suggesting that cultured trophoblasts may retain the regulatory effects of their *in situ* milieu even after several days of culture (Fig. 4).

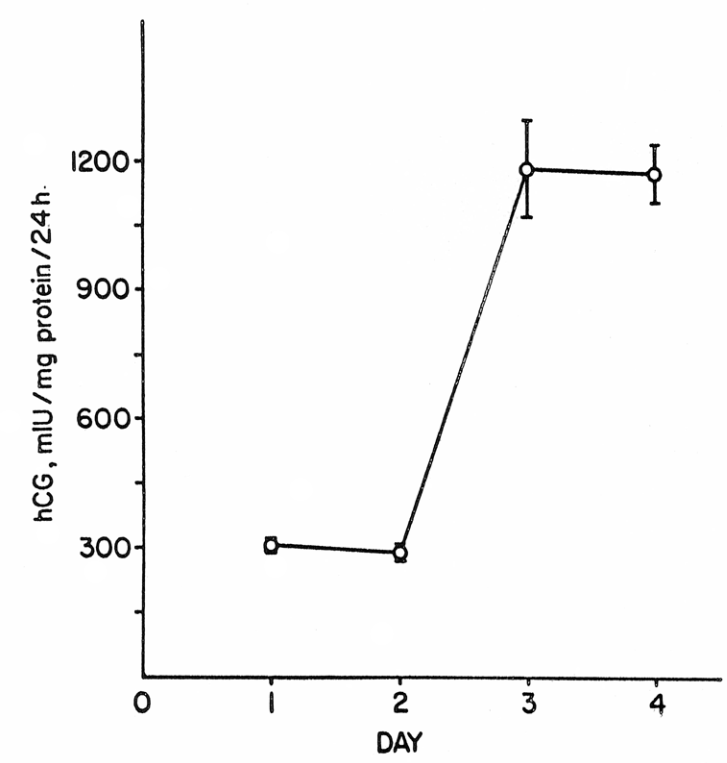

Figure 4. hCG secretion by trophoblasts in culture. Percoll-gradient purified cytotrophoblasts were cultured in DMEM media for four days. Media was changed daily and assayed for hCG by radioimmunoassay. hCG was not detectable at the time of initial plating (Kliman et al., 1986).

What regulates hCG synthesis and secretion in the trophoblast? Workers have attempted to discover what regulates hCG synthesis and secretion by examining likely factors *in vitro*. Table 1 summarizes our current knowledge of the regulatory factors that appear to modulate hCG secretion in trophoblasts. The sensitivity of human trophoblasts to exogenous regulation is clearly demonstrated by examination of the marked stimulatory effect of cAMP on hCG and progesterone secretion by cultured trophoblasts (Fig. 5).

