Choriocarcinoma Cells Increase the Number of Differentiating Human Cytotrophoblasts through an \textit{in Vitro} Interaction*

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Abraham Hochberg, Colin Sibley, Mary Pixley, Yoel Sadovsky, Brian Strauss, and Irving Boime

From the Departments of Pharmacology and Obstetrics and Gynecology, Washington University School of Medicine, St. Louis, Missouri 63110

The human placenta arises from the zygote through single cell intermediates called cytotrophoblasts that in turn give rise to a syncytiotrophoblast. In culture, mononucleated cytotrophoblasts exhibit little, if any, cell division but are converted to multinucleated cells. Choriocarcinoma, the malignant tumor of placental trophoblast, comprises a mixed population of dividing cellular intermediates that resemble cytotrophoblasts but are less differentiated. Because the choriocarcinoma intermediates arise from dividing cells, the tumor may contain one or more cell types in abundance not present in the population of isolated placental cells. To study placental differentiation through cell-cell interaction, choriocarcinoma cell lines were co-cultured with placenta-derived cytotrophoblasts, and placental hormone biosynthesis, as a marker of differentiation was examined. We reasoned that intermediates formed by the tumor might interact with and complement those intermediates in the placenta-derived cytotrophoblast population. Co-culturing either the JAr or JEG choriocarcinoma cell lines with cytotrophoblasts elevated the synthesis of the chorionic gonadotropin $\alpha$ and $\beta$ sub-units 10–20 fold, and human placental lactogen 5-fold. The effect was specific for these trophoblast-derived cells, since comparable quantities of Chinese hamster ovary or HeLa cells did not affect the placental cytotrophoblast culture. Further experiments suggested that the source of enhanced synthesis was the cytotrophoblasts. We propose that an interaction between cytotrophoblasts and choriocarcinoma cells occurs, which results in an increased number of differentiating cytotrophoblasts. Such co-cultures may represent a model system for examining choriocarcinoma cell interaction with normal cells, a process known to occur \textit{in vivo}. The data are also consistent with the hypothesis that the regulated chorionic gonadotropin production in the placenta is determined by interaction among trophoblast cells at different stages of differentiation.

Mononucleated cytotrophoblast cells, which are mitotically active, fuse to form a mitotically inactive, multinucleated syncytiotrophoblast. Morphological data suggest that there exist transitional or intermediate trophoblasts in this pathway (1–5). Human trophoblast in the placenta elaborates at least two major protein hormones, chorionic gonadotropin (CG)\(^1\) and placental lactogen (hPL) (5). CG consists of two non-identical subunits ($\alpha$ and $\beta$), and hPL is a single chain polypeptide, which shares greater than 90% homology with human growth hormone. Their temporal appearance in maternal serum during pregnancy is different; CG peaks in the first trimester, while hPL reaches maximal levels at term. It has been proposed that the extent of trophoblast differentiation is responsible for their stage-specific expression (5).

In culture, mononucleated cytotrophoblasts exhibit little, if any, cell division but are converted to multinucleated cells. An induction of CG and hPL parallels these morphological changes (5). Early in culture, when the trophoblasts are mononucleated, only the CG\(\alpha\) subunit is detected (6, 7). After a lag of several hours, CG\(\beta\) synthesis initiates; it is at this time that fusion is seen. Thus, we proposed that the CG\(\beta\) levels \textit{in vivo} are sustained by a pool of differentiating cytotrophoblasts and that the presence of these cells maintains an intermediate cell population synthesizing CG (5, 6). The expression of hPL is associated with a more advanced stage of differentiation, and in culture, hPL can be detected after multinucleated structures are formed (8).

Choriocarcinoma, the malignant tumor of trophoblast cells, comprises a mixed population of cellular intermediates that are in different stages of differentiation (9–13). There are clusters of cytotrophoblast-like cells and a minor population of multinucleated cells that are apparently immature syncytial elements. In contrast to trophoblasts, JAr cells divide in culture. Both in culture and \textit{in vivo}, choriocarcinoma cells apparently undergo a cell-cell fusion and differentiate into cells synthesizing CG; this occurs in less than 10% of the cells.\(^2\) Compared with cultured cytotrophoblasts, the extent of JAr cell differentiation is limited. Based on this point and the inability of cytotrophoblasts to divide in culture, we reasoned that if they were co-cultured with choriocarcinoma cells, the intermediates formed by the tumor might interact with and complement those intermediates in the cytotrophoblast population. Together, the two sets of cells would overcome the deficiency of the individual populations and allow the identification of trophoblast intermediates in the differentiation pathway. Here we show that co-cultures of JAr and JEG choriocarcinoma cell lines enhance the formation of CG- and hPL-producing cellular intermediates from quiescent pla-

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1 The abbreviations used are: CG, chorionic gonadotropin; hPL, human placental lactogen; Heps, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; CAT, chloramphenicol acetyltransferase.

corta-derived cytotrophoblasts. Moreover, this effect is specific for choriocarcinoma cells, since HeLa and Chinese hamster ovary cells did not affect the placental cytotrophoblast cultures. We conclude that choriocarcinoma cells can interact with and increase the differentiation of placental-derived cytotrophoblasts.

