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Biological Markers During Early Pregnancy: Trophoblastic Signals of the Peri-Implantation Period

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The peri-implantation period extends from the time the blastocyst is free in the uterus, through the processes of recognition and attachment, to the beginning of trophoblast differentiation and the interactions between the embryo and the uterine endometrium which initiate establishment of the hemochorial placenta. It is during the peri-implantation period that the embryo and hormonally regulated endometrial cells appear to be most sensitive to factors which introduce risk into the intrauterine environment.

There are no markers which can be used practically to assess pregnancy risk during the peri-implantation period of either human or laboratory rodents. Experimental studies, using *in vitro* laboratory models of differentiating trophoblast cells, have identified peptide hormone markers of pivotal developmental processes. Exposure of trophoblast during the expression of these processes could have severe and far-reaching effects individually and societally. While these trophoblast signals are limited in their utility with respect to health monitoring extrapolation of these findings to human pregnancy, the signals could serve to identify more practical and sensitive markers to assess risk in early gestation.

Human chorionic gonadotropin (hCG) has been used extensively as a marker to assess risk during the early stages of pregnancy. Extrapolation of experimental data indicates how hCG could be used more effectively in analyses of possible cause and effect relationships. The limitations of hCG as a marker for risk during the human peri-implantation period are discussed. Peptide hormones which could serve to assess risk during this critical period of extraordinary sensitivity to toxic factors are introduced.

Introduction

It is during the immediate peri-implantation period when the maternal uterine environment and the embryo are interacting to establish pregnancy that the uterus and embryo appear to be the most sensitive and susceptible to factors which bring the greatest risk to bear on the successful and uneventful completion of gestation. The data which support this peremptory statement have accumulated over the last two centuries. The chances of a couple of proven fertility to conceive offspring in any one menstrual cycle is about 25% (*1,2*). It remains more difficult to categorize to what extent this high failure rate is due to errors or dysfunctions in ovulation, fertilization, implantation, or subsequent development. In animal husbandry there is a high incidence of early embryonic loss, as great as 40% in pigs. It has been estimated that as many as 50% of presumed human conceptions may similarly be due to early embryonic losses (*2,3*).

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To an extent, the records of human *in vitro* fertilization/embryo transfer (IVF/ET) programs validate these estimates. The success rates, 15 to 20%, are marginally below the accepted norm. However, these rates are disconcerting when it is appreciated that the fertilized embryos that are transferred represent a selected population. The deficit is attributable to the failure of events that are related to implantation (*4,5*). Since errors in implantation appear to be one of the largest, if not the single largest, cause of failure in apparently reproductively competent individuals (Fig. 1), it is rational to suggest that this extraordinary sensitivity to risk could be compounded further by the introduction of xenobiotic agents into the intrauterine environment.

Laboratory Models

In the United States, solution of this problem cannot be aided by prospective experimentation. This option is effectively foreclosed by ethical constraints and legal restrictions (*6,7*). For this reason the study of early development in the human has necessarily become more dependent on knowledge derived from comparative studies than have other areas of human biology. Ma-

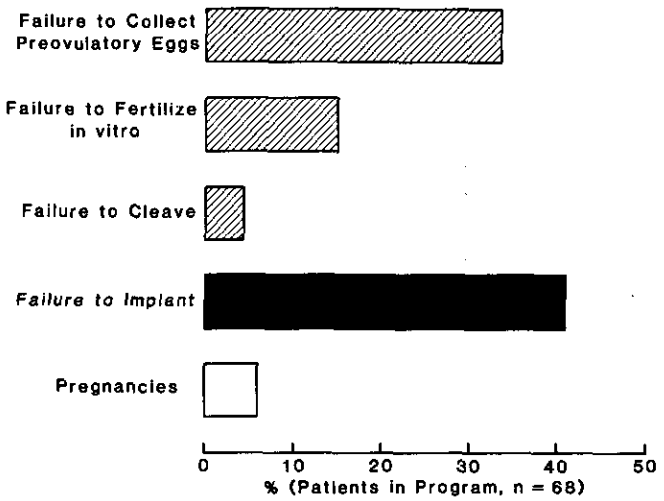

FIGURE 1. The relative contribution of different stages of very early gestation to the outcome of an early *in vitro* fertilization/embryo transfer program. This figure summarizes data that reported results in a treatment group that resulted in the first successful IVF/ET pregnancy (*4*). Of the 79 women monitored during the course of spontaneous menstrual cycles, 68 women underwent laparoscopy for attempted oocyte retrieval resulting in the delivery at term of two normal infants (*5*).

terial available for study is represented by examples of almost all the divergent evolutionary trials of the mammalian reproductive systems; as such, they are imperfect analogies of human reproduction. Nevertheless, divergence often being a better teacher than simulation, comparative analysis has provided the insight that has allowed common mechanisms characteristic of the preimplantation period to be identified (*8*).

It is important to appreciate that animal experiments, particularly *in vitro* laboratory models, are only models. The usefulness of these analogies is correlated to the extent the model mimics the specific process being studied and is thus a function of our understanding. The caveats inherent in the design and execution of laboratory models (*9*) limit, but do not discount, the opportunity to focus on a distinct target or unique toxin-specific cellular or molecular event that alters the reproductive or developmental outcome. The accuracy with which such events can be identified and the precision with which they can be analyzed is constitutive to the experimental model only to the degree that we understand the process under investigation and its putative role in the program of differentiation.

Xenobiotics and the Peri-Implantation Period

Given almost any definition of biomarkers it is evident that there are no truly specific or reliable markers for xenobiotic agents that can yet be exactly correlated with any of the cellular and molecular events of early mammalian development. It is true that there are data which permit the generalization that events of early development are adversely influenced by a variety of toxic agents (*10*). However, the data derive from studies that were not stringently designed or executed and often were analyzed retrospectively. Their usefulness in identifying specific sensitive loci is gratuitous. The possibility of overcoming such deficiencies is inhibited by the paucity of valid, stage-specific markers of reproductive and developmental events *per se*. The few putative early event markers that might prove useful (cleavage rates, compaction, blastocoelation, expression of embryonic mRNA, expression and organization of trophectodermal cytokeratins, etc.) have rarely, if ever, been used in studies of reproductive toxicology.

The identification of xenobiotics that produce human reproductive and developmental aberrations depends on the integrative analysis of at least three types of monitoring, i.e., environmental, biological, and health. The concept of a biological marker as a signal for a reproductive or developmental event that can be used to monitor the effect of a xenobiotic agent is well established. However, there are no markers of this sort, markers that are medically and economically effective and can satisfy the pragmatics of normal human life, available for the analysis of those events related to the peri-implantation period in human or other mammalian species. Thus, there remains a compelling need to identify valid and effective markers. The usefulness of such putative markers will be determined, in terms of any xenobiotic, by their ability to identify specific and/or sensitive targets, by the role of these targets in normal biology (e.g., will the preferential localization and accumulation of the xenobiotic by a specific cell be of any consequence), and by the nature and/or degree of the effect (i.e., death, anomaly, dysfunctions, quality of survival) (Fig. 16).

Implantation

Implantation of the mammalian embryo within the uterus of the maternal host is a unique interaction between two genetically dissimilar organisms. For most species, close coordination of changes in the developing embryo and the uterus, probably initiated while the embryo is still in the oviduct, is required. Disruption of this synchrony leads to failure of the implantation process. Synchrony (*11*) is the concept that defines the highly regulated integration of multistep developmental programs, proceeding independently in the intercommunicating epithelial and stromal cells of the uterine endometrium and in the embryo, that is obligatory for the blastocyst to interact with the sensitized uterine-epithelium at a species-specific time.

The idea that in response to changing ratios of progesterone and estrogen, the uterus matures from a hostile to a neutral, nonreceptive to a receptive environment for the blastocyst and that implantation is a property of the receptive uterus, is supported by physiological data (*12–14*) and confirmed by morphological (*15,16*), and to a lesser extent, by biochemical, correlates (*12,13,17–19*). While an obligatory role can be justified in the case of uterine development, the role of

these same hormones in embryo development is not secure. Whith only a single exception (*20*), *in vitro* blastocyst differentiation has been reported to proceed programmatically in the absence of steriod hormones (*21,22*).

Based on these earlier studies, we now possess a reasonable understanding of the conditions necessary to coordinate events within the female reproductive tract that bring the embryo into the uterine environment with the maximum opportunity for implantation (*13, 14,19,23*). However, these data are not adequate to define the cause-and-effect relationships that are the basis for the specific actions of hormones, drugs, and toxic agents. The questions now arising go beyond these historic approaches. In order to define the regulatory biology of blastocyst-endometrial interactions and how they may be altered by xenobiotic agents, it is necessary to bring new, more incisive methods developed in other disciplines, i.e., cell biology, immunology, molecular biology, etc., to bear on these investigations.

The placement of the trophectoderm (TE) cells of the blastocyst in intimate contact with the receptive uterine endometrium initiates the progressive phases of the implantation process (*19,21,23*). It is suggested that these phases are programatically directed by information exchanged directly via cell-cell communication (*24*), but also modulated by directive and permissive signals from stromal-epithelial intercommunication (*25*). These informational molecules are expressed in response to the same steroids which synchronized the blastocyst and uterus from the beginning.

In order to interpret specific implantation events in terms of cell-cell interactions, we have developed methods for the separation of homogeneous populations of the individual cell types involved directly in implantation (endometrial epithelial and stromal cells, blastocyst trophectoderm, and ectoplacental cone cells and trophoblast giant cells). We have also developed methods for the *in vitro* culture of these individual cell populations so that we can begin to analyze the biochemical mechanisms which regulate their differentiation and thereby their interactions (*27–30*).

To apply the concept of biomarkers to the interaction of xenobiotics and those processes that define early mammalian development, we formulated a specific research strategy. The plan is necessarily dependent on animal experimental models. The first goal of the plan is to identify markers causally related to events critical to embryo-uterine interactions. Cellular and biochemical methods have been developed to identify and validate structural and/or functional markers of the regulatory processes involved in the differentiation of each component (*28–30*). Construction of such a directory of experimental markers provides a focus to select the most reasonable candidates that could be examined in modes applicable to a nonlaboratory environment. The evolution of this strategy, which necessarily begins in the laboratory, enhances the feasibility of applying the conceptual triad of environmental, biological, and health monitoring to the action of xenobiotic agents on early development. For the purposes of this essay, we have chosen to discuss differentiation of the trophoblast (trophectoderm) of the blastocyst to demonstrate the productivity of this research strategy.