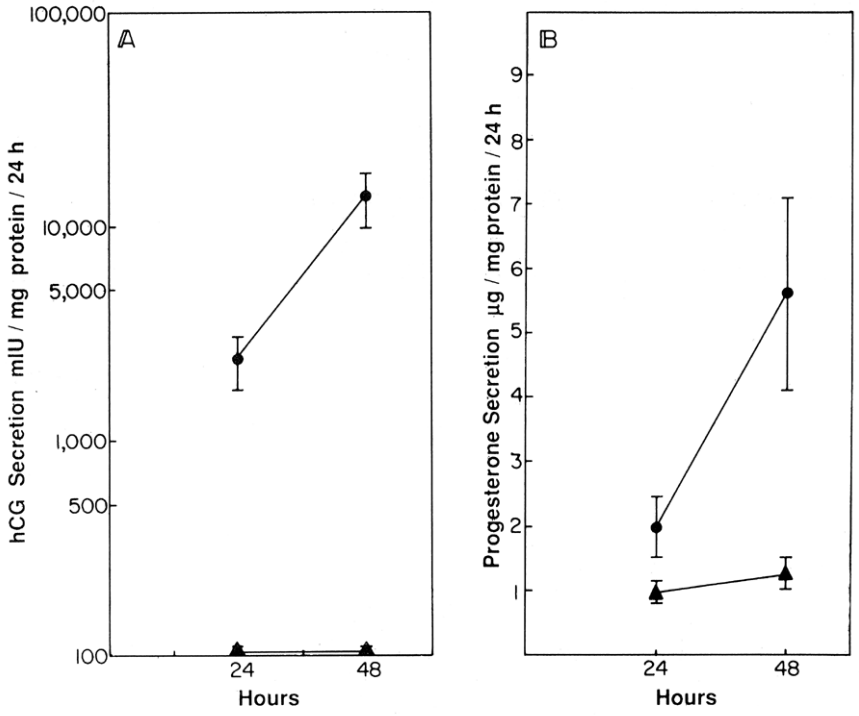

Figure 5. Effects of 8-bromo-cAMP on hCG and progesterone secretion by cultured cytotrophoblasts. Percoll-gradient purified cytotrophoblasts were cultured for 48 hours in the absence (▲) or presence (●) of 8-bromo-cAMP. hCG (A) and progesterone (B) were quantitated in the medium at 24 hour intervals. Values presented are the mean ± SE from six separate experiments. At each time point, 8-bromo-cAMP-treated cultures secreted significantly more ($p <$

0.014, by the Wilcoxon signed rank test) progesterone and hCG than did control cultures (Feinman et al., 1986).

Table 1

Regulation of trophoblast hCG secretion

Factor	Trophoblasts (Trimester)	Effect on hCG Secretion	References
cAMP	Term	Stimulates	(Feinman et al., 1986)
hCG	Term	Stimulate	(Shi et al., 1993)
GnRH	Term	Stimulates	(Belisle et al., 1989; Szilagyi et al., 1992)
GnRH	First, Term	Not clear	(Kelly et al., 1991)
ß-adrenergic agonists	First	Stimulates	(Oike et al., 1990)
Dexamethasone	Term	Stimulates	(Ringler et al., 1989a)
Inhibin	Term	Inhibits	(Petraglia et al., 1991; Petraglia et al., 1987; Petraglia et al., 1989)
Activin	Term	Potentiates GnRH simulation of hCG secretion	(Petraglia et al., 1991)
Activin	First	Stimulates	(Steele et al., 1993)
EGF	First, Term	Stimulates	(Maruo et al., 1987)
Thyroid hormone	First, Term	Stimulates	(Maruo et al., 1991)
Thyroid Stimulating Hormone	Term	Inhibits	(Beckmann et al., 1992)
Interleukin-1.	First	Stimulates	(Yagel et al., 1989b)
Interleukin-6	First	Stimulates	(Nishino et al., 1990)
Basement Membrane	First	Stimulates	(Truman and Ford, 1986)
Decidual Protein	Term	Inhibits	(Ren and Braunstein, 1991)
Prolactin	Term	Inhibits	(Yuen et al., 1986)

Human placental lactogen (hPL)

This potent glycoprotein is made throughout gestation, increasing progressively until the 36th week, where it can be found in the maternal serum at a concentration of 5-15 μg/ml, the highest concentration of any known protein hormone. The major source of hPL appears to be the villous syncytiotrophoblasts, where it is made at a constant level throughout gestation (Sakbun et al., 1987). In addition to the villous syncytiotrophoblast, hPL has been identified in invasive intermediate trophoblasts during the first trimester (Heyderman et al., 1981; Kurman et al., 1984), as well as the third trimester (Gosseye and van, 1992). In addition to identifying hPL within trophoblasts *in situ*, experiments have shown that cultured first trimester trophoblasts secrete hPL *in vitro*.(Dodeur et al., 1990) Sakbun et al. (Petraglia et al., 1989) have also identified hPL mRNAs in cultured trophoblasts. Hoshina et al. (Hoshina et al., 1984), working with choriocarcinoma cell lines, have proposed that hPL gene expression occurs after α-hCG and ß-hCG gene expression, suggesting that hPL is a product of a more differentiated trophoblast. Kliman et al. have also shown that intracytoplasmic α-hCG appears prior to intracytoplasmic hPL in cultured term trophoblasts (Kliman et al., 1987).

The factors that regulate hPL synthesis and secretion are not as well studied as for hCG. Kato and Braunstein (Kato and Braunstein, 1989) have demonstrated that the secretion of hCG and hPL are discordant during the first 5 days of term trophoblast culture, suggesting different regulatory pathways for these hormones. Dodeur et al. (Dodeur et al., 1990) demonstrated that dibutyryl cAMP stimulated hPL secretion from cultured first trimester trophoblasts. Maruo et al. (Maruo et al., 1987) have shown that EGF, in addition to increasing hCG secretion by cultured human trophoblasts, also augments hPL secretion by these cells. Handwerger et al. (Handwerger et al., 1987) showed that high density lipoproteins (HDL) stimulate the release of hPL from human placental explants, while Wu and Handwerger showed that HDL stimulates hPL release from cultured trophoblasts via a protein kinase-C-dependent pathway (Wu and Handwerger, 1992). Finally, Petit et al. (Petit et al., 1989) have demonstrated that angiotensin II stimulates hPL release by cultured trophoblasts, while opioids stimulate hPL release via a calcium influx mechanism (Petit et al., 1993).