Trophectoderm

The first cells to differentiate in the mammalian embryo are the trophectoderm (TE) cells. This commitment occurs at compaction of the cleavage stage embryo, at which time the blastomeres assume an inside-outside orientation (*31*). Although trophoblast cells do not contribute to the formation of the embryo, they become an integral part of the placenta (*21*). TE and its differentiated derivatives (trophoblast giant cells, TGC) are intimately involved not only structurally, but in most of the functions ascribed to the placenta, and are critical to viviparity (*32*). Thus, compaction and the subsequent process of blastocoelation are critical processes at which the adverse effect of toxic agents could provoke far-reaching influences on development.

In utero the mammalian embryo is comprised of an inner cell mass (ICM), the presumptive embryo, and a blastocoel; both are surrounded by an outer covering of trophoblast (TE) cells. The TE cells which cover the ICM are termed polar TE, while those which surround the blastocoel are termed mural TE. Preimplantation and early postimplantation TE cells are proliferative and diploid (2–4 $\bar{c}$).

When trophoblast cells lose contact with the ICM (or attach to the uterine epithelium), they lose their ability to divide. Unlike somatic cells which remain diploid after they cease to divide in G_2, the trophoblast cell, which also ceases division in G_2, becomes a giant cell and increases the DNA per cell (*33*). In the human this increase in DNA/trophoblast cell is accomplished mainly by cell fusion (cytotrophoblast→syncytiotrophoblast). In the mouse and the rat the increase in DNA/cell is nuclear. It takes place via endomitotic and endoreduplicative mechanisms (*34*) and not by fusion (*33*).

In utero blastocyst attachment (adhesion) is an initial step in implantation. It is important because it signals the start of those processes of differentiation which manifest themselves in the establishment of a definitive placenta. A highly regulated program of structural and functional differentiation is expressed during the interval defined by the attachment of blastocyst TE to receptive uterine epithelium and the apposition of TGC with elements of the maternal vascular system (*19,21,23*). These modifications in trophoblast function (Table 1) support the viability and the ordered patterns of embryonic growth and development. Progression of trophoblast through the remodeled substrate of the uterine decidual cells is assured by the secretion of elevated titers of progesterone by either the corpus luteum or the trophoblast cells. Since the mammalian species vary from each other at each step in the process of establishing the placenta, the studies reported here are limited mainly to rats and mice.

On the basis of preliminary studies, mainly in the

Table 1. Biochemical markers of trophoblast differentiation expressed during the peri-implantation period.

Class	Type
Deciduogenic stimulus	Initiates stromal cell differentiation (decidualization)
Endocrinological	Steroids (estrogen, progesterone, testosterone) Peptide hormones (hCG, rPL-1) Releasing factors
Immunological	Trophoblast *recognition antigens*
Invasiveness (controlled, self-limiting)	Cytoskeletal (adhesion→ migration) Matrix remodeling enzymes Cytolytic enzymes Phagocytosis

mouse (*21–23*), it was determined that of all the possibilities (Table 1), monitoring the synthesis and secretion of the steroid hormones and the peptide hormones would provide the best opportunities to identify markers unique to the differentiation of TE. The subjects of our initial studies were rat blastocysts and their trophoblast outgrowths cultured *in vitro*.

In Vitro Culture of Rat Blastocysts and Trophoblast Outgrowths

We have cultured rat blastocysts recovered from the uterus on day 4 of gestation (day of implantation). Blastocysts were cultured in groups of 10-20/35 mm culture dishes in 3 mL of NCTC-135 (Gibco) plus 10% heat-inactivated fetal calf-serum (FCS) and 1% streptomycin-penicillin. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Spent media was collected daily for analyses (*35*). Under these conditions the inner cell mass persists for only 60 to 72 hr, while the trophoblast tissue remains viable and continues to grow.

Steroidogenesis

Highly specific radioimmunoassays were used to determine progesterone, estradiol, and testosterone in the same sample of spent blastocyst media (*23,35*). Media without blastocysts provided control measurements.

Rat blastocysts secrete significant amounts of progesterone (0.1–0.5 pg/mL/blastocyst) during the initial phases of hatching (equivalent gestation day, EGD, 5) and outgrowth (EGD 6), increasing to 6 to 7 pg/mL/blastocyst (EGD 8–13) (Fig. 2). Progesterone then falls to a lower but still significantly elevated value (4.5 pg/mL/blastocyst) from EGD 14. The patterns of estradiol and testosterone by the rat blastocyst and trophoblast outgrowths can also be demonstrated (Fig. 2). The patterns of secretion of these latter two steroids have been too erratic to serve as reliable markers of trophoblast differentiation.

It is of interest to note that the production of progesterone by rat blastocyst trophoblast outgrowths occurs at the time during gestation (EGD 6–8) when plasma progesterone of the pregnant mother has already increased to values approaching 60 ng/mL (*23*). Maternal plasma progesterone plateaus at 90 to 120 ng/mL between EGD 10–12. In the presence of this great pool of maternal plasma progesterone, no role has yet to be identified for the progesterone (or estradiol and testosterone) contributed by the trophoblast cell. Possibly, trophoblast progesterone (as contrasted with the progesterone from the corpus luteum) plays a paracrine role in the ontogeny of decidual cell differentiation and/or an endocrine role in the trophoblast regulation of its estradiol receptor (*36*) or rat placental lactogen synthesis (*30*). If trophoblast steroidogenesis does have some regulatory role, it remains to be determined how critical this progesterone is to developmental programs and the relative sensitivity of trophoblast steroidogenesis to toxic insult compared to steroid hormone production by the corpus luteum.

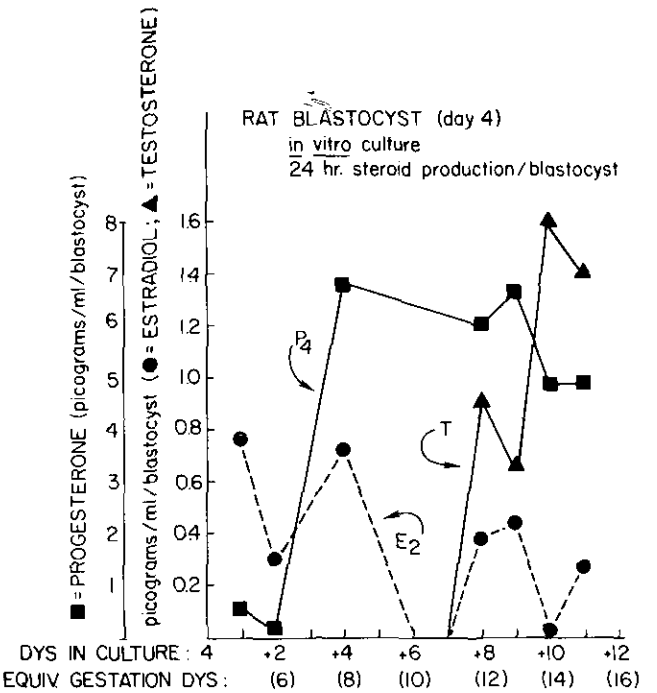

FIGURE 2. Steroid production by rat blastocyst outgrowths. Day 4 blastocysts (sperm positive = day 0, onset of pregnancy) were recovered from the uterus and cultured in groups of 10–15 in 3 mL NCTC-135 plus 10% fetal calf serum in 35 mm plastic dishes. Medium was changed daily. Hormones were assayed by specific radioimmunoassay of spent medium. Equivalent gestation day = the age of the blastocyst in culture equivalent to the age it would have been if left *in utero* (*23*).

Trophoblast (Placental) Peptide Hormones

At midpregnancy in the rat (EGD 11), the control of luteal progesterone secretion shifts from the pituitary to the placenta (*37*). The maintenance of the second half of pregnancy is dependent on the luteotropic and lactogenic activity of placental secretions. A hormone of

placental origin with both activities was identified in the serum of midpregnancy rats (*38*). Subsequently it has been shown that the hormone from placental extracts (lactogen) demonstrates two peaks of activity. A placental hormone with luteotropic activity was first detected in placental extracts at EGD 11. A second peak observed on EGD 16 to 17 was lactogenic and essentially devoid of luteotropic activity. It is now recognized that these two peaks of activity represent the production of two distinct peptide hormones synthesized by the placenta of both mice (*39*) and rats (*30*).

Since the presence of placental peptide hormone(s) prior to EGD 11 had not previously been reported, it became of interest to determine if trophoblast outgrowths of day 4 rat blastocysts would differentiate in culture to express placental lactogen (4 + 6–7 days). The presence of small but detectable amounts of placental lactogen in the spent medium of rat blastocyst cultures at EGD 6 to 8 was surprising but rational. The production of placental lactogen by blastocyst outgrowths peaks on EGD 9 and then decreases with time in culture (Fig. 3). It was not possible using the radioreceptor assay (*40*) and other tests available at that time to distinguish whether this secretory product represented only the early luteotropic activity or was a mixture of both activity peaks. Nor could it be determined which particular cell type was the source of secretion.

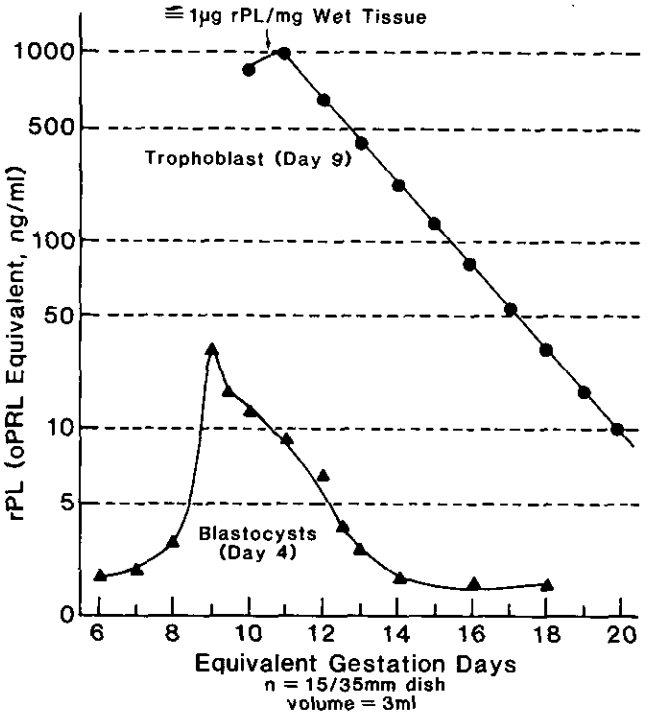

FIGURE 3. Rat placental lactogen (rPL) assayed by a radioreceptor assay (*40*) and expressed in terms of ovine prolactin (oPRL) equivalents. Blastocysts were recovered from uteri on day 4 and cultured in groups of 15/35 mm culture dishes in 3 mL NCTC-135 medium. rPL was measured in 24 hr spent medium. Trophoblasts (mural, polar) were dissected from conceptuses on EGD 9. Trophoblasts from 15 conceptuses were cultured *in vitro* and rPL was assayed on 24 hr spent medium.

To help resolve the issue of specific cell site of placental lactogen secretion and improve the utility of this model as a monitor for the action of xenobiotic agents, trophoblast cells were dissected from rat embryos at day 10 of pregnancy. The majority of separated cells was composed of primary and secondary trophoblast giant cells, but included representatives of polar trophectoderm and ectoplacental cone cells and their derivatives. After harvest the cells were dissociated in Dulbecco's PBS lacking Ca^{2+} containing trypsin (0.5%)-EDTA (0.2%) plus 200 units DNAse before culture (*26,27*). The same radioreceptor assay used to study placental lactogen (PL) production by rat blastocysts was used to estimate hormone production by trophoblast cells. Under these conditions the peak production by day 10 trophoblast cells occurs at EGD 11. The peak value (Fig. 3) represents secretion of a PL hormone of almost 300 ng/day/trophoblast; a value greater than that expected on the basis of plasma titers.

The loss of secretory capability in either culture situation (Fig. 3) cannot be explained at this time, but the possibility that this loss is due to culture conditions must be considered. Equally unexplained are the differences between the secretory physiology of blastocyst outgrowths and trophoblast cells. Not only were the peaks of PL activity expressed at different times, but trophoblast cells have significantly greater secretory capacity than blastocyst outgrowths at equivalent developmental ages (approximately 100-fold at EGD 11). These differences may be related to the gestation age at which the cell or its progenitor was placed in culture. However, the data argue in favor of some regulatory factor(s) operable *in vivo* between EGD 4 and 10 that may contribute to the capacity of trophoblast cells to secrete PL in sufficient titers to maintain luteal steroidogenesis by EGD 11 when pituitary factors have been withdrawn. If such factors exist, they have not yet been identified, but their existence suggests an important control point that could be perturbated by toxic agents.

These data suggested further two modifications in the research plan. The first was to focus attention on the secretion of the placental hormones because of the importance of their putative actions and their apparently specific relationship to the trophoblast cell. Emphasis was placed on PL secretion and its specific secretory cell, rather than continuing analysis of progesterone. The decision was based on observations that progesterone synthesis was not exclusive to trophoblast and no leads to the function of trophoblast progesterone exist. The second modification was to enhance the study of PL by improving the methods used to separate the suspected endocrine-competent trophoblast giant cells from their undifferentiated trophectoderm and ectoplacental cone percursors.

Trophoblast Giant Cells

Differentiation of the fetal placenta is essential to embryonic development in mammals. In rodents the

trophoblast giant cells are an integral component of the fetal placenta. *In vivo* and *in vitro* primary and secondary TGC derive from mural and polar blastocyst TE, respectively. Cells of the ectoplacental cone (EPC) derive from polar TE (*33*) and serve as precursors for additional secondary TGC. TGC cease to divide as a primary step in their differentiation from TE or EPC. A proportion (15–25%) of the TE cells become binucleate or multinucleate. Morphologically TGC are nondividing, polytene giant cells. The c̄ number (haploid DNA) in TGC increases from 2 to 4 to as much as 1024 as a result of endoreduplication (*41*). In human trophoblast cells only a fraction (estimated at no more than 40%) become endoreduplicative (*42*). DNA content of endocrine-competent human trophoblast cells increases by fusion or syncytialization of cytotrophoblasts, rather than by endoreduplication. Functionally, TGC express secretory programs for proteolytic enzymes (proteases, collagenases, elastases), steroid hormones (progesterone, testosterone, estrogen), and at midgestation a peptide hormone(s), placental lactogen(s) (Table 1). Rodent trophoblast, unlike human, does not appear to produce a chorionic type of gonadotropin.

Placental Hormones

Placental peptide hormones display characteristics similar to pituitary prolactin (PRL) and growth hormone (GH). For this reason they have been termed placental lactogens (PL) or chorionic somatomammotropins (*43,44*). PL is the dominant trophic hormone affecting fetal development during the latter half of pregnancy (*45*). PLs may directly regulate the development of fetal tissues, as demonstrated by the stimulatory actions of ovine PL on amino acid transport and ornithine decarboxylase activity in fetal tissues (*46,47*), or indirectly, via alterations in maternal protein, carbohydrate, and lipid metabolism (*48*).

The biology and biochemistry of PLs vary among species, but all appear to share a common feature. They are secretory products of placental giant cells. In the human, PL has been localized to the syncytiotrophoblast (*49*), in sheep, to the trophoblast binucleate cells (*50–52*) and in the rat, to the trophoblast giant cells (*30,35*). These PL secretory cells differentiate from readily identifiable precursor cell populations: human, cytotrophoblast (*53*), sheep, uninucleate cells (*54*); mouse, trophectoderm and ectoplacental cone cells (*33,55*). Trophoblast giant cell differentiation has not been rigorously studied. The most thorough investigations have been done in the rodent, primarily the mouse, in which Rossant and her co-workers (*55–57*) have analyzed the transformation of ectoplacental cone cells to differentiated trophoblast giant cells.

The rat and mouse produce two types of PLs (*30, 58,59*). These hormones can be distinguished biochemically, immunologically, and by their temporal appearance during pregnancy. The early form (PL-1) is present during midpregnancy (days 9–11 in the mouse; days 10–12 in the rat), and the late form (PL-2) predominates during the latter half of pregnancy. PL-1 has a larger molecular weight and is a more acidic protein than PL-2 (*30*). Both are active in radioreceptor assays and bioassays for lactogenic hormones (*30*); however, neither is active in a growth hormone radioreceptor assay. Serum PL–2 levels have been measured in the mouse throughout pregnancy and during the periparturitional period (*60,61*). The ovaries have an inhibitory influence on serum PL-2 levels, while the fetus has a trophic influence (*62*). Distinct genetic differences in the serum profiles of PL-2 have been reported (*60*). In addition, serum PL-2 levels appear to be elevated in genetically dwarf mice, implicating a role for the anterior pituitary in the regulation of serum PL-2 levels (*63*). That PL was a product of the trophoblast rather than other components of the placenta was first demonstrated by the previously discussed studies of PL production by blastocyst trophoblast outgrowth. In order to show more directly that TGC, *per se*, were the site of placental hormone production, the cells were dissected as essentially homogeneous single cell-type populations from rat conceptuses at gestation days 9, 10, 11, or 12 (*27,28,30*).

For each conceptus the TGC were separated into mural and polar giant cells. A third group of cells, central TGC, was established to avoid mixing of the mural and polar samples at the time of dissection (Fig. 4). The methods for dissection and culture have been described previously (*27,28,30*). The rPL profile of spent culture media was characterized by gel filtration chromatography and SDS-gel electrophoresis. The relative activity of media fractions was determined by the prolactin radioreceptor assay (*40*).

In utero, mural TE are the first to cease cell division. They begin to differentiate into primary trophoblast

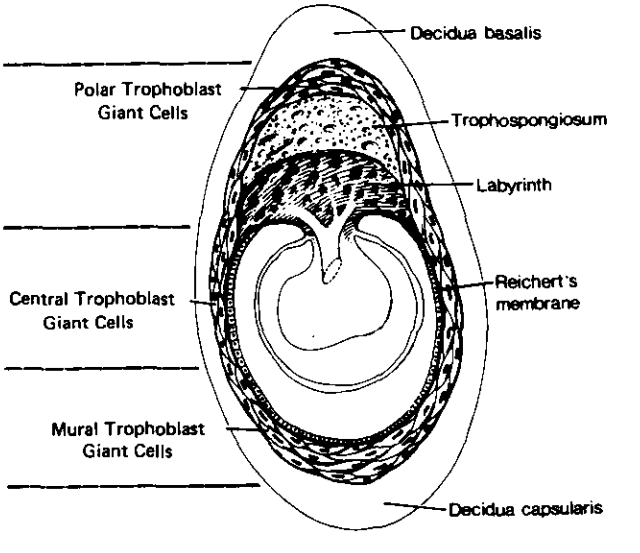

FIGURE 4. Schematic diagram of a midpregnant rat conceptus. The dashed lines indicate the regional limits of dissection for the harvest of the individual groups of trophoblast giant cells (*30*). This diagram is based on information presented by Davies and Glasser (*65*).

giant cells as a consequence of their attachment to the receptive uterine epithelial cells of the antimesometrial uterus. Polar trophectoderm cells remain diploid and continue to proliferate as the embryo elongates during formation of the egg cylinder (*33,64*). Approximately 48 to 60 hr later, as proliferative pressure moves polar TE cells away from the ICM and they begin to attach to the mesometrial epithelial cells, the polar TE cease to divide. They begin to differentiate into secondary trophoblast giant cells. The existence of two placental lactogens with different peaks of activity and two apparently related different TGC suggested that 1° TGC might be the source of the early PL (rPL-1) and that the later rPL-2 was a secretory product of the 2° TGC. If this were proven then there might possibly be two separate targets, each with its unique secretory product and each with possibly a different sensitivity to xenobiotic agents. To examine the relationship between TGC types and rPL-1 and rPL-2, the different populations of TGC were dissected free from the rat conceptus on either gestation day 9, 10, 11 or 12. The spent media of *in vitro* cultures was monitored for the qualitative secretion of the placental lactogens.

Analysis of 24 hr spent medium from mural, polar, and central TGC of day 11 rat conceptuses (Fig. 5) showed that the ratio of PL-1 to PL-2 released by TGC was similar, regardless of their location in the conceptus. On any one developmental day, the only regional differences are quantitative rather than qualitative. The output of total PL and of testosterone by mural TGC was almost three times greater than polar TG cells (*30*). Mural TGC output of progesterone was approximately eightfold greater than polar TGC.

The type of PL released by TGC proved to be correlated with the day of development. Thus, TGC placed in culture on EGD 10 release predominantly PL-1, while TGC cells isolated on EGD 11 release predominantly PL-2. This transition is reflected in plasma patterns. This switch from PL-1 to PL-2 which occurs *in vivo* cannot be reproduced under the *in vitro* conditions currently in use (Fig. 6).