TROPHOBLAST TOXICITY ASSAY

Utilizing the *in vitro* assay systems and the secreted products described above, an assay to assess the toxicologic potential of chemicals, both natural and synthesized, can be developed. The Trophoblast Toxicity Assay focuses on the three major differentiated forms of trophoblasts described above. To perform this assay, we have chosen characteristic markers for each of these trophoblasts: amino acid uptake, and secretion of ß-hCG and progesterone for villous syncytiotrophoblasts; trophouteronectin secretion for anchoring trophoblasts; and PAI-1 secretion for invasive intermediate trophoblasts. We have studied all of these products and have utilized standard assay methods to measure these trophoblast markers. The goal of the Trophoblast Toxicity Assay will be to evaluate the effects of chemicals on the ability of cultured human trophoblasts to take up amino acids and secrete these markers. Dose-response curves will be generated for agent as a function of trophoblast differentiation in culture over a 72 to 96 h period. Statistical methods will be employed to accurately evaluate the differences seen in the treated cultures compared to controls. In concert with the biochemical aspects of the Trophoblast Toxicity Assay, the cultures will be evaluated cytologically to assess for gross effects of these chemicals, and immunocytochemically to assess for cell to cell variability for each of the markers examined.

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CONCLUSIONS

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REFERENCES

Bax CM, Ryder TA, Mobberley MA, Tyms AS, Taylor DL, Bloxam DL, Ultrastructural changes and immunocytochemical analysis of human placental trophoblast during short-term culture. Placenta 1989 10: 179-194.

Beckmann MW, Wurfel W, Austin RJ, Link U, Albert PJ, Suppression of Human Chorionic-Gonadotropin in the Human Placenta at Term by Human Thyroid-Stimulating Hormone Invitro. Gynecologic and Obstetric Investigation 1992 34: 164-170.

Belisle S, Bellabarba D, Gallo Payet N, Lehoux JG, Guevin JF, On the role of luteinizing hormone-releasing hormone in the in vitro synthesis of bioactive human chorionic gonadotropin in human pregnancies. Canadian Journal of Physiology and Pharmacology 1986 64: 1229-1235.

Belisle S, Petit A, Bellabarba D, Escher E, Lehoux JG, Gallo Payet N, Ca2+, but not membrane lipid hydrolysis, mediates human chorionic gonadotropin production by luteinizing hormone-releasing hormone in human term placenta. Journal of Clinical Endocrinology and Metabolism 1989 69: 117-121.

Blay J, Hollenberg MD, The nature and function of polypeptide growth factor receptors in the human placenta. Journal of Developmental Physiology 1989 12: 237-248.

Branchaud C, Goodyer CG, Guyda HJ, Lefebvre Y, A serum-free system for culturing human placental trophoblasts. In Vitro Cellular and Developmental Biology 1990 26: 865-870.

Conley AJ, Mason JI, Placental Steroid-Hormones. Baillieres Clinical Endocrinology and Metabolism 1990 4: 249-272.

Cunningham FG, MacDonald PC, Gant NF, Leveno KJ, Gilstrap LCI, Hankins GDV, et al. Williams Obstetrics. Stamford: Appleton & Lange, 1997.

Dodeur M, Malassine A, Bellet D, Mensier A, Evain Brion D, Characterization and differentiation of human first trimester placenta trophoblastic cells in culture. Reproduction, Nutrition, Development 1990 30: 183-192.

Feinberg R, Kliman HJ, Lockwood C, Oncofetal fibronectin: A trophoblast "glue" for human implantation? American Journal of Pathology 1991 138: 537-543.

Feinberg R, Kliman HJ, Human trophoblasts and tropho-uteronectin (TUN): A model for studying early implantation events. Assisted Reproduction Reviews 1993 3: 19-25.

Feinberg RF, Kliman HJ, Wang CL, Transforming growth factor-beta stimulates trophoblast oncofetal fibronectin synthesis in vitro: implications for trophoblast implantation in vivo. Journal of Clinical Endocrinology and Metabolism 1994 78: 1241-1248.