Differences in the quantity of hormone production could be an effect of local environment, i.e., polar TGC interface with maternal decidua basalis and the mesenchymally induced trophospongiosum of the chorioallantoic placenta, while mural TGC are situated between the decidua capsularis and the parietal endoderm (*65*). Production could be mediated by signal differences from the different environments and/or their interpretation by the respective TGC. This suggests that residual effects of xenobiotic agents in undifferentiated uterine stromal cells might exercise some influence on trophoblast endocrine function when these cells later decidualize. The absence of qualitative regional effects confirms that all TGC are essentially similar; functional differentiation of an individual TGC results in the sequential expression of PL-1 and PL-2 by the same cell. Thus there is only one, not two, trophoblast targets and the response of that target to toxic exposure is relevantly influenced by the developmental stage at which the trophoblast cell is placed at risk.

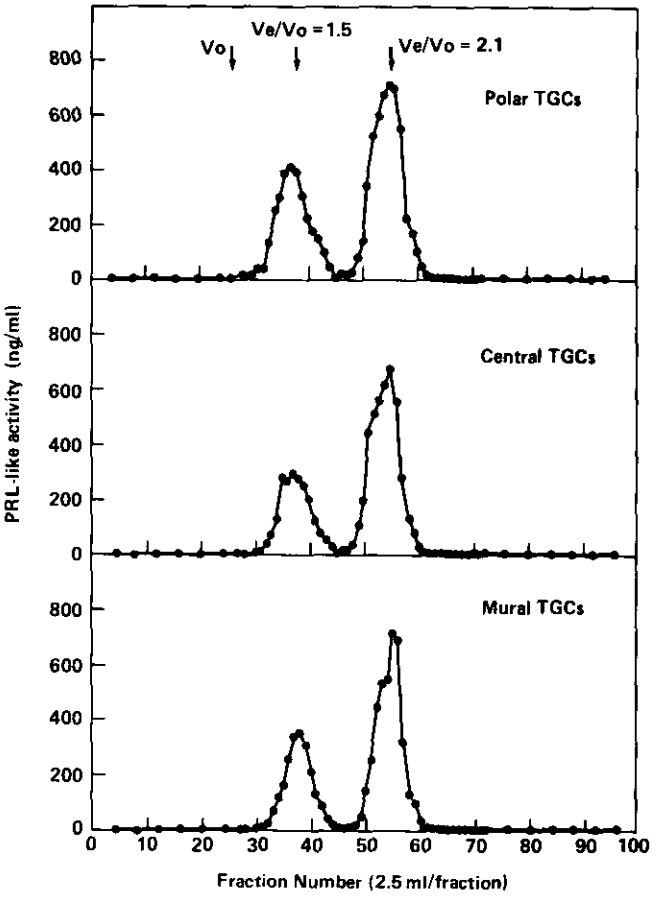

FIGURE 5. Elution profiles of spent media from day-11 trophoblast giant cell cultures. The trophoblast giant cells were isolated from polar, central, and mural regions of the conceptus (Fig. 4) and incubated for 24 hr. These elution profiles were obtained by gel filtration of samples on Sephadex G-100. The rPL activity of the eluates, expressed in terms of oPRL equivalents, was assayed by the PRL radioreceptor assay (*40*).

TGC Cytoskeleton

Because morphological and functional differentiation have been linked to alterations in the expression of cytoskeletal proteins, we have analyzed microtubules and intermediate filaments in endocrine-competent TGC (*66*) in order to identify additional markers.

Microtubules. Tubulin and polymerized microtubules have been identified in TGC, but the microtubule organizing center (MTOC) has yet to be described. When TGC (day 10 + 48 hr *in vitro*) were cultured in the presence of colcemid (0.06 μg/mL), their microtubules were completely disrupted by 4 hr. Preliminary studies showed a 75% decrease in the production of rPL over the next 24 hr. Indirect immunofluorescent staining revealed multiple punctate sites interacting qualitatively with human autoantibodies to centrosomes, with antibodies to purified tubulin, but not kinetochore

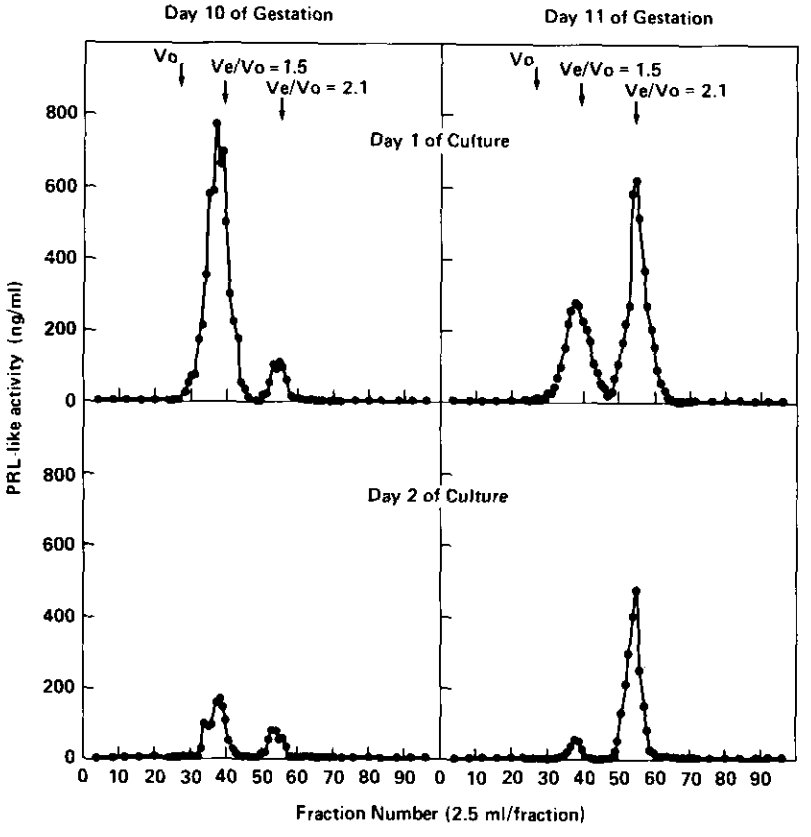

FIGURE 6. *Elution profiles of spent media from day-10 and day-11 trophoblast giant cell cultures. The trophoblast giant cells were dissected* from the mural region of the conceptus (Fig. 4) and cultured for 48 hr (upper panel: 0–24 hr: lower panel: 24–48 hr). Media was harvested at 24 hr intervals and chromatographed on Sephadex G-100. The rPL activity of the eluates was assayed using the PRL radioreceptor assay (*40*).

antibodies. These sites occur throughout the cell; they were located in the perinuclear position where centrioles, which conventionally serve as MTOCs, are found. Removal of colcemid resulted in the reassembly the cytoplasmic microtubule complex coincident with a 60% recovery in rPL production during the next 24 hr. Under reassembly conditions, tubulin repolymerizes at each of the multiple cytoplasmic sites. Thus TGC from TE are unlike most other cells. They possess a decentralized system for microtubule renucleation occurring at multiple sites throughout the cytoplasm. Microtubules may represent a sensitive and ubiquitous target for xenobiotic agents. Disruption in microtubule assembly could have far-reaching effects on cell function.

Intermediate Filaments. In rodents, TE are the first embryonic cells to exhibit intermediate filament proteins (*66*). Mouse preimplantation blastocyst TE displayed two intermediate filament proteins (54 Kd, 46 Kd) identified as cytokeratins. In contrast, midgestation TGC displayed not only the 54 Kd and 46 Kd intermediate filament proteins, which were major components of their cytokeratin profiles, but 52 Kd, 45 Kd, 43 Kd and 40 Kd species. Of these additional keratins, the 52 Kd and 40 Kd were more prominent (*28*).

Analysis by two-dimensional polyacrilamide gel electrophoresis, partial proteolytic digests, coupled with immunoprobing of Western blots and with indirect immunofluorescence staining of TGC, showed that the constitutive intermediate filament proteins were cytokeratins and that this profile is shared by simple epithelial cell types (vs. stratified squamous) represented by liver, lung, pancreatic islets, parietal endoderm, and uterus. Detectable amounts of the 52 and 40 Kd keratins are found in day 9 TGC; the entire complex is present in day 12 TGC. During this transition period, the individual cytokeratin species elaborate isoelectric variants. Relative to total protein, intermediate filament protein increases over the same period. This increase in intermediate filament protein is reflected by indirect immunofluorescence staining. Indirect immunofluorescence arrays display an increase in the profusion and complexity of the cytokeratin filament network in day 11 and 12 TGC compared to day 9 TGC and blastocyst outgrowths. While no specific function has yet been assigned to any of the expressed proteins of the various cytokeratin gene families, these additional landmarks may eventually come to be of significance in monitoring the functional and morphological differentiation of specialized cells (*67*), i.e., EPC cells and other stem or precursor cell populations.

In vitro analysis of the morphological and functional differentiation of blastocyst outgrowths and isolated

TGC have been effective in terms of identifying valid biological markers of processes essential to establishment of the fetal placenta. While other markers (Table 1) have not been discounted, these experiments have recognized placental lactogens as the unique candidates. Identification of the differentiated trophoblast cell (TE → TGC) as the single cellular source of these hormones is also an important observation. Further studies to assess the risk of a presumptively pregnant female can now focus on the development of the trophoblast and its expressed secretions.

The expression of PL-1 during the period of organogenesis may be only coincidence, but the possibility that PL-1 and organogenesis may be more closely related, possibly via a reciprocal relationship with insulin growth factors (*68*), suggests that in spite of the potential for repair (endoreduplication of DNA, transformation of ectoplacental cone cells), early exposure of an individual (from the determination of TE at compaction through the initial turn-on of the PL genes) may produce consequences more reaching than if exposure occurred as TGC differentiation (and organogenesis) was terminating (relative to the onset of the period of fetal growth).

These data give rise to a number of questions that should be resolved before research strategies designed to evaluate the use of these markers as health monitors can be used. The implied importance of DNA adducts (*69*) suggests that the relationship of increased DNA per trophoblast cell to the expression of PL hormones and other developmental programs may be critical in the response of the trophoblast cell to xenobiotic agents. This is a difficult issue to resolve because TGC are almost terminally differentiated at the time of harvest, and neither TGC nor the outgrowths provide sufficient biological material for analysis.

Ectoplacental Cone Cells

Ectoplacental cone (EPC) cells arise from polar TE. They are diploid and proliferative. Because of their numbers and because they endoreduplicate and differentiate in culture, they represent a model that could resolve some of the questions posed in previous experiments. Mouse EPC cells (Fig. 7) transplanted ectopically or cultured *in vitro* transform into giant cells. Rossant and her colleagues (*55–57*) have analyzed the transformation of EPC cells to differentiated trophoblast giant cells (TGC). Mouse EPC cells transplanted ectopically or cultured *in vitro* transform into giant cells (*57*). The transformed giant cells endoreduplicate their nuclear DNA; they are polyploid and synthesize proteins that are characteristic of TGC *vis-à-vis* EPC (*57*). The presence of the inner cell mass adjacent to the EPC cells is believed to maintain them in a proliferative state and inhibit the transformation to TGC (*56*). As gestation progresses, some EPC cells are pushed further from the inner cell mass or its derivatives and transform into TGC. Thus, EPC cells serve as a reservoir of precursor cells that contribute to the growth and expansion of trophoblast during the second-half of pregnancy.

While it has been demonstrated that TGC derived from TE produce steroid and peptide hormones (*23*), a question arose as to whether all giant cell derivatives of trophectodermal origin acquired endocrine competence. Thus, if EPC are, in fact, a precursor reservoir for giant cells, can these morphological and biochemical transformants also subserve the critical functional role of the 1° and 2° trophoblast giant cells, the production of PL?

Ectoplacental cone cells can be harvested (Fig. 7) from rat conceptuses on days 9 to 12 of pregnancy, cultured *in vitro* and observed to change. A proportion become bi- or multinucleate (Fig. 8). The majority of cells become giant, polyploid, and endoreduplicate during the culture period (Figs. 9 and 10). Endoreduplication of rat EPC cells *in vitro* mimics the patterns observed when mouse EPC (*57*) were placed in culture. Ectoplacental cone cells obtained from days 9 to 12 of gestation all show similar morphological and constitutive biochemical changes during their transition to TGC. Functionally, EPC cells from day 12 conceptuses become giant cells that are capable of producing PL-2 (Figs. 11 and 12). For a number of technical reasons, including the lack of an accurate, sensitive radioimmunoassay, we have not yet been able to unequivocally identify the secretory product elaborated by cultures of day 10 EPC cells. Of potential far-reaching importance has been the observation (Munir and Glasser, in preparation) that no PL can be detected by the PRL radioreceptor assay in either cultures of day 10 or day 12 EPC until the second round of endoreduplication has been completed (Fig. 13). The unavailability of specific probes does not permit us to determine if PL is a product expressed only by trophoblast cells of $8n$ or greater, or whether this event represents some critical change in the microenvironment of the culture.

Pre- and postimplantation precursor cells are diploid and dividing (*56,64*). When these cells lose contact with the inner cell mass and its derivatives (*54,56*), they lose their ability to divide and become giant with nuclear DNA contents greater than 4c. The mechanism of nuclear DNA replication in the rodent TGC is via endoreduplication (*34,41,70,71*). Using available methodology, Sherman et al. (*70*) concluded that the accumulation of DNA in the TGC was not due to a disproportionate amplification of satellite sequences. Constancy in the ratio of satellite to main band DNA implied that all genes are replicated to the same extent; specific genes were not being amplified, nor was satellite DNA being underreplicated.

Very little information has been developed since the advent of recombinant DNA methodology regarding the organization of the TGC genome or gene expression of genomic DNA which can increase from 2 to 1024c. Extensive endoreduplication during the course of normal differentiation make TE, EPC, and TGC unique mammalian cells and thereby unique models to study gene expression. Analysis of interspersed repetitive DNA

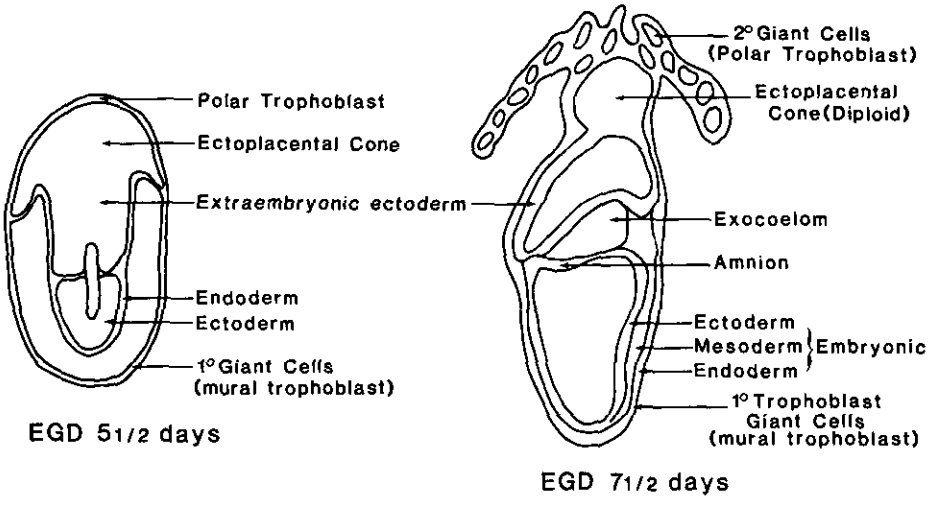

FIGURE 7. Schematic representation of the mouse embryo at 5.5 and 7.5 equivalent gestation days. Adapted from Johnson and Rossant (*57*). Dissection of day 10, 11, and 12 EGD rat ectoplacental cone cells was guided by the diagram of the 7.5 day mouse embryo.

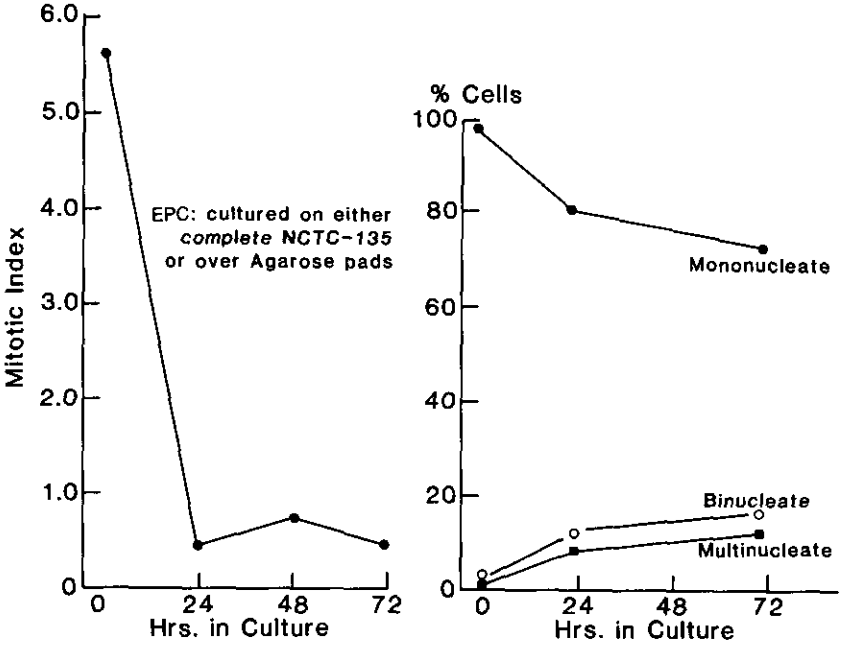

FIGURE 8. The mitotic index of ectoplacental cone cells isolated from EGD 11 rat conceptuses. EPC cells were cultured in plastic culture dishes in either NCTC-109, or unattached over agarose pads. The number of nuclei per cell was plotted over the same period and expressed as a fraction of the entire study population.

sequence arrangement correlated with the intricate program of differential biochemical expression of endoreduplicated TGC DNA offers a unique opportunity to study the cellular and molecular basis of differentiation.

Regulation of Trophoblast Hormone Synthesis

Relative to the successful regulation of early postimplantation interactions between the embryo and the decidualizing stroma, what advantage is nuclear DNA endoreduplication (or syncytialization) to giant cell differentiation? Even if modern recombinant DNA technology confirms that PL expression is a correlate of replication of the entire trophoblast genome rather than amplication (*41,70*), we are left with relevant unanswered questions. We still must seek the signals that initiate PL-1 synthesis in the enlarging genome, that direct the sequence rearrangements obligatory for the PL-1 to PL-2 transition, and that initiate PL-2 synthesis. These questions are applicable to analysis of human

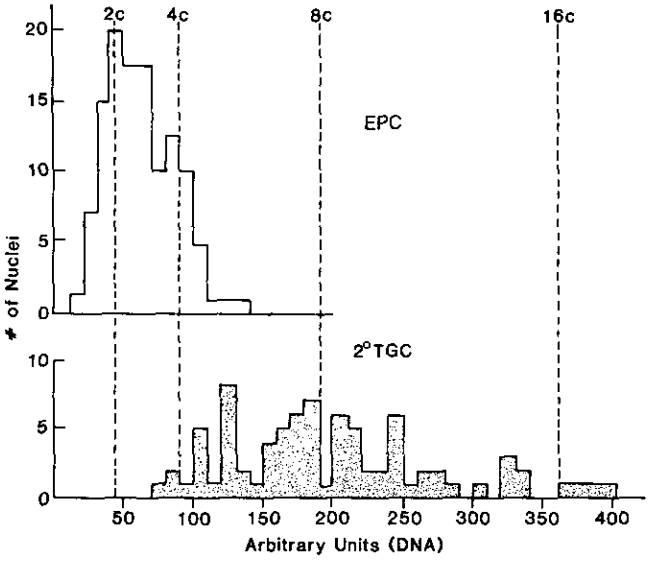

FIGURE 9. Histogram of cytophotometric analysis of Feulgen-stained DNA per nucleus in ectoplacental cone (epc) and secondary trophoblast giant (TGC) cells freshly dissected on EDG 11. The values for control, 2c, 4c, and 8c amounts of DNA were obtained from control liver samples.

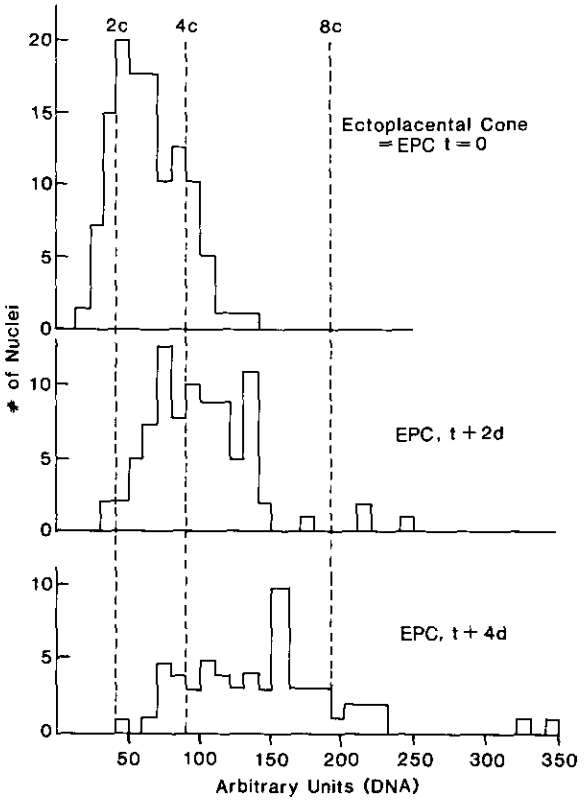

FIGURE 10. Histogram of cytophotometric analyses of Feulgen-stained DNA per nucleus in freshly dissected EGD 11 ($t = 0$) rat ectoplacental cone cells. Cells were placed into culture and the DNA per cell analyzed after +2d and +4d. The endoreduplicative shift in DNA/nucleus correlates with increases in nuclear and cytoplasmic volumes in this nondividing cell population.

trophoblast, for they identify regulatory foci coincident to differentiation of cytotrophoblast at which point xenobiotic agents could interrupt or redirect normal development.