Feinberg RF, Wun TC, Strauss JF, Queenan JT, Kliman HJ, Kao LC, et al., Plasminogen-activator inhibitor type-1 and type-2 in human trophoblasts - PAI-1 is an immunocytochemical marker of invading trophoblasts. Laboratory Investigation 1989 61: 20-26.

Feinman MA, Kliman HJ, Caltabiano S, Strauss JFd, 8-Bromo-3',5'-adenosine monophosphate stimulates the endocrine activity of human cytotrophoblasts in culture. Journal of Clinical Endocrinology and Metabolism 1986 63: 1211-1217.

Fisher SJ, Cui TY, Zhang L, Hartman L, Grahl K, Zhang GY, et al., Adhesive and degradative properties of human placental cytotrophoblast cells in vitro. Journal of Cell Biology 1989 109: 891-902.

Fisher SJ, Sutherland A, Moss L, Hartman L, Crowley E, Bernfield M, et al., Adhesive interactions of murine and human trophoblast cells. Trophoblast Research 1990 4: 115-138.

Gosseye S, van dF, HPL-positive infiltrating trophoblastic cells in normal and abnormal pregnancy. European Journal of Obstetrics, Gynecology, and Reproductive Biology 1992 44: 85-90.

Hamasaki K, Ueda H, Okamura Y, Fujimoto S, Double immunoelectron microscopic labeling of human chorionic gonadotropin and human placental lactogen in human chorionic villi. Sangyo Ika Daigaku Zasshi 1988 10: 171-177.

Handwerger S, Quarfordt S, Barrett J, Harman I, Apolipoproteins AI, AII, and CI stimulate placental lactogen release from human placental tissue. A novel action of high density lipoprotein apolipoproteins. Journal of Clinical Investigation 1987 79: 625-628.

Heyderman E, Gibbons AR, Rosen SW, Immunoperoxidase localisation of human placental lactogen: a marker for the placental origin of the giant cells in 'syncytial endometritis' of pregnancy. Journal of Clinical Pathology 1981 34: 303-307.

Hoshina M, Hussa R, Pattillo R, Camel HM, Boime I, The role of trophoblast differentiation in the control of the hCG and hPL genes. Advances in Experimental Medicine and Biology 1984 176: 299-312.

Jara CS, Salud AT, Bryantgreenwood GD, Pirens G, Hennen G, Frankenne F, Immunocytochemical localization of the human growth hormone variant in the human placenta. Journal of Clinical Endocrinology and Metabolism 1989 69: 1069-1072.

Jones CT, Endocrine function of the placenta. Baillieres Clinical Endocrinology and Metabolism 1989 3: 755-780.

Kato Y, Braunstein GD, Discordant secretion of placental protein hormones in differentiating trophoblasts in vitro. Journal of Clinical Endocrinology and Metabolism 1989 68: 814-820.

Kato Y, Braunstein GD, Purified first and third trimester placental trophoblasts differ in in vitro hormone secretion. Journal of Clinical Endocrinology and Metabolism 1990 70: 1187-1192.

Kawagoe K, Akiyama J, Kawamoto T, Morishita Y, Mori S, Immunohistochemical demonastration of epidermal growth factor (EGF) receptors in normal human placental villi. Placenta 1990 11: 7-15.

Kelly AC, Rodgers A, Dong KW, Barrezueta NX, Blum M, Roberts JL, Gonadotropin-releasing hormone and chorionic gonadotropin gene expression in human placental development. DNA and Cell Biology 1991 10: 411-421.

Kliman HJ, Feinberg RF. Trophoblast differentiation. In: Barnea E, Hustin J and Jauniaux E, editors. The First Twelve Weeks of Gestation. New York: Springer-Verlag, 1992.

Kliman HJ, Feinman MA, Strauss JF, Differentiation of human cytotrophoblasts into syncytiotrophoblasts in culture. Trophoblast Research 1987 2: 407-421.

Kliman HJ, Nestler JE, Sermasi E, Sanger JM, Strauss JFd, Purification, characterization, and in vitro differentiation of cytotrophoblasts from human term placentae. Endocrinology 1986 118: 1567-1582.

Kurman RJ, Main CS, Chen HC, Intermediate trophoblast: a distinctive form of trophoblast with specific morphological, biochemical and functional features. Placenta 1984 5: 349-369.

Loke YW, New developments in human trophoblast cell culture. Colloque INSERM 1990 199: 110-116.

Loke YW, Gardner L, Grabowska A, Isolation of human extravillous trophoblast cells by attachment to laminin-coated magnetic beads. Placenta 1989 10: 407-415.