There is not much more useful data known about the biochemical or physiological regulation of PL synthesis and secretion. This situation is not due to lack of work but rather due to the types of experiments conducted. Only recently has research veered from the analysis of animals, whole tissues, or complex populations of heterologous placental cells. The secretion of PL-2 is influenced by the presence of the fetus (stimulatory) and ovarian and adrenal steroids (inhibitory) (*72*). When rat fetuses are removed from the placenta (fetectomy) (*65*) at day 14, serum PL-2 levels are markedly depressed. The stimulatory role of the fetus was confirmed in a report that established the requirement for progesterone in maintaining PL-2 secretion and suggested that PL-1 terminates PRL surges at midpregnancy (*73*). Although the absence of progesterone may arrest PL-2 secretion, its effect may depend on the presence of a particular target cell. Of interest is our observation that progesterone, 1×10^{-6}M, inhibited (75–80%) PL-2 production by terminally giant cells differentiating from day 12 EPC, but did not influence PL-2 production by differentiated TGC (Fig. 14) (Soares and Glasser; Munir and Glasser, unpublished observations). Identification of the mechanism of progesterone regulation and the basis for the differential response of day 11 vs. 12 EPC depends on molecular probes that are not yet available.

Extrapolation to Human Trophoblast

The experiments with *in vitro* models of trophoblast cells have proven productive. These studies have identified the importance of the postmitotic differentiation of a particular class of epithelial cells, i.e., the trophoblast cell and its derivatives, in the control of normal mammalian development. Markers of structural and functional events critical to this problem of differentiation have also been reported (*28–30*). It is appropriate to ask whether these results can be extrapolated to the analysis of early human development. Second, would such extrapolation improve our understanding of the early communication between the human embryo and the maternal host to the point of identifying markers to monitor exposure to xenobiotic agents?

It should be recognized that while the rodent trophoblast experiments have enlarged our understanding of certain aspects of postimplantation biology, they have not yielded markers that could reliably signal the status of the trophoblast during the high risk pre- and peri-implantation period in either mouse, rat, or human. These species represent animals in which the residence time of the free blastocyst *in utero* is rather short. This time is in contrast to certain farm animals (sheep, pig, cow) in which the uterine residence time of the free blastocyst is long (> 12 days) and the blastocysts synthesize and secrete gonadal signals which mark the immediate preimplantation period (*74*). Suggestions that

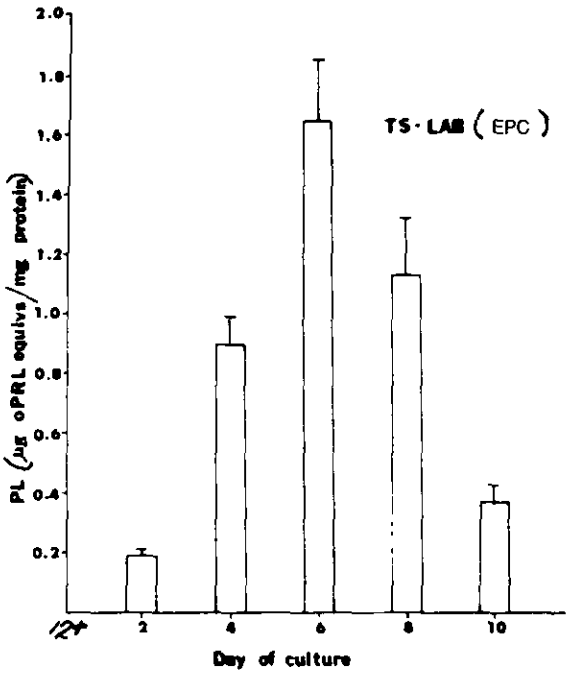

FIGURE 11. *rPL production by ectoplacental cone cells dissected* from EGD 12 rat conceptuses and cultured *in vitro* in NCTC 109. rPL, expressed as o-PRL equivalents, was assayed in spent medium by radioreceptor assay.

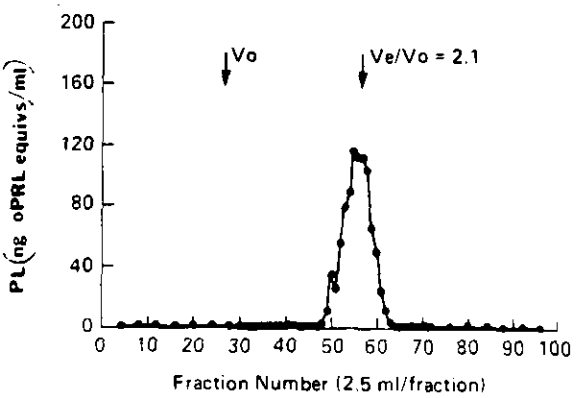

FIGURE 12. Gel chromatographic analysis (Sephadex G-100) of spent medium from rat ectoplacental cells (EGD 12) cultured *in vitro*. The Ve/Vo ratio of 2.1 established the active fraction as rPL-2 (Fig. 5).

the short *in utero* residence blastocysts also generate steroid implantation signals (*75*) have not been confirmed (*74*).

A practical marker of postimplantation trophoblast development in the human does exist. The radioimmunoassay of chorionic gonadotropin (hCG) provides useful general information. In an effort to identify additional markers that provide more precise and sensitive indices of early human gestation, investigators have called on contributions from immunochemistry, biophysics, and bioengineering. We would ask if our studies in cell biology also contribute to these efforts in any

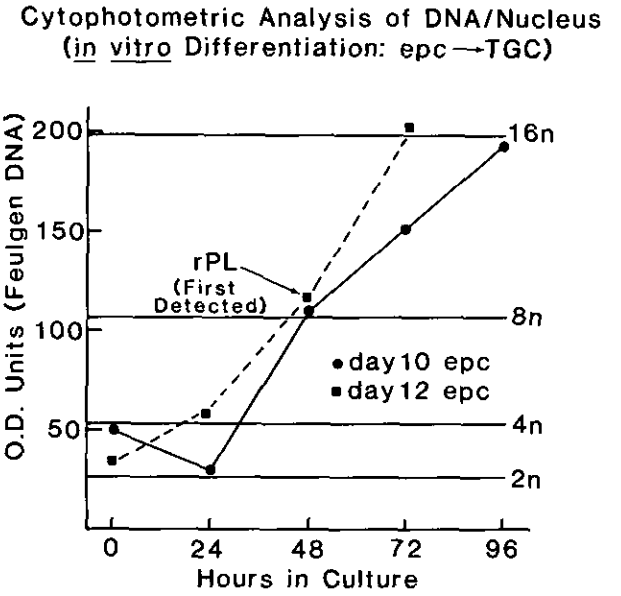

FIGURE 13. Comparison of DNA/nucleus in cultured rat EGD 10 and EGD 12 ectoplacental cone cells as a function of time in culture. The time when rPL is first detected by oPRL-RRA is noted. Three separate experiments are represented by these data.

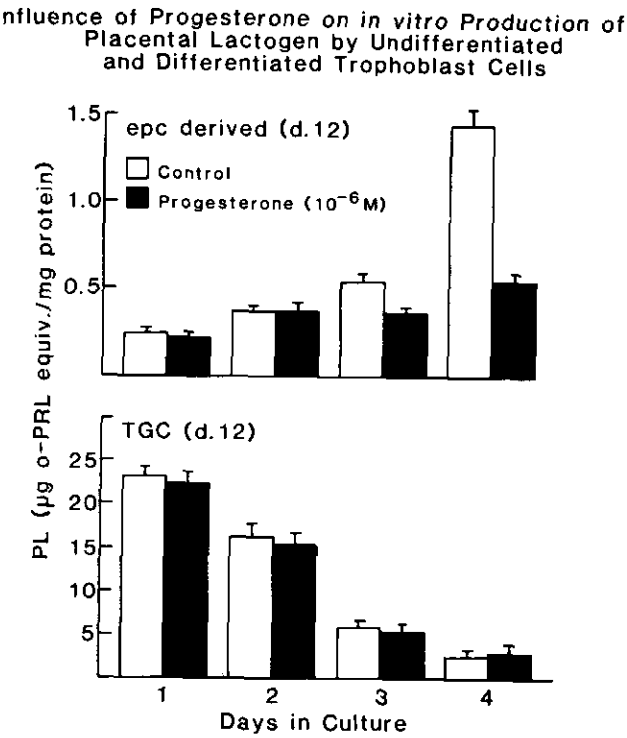

FIGURE 14. The *in vitro* production of rPL by undifferentiated trophoblast cells (EGD 12 ectoplacental cone cells) is down-regulated by the inclusion of progesterone in the culture medium (upper panel). The same culture conditions have no influence on the relative rPL production by terminally differentiated trophoblast cells (EGD 12 trophoblast giant cells; lower panel).

way; either in reevaluating the usefulness of existing markers, i.e., hCG, or providing directions to the discovery of new ones.

Human Chorionic Gonadotropin as a Marker

Human chorionic gonadotropin (hCG) is the confirmed practical marker for development of human trophoblast cells. hCG is a glycosylated (30%) peptide hormone (M.W. 36,700). The hormone is composed of two nonidentical subunits that are noncovalently linked. The α subunit is similar to the α subunits in FSH, LH, and TSH, differing from the others only in its carbohydrate composition. The β subunit of hCG is responsible mainly for conferring biological activity. β-hCG differs from β subunits of FSH, LH, and TSH both in its amino acid and its carbohydrate composition. Both subunits are required for biological activity, as neither subunit alone binds to hCG receptors. Specific antibodies have been raised against the α and β subunits and the intact hormone. Radioimmunoassays of increasing specificity, precision, and accuracy have been used to measure plasma levels of hCG (*76,77*). The major recognized action of hCG is its role in the regulation of steriodogenesis in the corpus luteum. Thus, hCG serves a pivotal function in the maintenance of pregnancy. hCG has also been implicated in the regulation of steriodogenesis in the fetal testis and possibly the fetal adrenal. Its role in the regulation of placental steriodogenesis is controversial.

The widespread use of the radioimmunoassay (RIA) for hCG derives from the ready availability of specific antibodies at reasonable cost, the relative ease of the assay, the extensive background on interpretation of plasma titers, plus the absence of and the need for more specific markers. The RIA for hCG has received general acceptance as an indicator of pregnancy and some of the pathophysiological events that may be associated with it. The biological rationale for the hCG-RIA is that a rise in plasma hCG reflects the presence of a functional tropohblast and thereby verifies the existence of a presumed pregnancy (*77*). A decline in the rising hCG titers signals the interruption of pregnancy but does not identify the cause or the site of interruption. All this is certified by an extensive literature. This has made way for a general approval of this method, so it now has been proposed to extend hCG-RIA to monitor the sequelae of environmental exposure of populations that include reproductively competent women (National Research Council Committee on Biomarkers in Reproductive and Developmental Toxicology).

Human Trophoblast Differentiation and hCG Synthesis

Syncytialization (fusion of cytotrophoblast cells) characterizes the development of the definitive human hemochorial placenta (*78*). *In situ* hybridization has localized the α subunit mRNA to cytotrophoblast, syncytiotrophoblast, and the intermediate forms that represent the transition between the two definitive cell types (Table 2). However, the β subunit mRNA can be localized only to the intermediate form and syncytiotrophoblast (*79*). This confirms the immunocytochemical data that localizes the intact hCG molecule in the syncytiotrophoblast but not the cytotrophoblast (*80,81*). This strongly suggests that trophoblast differentiation must be in progress before the β subunit becomes available for dimerization with the α subunit and the rapid secretion of the intact hCG molecule. It is notable that increase in DNA content per trophoblast cell (regardless of the mechanism by which this is accomplished) is a correlate of both hCG secretion by syncytiotrophoblast and PL secretion by rat giant cells (Table 2). Since both synthesis and secretion of mature placental peptide hormone (hCG, PL) and the increase in DNA/cell occur during differentiation of trophoblast cells, it is reasonable to inquire as to the nature and importance of these relationships. If alterations in DNA are integral to the actions of a xenobiotic agent (*69*), changes such as those occurring in trophoblast cells could dictate limits of sensitivity and response.

Recombinant DNA technology has been used, to an extent, to study the regulation of placental peptide hormones. However, recombinant DNA technology has not rigorously addressed the questions of gene expression relative to the nature of genome replication or the relationship, if any, between the first hormone (hCG, rPL-1) and second hormones (hPL, rPL-2) expressed by the differentiating trophoblast cell.

An *In Vitro* Model to Study Human Trophoblast Differentiation

There is an experimental model that can be adapted to study the differentiation of the human trophoblast cell. Any variety of methods, including recombinant DNA technology, can be used to analyze the transition from cytotrophoblast to syncytiotrophoblast. The system would also be excellent for investigating the consequences of introducing xenobiotic agents and different steps in trophoblast differentiation. Friedman and Skehan (*42*) described a directory of morphological and functional properties that characterized the transition of cytotrophoblastlike (CTL) cells of the BeWo choriocarcinoma cell line to syncytiotrophoblastlike (STL) cells. Cytologically, CTL and STL were identical to their counterparts *in utero*.

BeWo CTL cells constitute 96 to 99% of the cell types of the stored cell line (Table 3). Cultured in the presence of subthreshold levels of methotrexate, the CTL cells will differentiate. At the end of a 96-hr culture period, over 90% of the cells express STL morphology and function. When methotrexate is removed, the STL cells shuttle backwards to become CTL. It should be noted that although the presence of methotrexate suppressed DNA synthesis, there is still a 66% increase in DNA/

Table 2. Relationship between expression of early placental peptide hormone (hCG, rPL) and DNA/cell during differentiation of human and rat trophoblast.

		Cytotrophoblast	Intermediate	Syncytiotrophoblast
Human	α-hCG mRNA	+	+	+
	β-hCG mRNA	−	+	+
	α-hCG synthesis	+	+	+
	β-hCG synthesis	−	+	+
	Intact hCG	−	+	+
	DNA (pg/cell)	20		33

Rat ectoplacental cone cells	EPC		EPC giant cell derivatives				
DNA[c]	2	2/4	4	4/8	8	8/16	16
rPL	−	−	−	−	+	+/++	+++

Table 3. Characteristics of an *in vitro* experimental model expressed during differentiation of BeWO choriocarcinoma cell-line *cytotrophoblastlike (CTL) to syncytiotrophoblastlike* (STL) cells.[a]

CTL[b] 96–99%	(+) → 1 μM Methotrexate ← (−)	STL[b] 90+%
Proliferative		Nondividing
Mononucleate		Multinucleate
Moderate size		Giant (increase 3–10)
Mononucleate		Multinucleate
Cytoplasmic filaments		(↓)
Ruffles	*(Transitional or*	(↓)
Desmosomes	intermediate types	(↓)
Fascia adherens *junction*	were not studied)	(↓)
Microvilli		(↑)
Golgi		(↑)
Vesicles		(↑)
Granules		(↑)
Smooth endoplasmic *reticulum, dilated*		(↑)
DNA: 20 pg/cell		33 pg/cell (+66%)[c]
Protein: 169 pg/cell		1163 pg/cell (+700%)
hCG (+)		(+++)

[a] Friedman and Skehan (*42*).
[b] CTL and STL *similar to in utero cytotrophoblast and syncytiotrophoblast.*
[c] 40% STL appear to be endoreduplicative.

cell coincident with the expression of increased levels of hCG.

Essentially no work has been done with this extremely promising model, either in terms of cell invasiveness or the synthesis of hCG. Nor has there been any analysis of the intermediate forms in either direction. A significant data base drawing on studies of the molecular biology of gene regulation of hCG (*82*), the endocrine physiology of response to cAMP, the molecular basis for second messenger response (*83*), and the cell biology of cytoskeletal changes (*42,66*) could be applied to this experimental system, which would add a dimension that could expand the utility of hCG as marker of risk exposure in early pregnancy.

The effectiveness of hCG as marker for early postimplantation development would be enhanced if the regulatory relationships between the undifferentiated (cytotrophoblast) and differentiated (syncytiotrophoblast) trophoblast cells and their intermediates could be resolved. Since the sensitivity and the response of each cell variant to normal regulatory and xenobiotic agents may prove to be different, understanding the contribution of each to the form and function of the definitive placenta is critical to the assessment of environmental risk.

Limits of hCG as a Marker

The utility of hCG as either a biological or health marker is limited by the onset of its appearance at sites accessible to sampling. Human chorionic gonadotropin can first be reliably detected 10 days after the midcycle LH surge (*84*), 3 to 4 days after the blastocyst has implanted on the uterine endometrium (Fig. 15). Given this chronology, it is possible that intact hCG (and its subunits) could be expressed prior to day 10. Because of its ubiquity, measurement of the α subunit (expressed by cytotrophoblasts before they initiate differentiation) would be equivocal. The earliest measurement of the intact hormone would depend on the differentiation of sufficient numbers of intermediate and terminal syncytiotrophoblast cells to produce sufficient hCG to be detected by the sensitivity of the assay.

Therefore, there is no effective biological marker, including hCG, to monitor events associated with transition from morula to blastocyst, entry into the uterus, and the various pre- and peri-implantation events that occur during days 5 to 10 (Fig. 15). The report (*85*) that a luteotropic hCG-like factor is produced by the human blastocyst and is detectable in the immediate preimplantation period (day 6) has never been confirmed. Aggressive prospective research, largely dependent on animal and/or experimental models, is required to identify trophoblast signals that might be expressed during this critical, high-risk period (days 5–7, 5–10).

At present, the most promising marker to characterize this developmental period is an endometrial signal, the luteotropiclike peptide product of the predecidual cells of the late secretory uterine stroma (*86*). Decidual cell signals have also been identified in the rat (*87*).

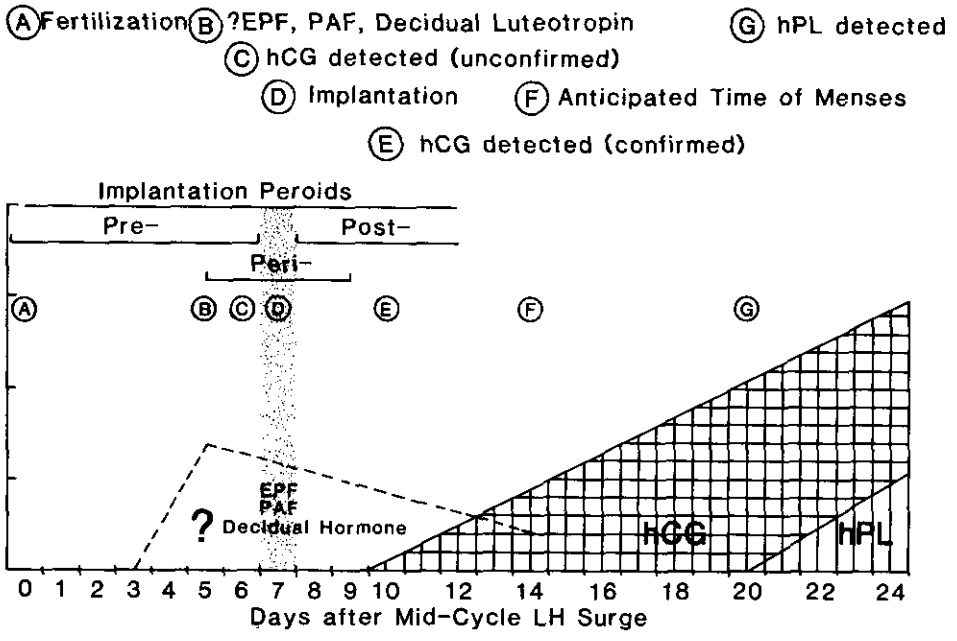

FIGURE 15. This diagram depicts some of the major events occurring *in utero* that define the peri-implantation period in the human. Note the absence of confirmed markers available during that period. Sensitive radioimmunoassays of hCG, using specific monoclonal antibodies, do not detect hCG until day 10, at least 3 days after implantation and initiation of trophoblast differentiation. Early pregnancy factor (EPF) and pregnancy-associated factor (PAF) are trophoblast (?) products that could be used as markers of the risk status of the embryo. The decidual luteotropic hormone could possibly serve as a marker of the condition of the endometrium.