Lysiak J, McCrae K, Lala PK, Localization of transforming growth factor-beta at the human fetal-maternal interface: role in trophoblast growth and differentiation. Biology of Reproduction 1992 46: 561-572.

Maruo T, Matsuo H, Mochizuki M, Thyroid hormone as a biological amplifier of differentiated trophoblast function in early pregnancy. Acta Endocrinologica 1991 125: 58-66.

Maruo T, Matsuo H, Oishi T, Hayashi M, Nishino R, Mochizuki M, Induction of differentiated trophoblast function by epidermal growth factor: relation of immunohistochemically detected cellular epidermal growth factor receptor levels. Journal of Clinical Endocrinology and Metabolism 1987 64: 744-750.

Maruyama I, Bell CE, Majerus PW, Thrombomodulin is found on endothelium of arteries, veins, capillaries, and lymphatics, and on syncytiotrophoblast of human placenta. Journal of Cell Biology 1985 101: 363-371.

Milwidsky A, Finciyeheskel Z, Yagel S, Mayer M, Gonadotropin-Mediated Inhibition of Proteolytic-Enzymes Produced by Human Trophoblast in Culture. Journal of Clinical Endocrinology and Metabolism 1993 76: 1101-1105.

Moll UM, Lane BL, Proteolytic Activity of 1st trimester placenta- localization of interstitial collagenase in villous and extravillous trophoblast. Histochemistry 1990 94: 555-560.

Nachtigall MJ, Kliman HJ, Feinberg RF, Meaddough EI, Arici A. Potential role of leukemia inhibitory factor (LIF) in human implantation. 41st Annual Meeting. Chicago, Illinois: Society for Gynecoloic Investigation, 1993: 96.

Nishino E, Matsuzaki N, Mashuhiro K, Kameda T, Taniguchi T, Takagi T, et al., Trophoblast-derived interleukin-6 (IL-6) regulates human chorionic gonadotropin release through IL-6 receptor on human trophoblast. Journal of Clinical Endocrinology and Metabolism 1990 71: 436-441.

Ohlsson R, Larsson E, Nilsson O, Wahlstrom T, Sundstrom P, Blastocyst implantation precedes induction of insulin-like growth factor II gene expression in human trophoblasts. Development 1989 106: 555-559.

Ohtani H, Maruyama I, Yonezawa S, Ultrastructural immunolocolization of thrombomodulin in human placenta with microwave fixation. Acta Histochemicay 1989 22: 393-395.

Oike N, Iwashita M, Muraki T, Nomoto T, Takeda Y, Sakamoto S, Effect of adrenergic agonists on human chorionic-gonadotropin release by human trophoblast cells obtained from 1st-trimester placenta. Hormone and Metabolic Research 1990 22: 188-191.

Petit A, Gallo PN, Bellabarba D, Lehoux JG, Belisle S, The modulation of placental lactogen release by opoids: a role for extracellular calcium. Molecular and Cellular Endocrinology 1993 90: 165-170.

Petit A, Guillon G, Tence M, Jard S, Gallo Payet N, Bellabarba D, et al., Angiotensin II stimulates both inositol phosphate production and human placental lactogen release from human trophoblastic cells. Journal of Clinical Endocrinology and Metabolism 1989 69: 280-286.

Petraglia F, Angioni S, Coukos G, Uccelli E, DiDomenica P, De RBM, et al., Neuroendocrine mechanisms regulating placental hormone production. Contributions to Gynecology and Obstetrics 1991 18: 147-156.

Petraglia F, Calza L, Garuti GC, Giardino L, D eRB, Angioni S, New Aspects of placental endocrinology. Journal of Endocrinologic Investigation 1990 13: 353-371.

Petraglia F, Sawchenko P, Lim AT, Rivier J, Vale W, Localization, secretion, and action of inhibin in human placenta. Science 1987 237: 187-189.

Petraglia F, Vaughan J, Vale W, Inhibin and activin modulate the release of gonadotropin-releasing hormone, human chorionic gonadotropin, and progesterone from cultured human placental cells. Proceedings of the National Academy of Sciences of the United States of America 1989 86: 5114-5117.

Pijnenborg R, Trophoblast invasion and placentation in the human- morphological aspects. Trophoblast Research 1990 4: 33-47.

Posner BI, Insulin receptors in human and animal placental tissue. Diabetes 1974 23: 209-217.