There are no trophoblast signals characteristic of this period, although EPF (early pregnancy factor) and PAF (pregnancy-associated factor) have been touted as candidates to mark this sensitive period of development (Fig. 15). The value of EPF and PAF would be augmented if the sites of their production could be certified and if their expression could be demonstrated to be a correlate of, rather than coincident with, a pivotal step in development. The potential importance of these possible markers deserves stringent study and support.

Future Studies

The research plan designed to identify trophoblast signals that might be used to monitor the influence of xenobiotic agents on early gestation is depicted in Figure 16. The ability to distinguish between alive and dead embryos might satisfy first efforts; however, lethality is a rudimentary marker with relatively little information value. Peri-implantation death carries a minimum burden, as such losses could occur without altering the scheduling of the normal menstrual (reproductive) cycle. The reality is that chronic recurrence of this event could be misinterpreted as some form of infertility rather than a more direct penalty of toxic exposure. Finally, however, markers are required to approximate injury, repair, and the nature of degree of altered function in those conceptuses that do not die immediately and in those that survive. The survival of individuals injured *in utero* exacts onerous psychological and socioeconomic burdens.

The research plan was designed for analysis of *in vitro* experimental models and is based on the episodic exposure of a target cell to any particular agent. Chronic exposures may represent more of a real-life situation. However, an experimental model is better served by episodic exposures that permit the pharmacodynamics and the sensitivity of a single target-cell to be analyzed at any single stage of its differentiation. It is then possible to monitor the sequelae of an episodic exposure at that stage of cell development.

In terms of generating trophoblast signals, injury to the undifferentiated trophoblast (human cytotrophoblast, rodent trophectoderm) is expected to produce more serious deficits and far-reaching effects on the products of conception than injury at later developmental stages. The undifferentiated trophoblast cell can be regarded as a determined stem cell (Fig. 16) that becomes increasingly committed to the trophectodermal epithelial lineage as it develops from compaction to the early blastocyst. The consequences of injury are factors of the sensitivity of the target cell and pharmacokinetic principles. The greater the number of target cells that can differentiate, i.e., human intermediate and synctiotrophoblast, rodent trophoblast giant cells, the fewer the earlier *in utero* deaths.

There are no studies on the transitional forms of differentiating human trophoblast, but studies of rat or mouse offer opportunities to investigate the relationships between differentiation and sensitivity. In the rodent there are three variants of the undifferentiated trophoblast cell in the blastocyst. Because of their lo-

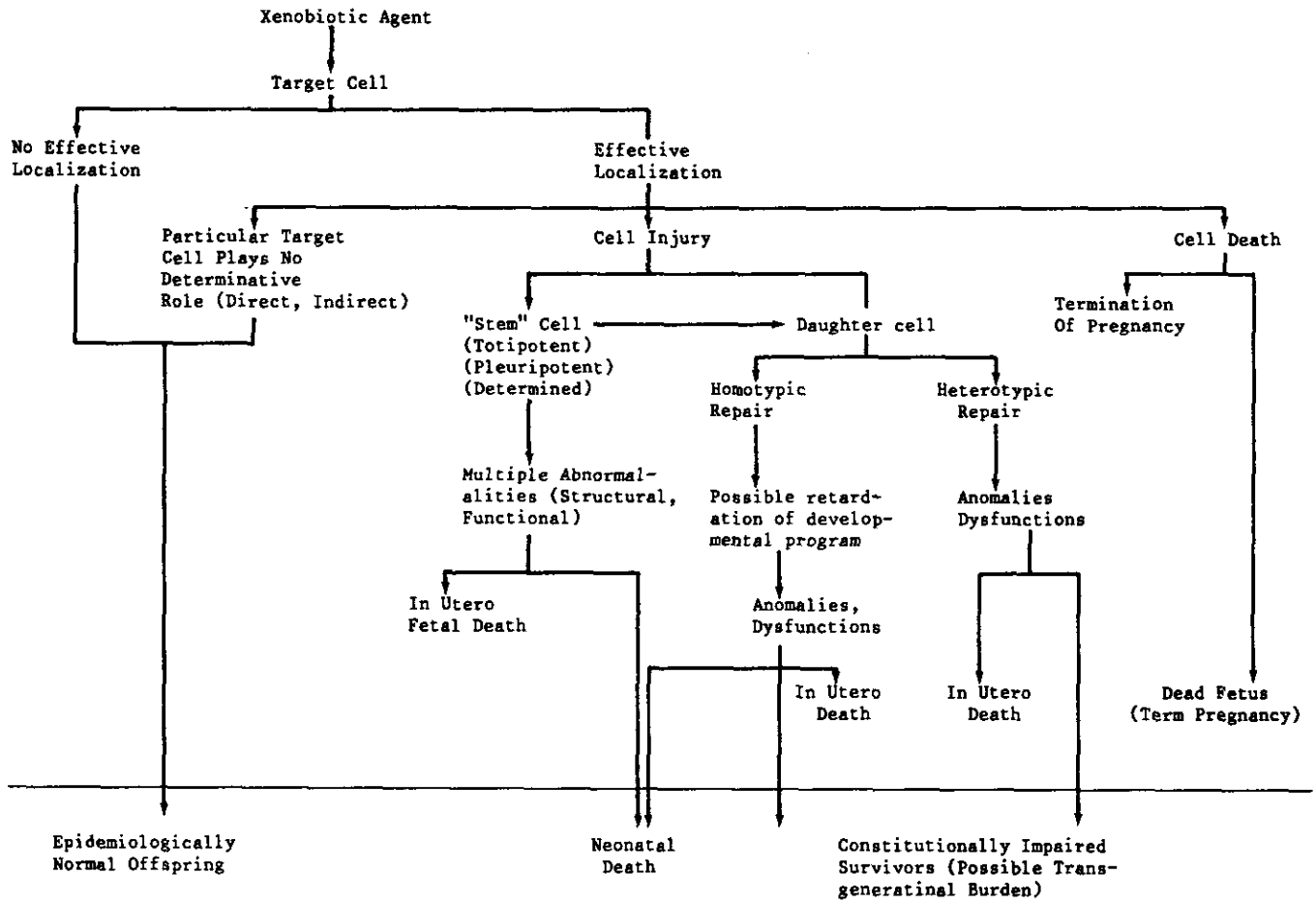

FIGURE 16. A scheme used to track the possible sequelae of exposure of a target cell to effective concentrations of a xenobiotic agent. In comparison with *in utero* cell death, the greatest burden and the most far-reaching individual and population effects are related to the nonrepairable injury of undifferentiated (stem) trophoblast cells, i.e., human cytotrophoblast; rodent trophectoderm. Also at considerable risk are the differentiated derivatives of the stem cell pool (daughter cells), i.e., human syncytiotrophoblast; rodent trophoblast giant cells. The putative sensitivity of these cells may be related to the increase in DNA per cell that characterizes their differentiation and the pivotal role these cells play in development because of their morphological and functional contributions to the definitive placenta. See text for further discussion.

cation and proliferative rates, mural TE, polar TE and EPC cells may have differing sensitivities to a particular xenobiotic agent. Thus, if the blastocyst is exposed in the interval immediately preceding attachment to the uterine epithelium, the initial stages of trophoblast differentiation and thereby the synthesis and secretion of the early trophoblast peptide hormone (human hCG, rodent PL-1) would be seriously affected, primarily because of interference with mural TE. If exposure was severe enough (concentration, duration) that a sufficient number of TGC did not develop, then pregnancy would terminate at the immediate postimplantation period. However, TGC from polar TE and EPC differentiate at later developmental periods, so that if the threshold requirement subsequent to attachment at the abembryonal/antimesometrial site were satisfied, sufficient recovery in the precursor pool of secondary TGC could take place. Depending on the extent of interference with the expression of other regulating signals (hPL, rodent PL-2, signals for gastrulation, specific organogenetic switches), consequences would be most probably noted as fetal and neonatal death.

The spectrum of effects is shifted if critical exposure occurs after differentiation has been initiated (e.g., at the daughter cells stage) (Fig. 16). Again, there is no literature regarding derivatives of undifferentiated cytotrophoblast or TE cells. In the human the effect of critical exposure of syncytiotrophoblast cells would be the result of multiple factors. If a sufficient number of these cells survived functionally to maintain the pregnancy, the consequences would derive from the competence to initiate other endocrine functions (hPL, steroidogenesis), the ability to serve the cell-cell interactions required for villous formation, etc. Changes would be modulated by the capacity of the proliferating pool of recovering trophoblast cells to affect homotypic repair and could carry development well into the fetal period. Term pregnancies of dead fetuses (*88*), neonatal

deaths, and the birth of impaired survivors are lasting penalties of postimplantation exposure.

In the rodent, the responses of presumptive placenta to a xenobiotic agent critically localized in the differentiating trophoblast cell might be expected to be more variable. Once differentiation has been initiated, the TGC are postmitotic, and as development proceeds, increasingly terminally differentiated. Without prospective research it is not possible to predict how a DNA adduct would be processed by an endoreduplicating nuceus or whether repair, if it does occur, is homotypic or heterotypic. The contribution from the EPC reservoir of diploid proliferative cells would be another factor for which no estimates exist. Not only are these cells a continuing source of TGC (PL-2), but formation of the definitive trilaminar trophoblast (*65*) depends on their responses to inductive signals emanating from advancing fetal mesenchyme. The net effect of early postimplantation exposure in the rodent is similar to the human, e.g., fetal deaths, neonatal death, and an increased incidence of burdened survivors.

Integrated with the use of acceptable biological markers, which can be used experimentally, a series of episodic exposures of rodent trophoblast cells at different stages of differentiation can generate a pattern of stage-specific responses. In the idiom of these experiments the risk of each developmental stage can be more specifically assessed. These relative values could be extrapolated to early human trophoblast differentiation. It is possible that such extrapolative exercises could provide greater clarity and direction to estimates of risk in human population. At the minimum, they indicate how dissatisfied we should be with our present knowledge and provide direction for future efforts.

NOTE ADDED IN PROOF: Kliman, H. J., Nestler, J. E., Sermasi, E., Sanger, J. M., and Strauss, J. F. III [Endocrinology 118: 1567–1582 (1986)] have recently reported on the purification, characterization, and *in vitro* differentiation of cytotrophoblasts from human term placentae. This cell separation has the same potential as the Friedman and Skehan model (*42*), which uses BeWo choriocarcinoma cells plus the advantage of using primary, albeit term, cells. Neither model, both of which support the same concept relating DNA/cell to the expression of mature hCG, has been confirmed or challenged experimentally.

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