Queenan JT, Jr., Kao LC, Arboleda CE, Ulloa Aguirre A, Golos TG, Cines DB, et al., Regulation of urokinase-type plasminogen activator production by cultured human cytotrophoblasts. Journal of Biological Chemistry 1987 262: 10903-10906.

Ren SG, Braunstein GD, Decidua produces a protein that inhibits choriogonadotropin release from human trophoblasts. Journal of Clinical Investigation 1991 87: 326-330.

Ringler GE, Kallen CB, Strauss JFd, Regulation of human trophoblast function by glucocorticoids: dexamethasone promotes increased secretion of chorionic gonadotropin. Endocrinology 1989a 124: 1625-1631.

Ringler GE, Kao LC, Miller WL, Strauss JFd, Effects of 8-bromo-cAMP on expression of endocrine functions by cultured human trophoblast cells. Regulation of specific mRNAs. Molecular and Cellular Endocrinology 1989b 61: 13-21.

Ringler GE, Strauss JFd, In vitro systems for the study of human placental endocrine function. Endocrine Reviews 1990 11: 105-123.

Robertson WB, Khong TY, Brosens I, De Wolf F, Sheppard B, Bonnar J, The placental bed biopsy: review from three European center. American Journal of Obstetrics and Gynecology 1986 155: 401-412.

Sakbun V, Koay ES, Bryant Greenwood GD, Immunocytochemical localization of prolactin and relaxin C-peptide in human decidua and placenta. Journal of Clinical Endocrinology and Metabolism 1987 65: 339-343.

Shi QJ, Lei ZM, Rao CV, Lin J, Novel role of human chorionic gonadotropin in differentiation of human cytotrophoblasts. Endocrinology 1993 132: 1387-1395.

Shorter SC, Jackson MC, Sargent IL, Redman CW, Starkey PM, Purification of human cytotrophoblast from term amniochorion by flow cytometry. Placenta 1990 11: 505-513.

Sirinathsinghji DJ, Heavens RP, Stress-related peptide hormones in the placenta: their possible physiological significance. Journal of Endocrinology 1989 122: 435-437.

Steele G, Currie W, Yuen B, Jia X, Perlas E, Leung P, Acute stimulation of human chorionic gonadotropin secretion by recombinant human activin-A in first trimester human trophoblast. Endocrinology 1993 133: 297-303.

Stewart CL, A cytokine regulating embryo implantation. New York Academy of Sciences 1994.

Szilagyi A, Benz R, Rossmanith WG, The human first-term placenta in vitro: regulation of hCG secretion by GnRH and its antagonist. Gynecological Endocrinology 1992 6: 293-300.

Truman P, Ford HC, The effect of substrate and epidermal growth factor on human placental trophoblast cells in culture. In Vitro Cellular and Developmental Biology 1986 22: 525-528.

Truman P, Ford HC, Pomare L, Human Placental Cytotrophoblast Cells - Identification and Culture. Archives of Gynecology and Obstetrics 1989 246: 39-49.

Ulloa-Aguirre A, August AM, Golos TG, Kao LC, Sakuragi N, Kliman HJ, et al., 8-Bromo-adenosine 3',5'-monophosphate regulates expression of chorionic gonadotropin and fibronectin in human cytotrophoblasts. Journal of Clinical Endocrinology and Metabolism 1987 64: 1002-1009.

Uzumaki H, Okabe T, Sasaki N, Hagiwara K, Takaku F, Tobita M, et al., Identification and characterization of receptors for granulocyte colony-stimulating factor in human placenta and trophoblastic cells. Proceedings of the National Academy of Sciences of the United States of America 1989 86: 9323-9326.

Wu YQ, Handwerger S, High density lipoproteins stimulate molecular weight 80K protein phosphorylation in human trophoblast cells: evidence for a protein kinase-C-dependent pathway in human placental lactogen release. Endocrinology 1992 131: 2935-2940.

Yagel S, Casper RF, Powell W, Parhar RS, Lala PK, Characterization of pure human first-trimester cytotrophoblast cells in long-term culture: growth pattern, markers, and hormone production. American Journal of Obstetrics and Gynecology 1989a 160: 938-945.

Yagel S, Lala PK, Powell WA, Casper RF, Interleukin-1 stimulates human chorionic gonadotropin secretion by first trimester human trophoblast. Journal of Clinical Endocrinology and Metabolism 1989b 68: 992-995.

Yuen BH, Moon YS, Shin DH, Inhibition of human chorionic gonadotropin production by prolactin from term human trophoblast. American Journal of Obstetrics and Gynecology 1986 154: 336-340.