MATERIALS AND METHODS

Cell Culture—The following cell lines were used: JAr and JEG choriocarcinoma cell lines, human fibroblasts, HeLa cells, and Chinese hamster ovary cells. They were maintained in Medium 199 containing 10% fetal calf serum, 25 mM Hepes (pH 7.4), penicillin (100 μg/ml), and streptomycin (100 μg/ml). Cytotrophoblast cells were purified from human term placenta by a modification (8) of the procedure of Kliman et al. (7). Briefly, 100-200 g of minced placenta, scraped free of blood vessels and connective tissue, was incubated in 500 ml of sterile Hanks’ buffered salt solution containing 25 mM Hepes (pH 7.4), 0.1 mM CaCl2, 0.8 mM MgSO4, penicillin, and streptomycin to which 500 mg of trypsin (Sigma) and 70 mg of DNase (Sigma) were added. After incubation for 30 min at 37 °C, the solution was filtered and trypsin activity inhibited by the addition of newborn calf serum (20% final concentration). Cytotrophoblast cells were obtained by centrifugation for 30 min at 3000 rpm through a discontinuous Percoll gradient (5-70%) prepared in Hanks’ buffered salt solution and before collection of the fraction banding between 35 and 55%. The cells were washed with Hanks’ buffered salt solution and then plated in Medium 199, as described above. From a separate set of cultures, choriocarcinoma cell lines harvested 72 h after plating were added to the cytотrophoblasts in a ratio of 1:10 and mixed in 6-well dishes.

Protein Synthesis and Immunoprecipitation of CG Subunits and hPL—Cells were labeled in Medium 199 minus cysteine containing 10% dialyzed calf serum and 25 μCi/ml [35S]cysteine (Amersham Corp. or ICN) for 24 h. The medium was then removed, and labeled CG subunits and hPL were immunoprecipitated and resolved in sodium dodecyl sulfate gels (14). The hPL antiserum was generously supplied by Dr. Stuart Handwerger (Department of Pediatrics, University of Cincinnati).

To measure specific activity of incorporation into total protein, the cells were washed twice with phosphate-buffered saline and incubated at 37 °C for at least 10 min in distilled H2O2 before they were scraped from the plate and frozen at 29 °C. After thawing, an aliquot of this lysate was taken for protein determination using the Bio-Rad protein assay reagent (catalog no. 500-0006). [35S]Cysteine incorporation was determined in a second aliquot by trichloroacetic acid precipitation.

Immunocytochemistry—Cells cultured in 6-well (35-mm) dishes were washed twice with phosphate-buffered saline and fixed for 10 min with 0.1 M KPO4 buffer containing 15% picric acid and 2% formaldehyde (pH 7.5). The cells were then plated in Medium 199, as described above. From a separate set of cultures, choriocarcinoma cell lines harvested 72 h after plating were added to the cytотrophoblasts in a ratio of 1:10 and mixed in 6-well dishes. From a separate set of cultures, JAr line, we analyzed co-cultures of another choriocarcinoma line, JEG. The ratio of these cells to the cytотrophoblasts was 1:10. CG synthesis was increased over 10-fold in the co-cultures (lane 5) compared with the JEG cells (lane 2). Addition of comparable numbers of non-trophoblast cells such as human fibroblasts or Chinese hamster ovary (not shown) and HeLa to cytотrophoblasts in a 1:10 ratio had no effect. Thus, stimulation of CG production in co-cultures was restricted to choriocarcinoma cells.

RESULTS

When normal human cytотrophoblasts are cultured in vitro, the CGα subunit appears before the CGβ subunit. These events are correlated with the transition from single mononucleated cells to multinucleated structures, and little, if any, cell division is observed. In contrast, choriocarcinoma cells divide in culture. Although they are in different states of differentiation, choriocarcinoma and cytотrophoblast cells synthesize CG. To assess potential interaction between choriocarcinoma cells and cytотrophoblasts, both cell populations were co-cultured. CGβ subunit synthesis was assayed as a marker of differentiation.

Cytотrophoblasts (5 × 106) were co-cultured with 5 × 104 JAr cells for 72 h, and the medium was supplemented with [35S]cysteine for an additional 24 h (Fig. 1). Equal amounts of media were immunoprecipitated with CGα-specific antiserum, and the proteins were resolved in sodium dodecyl sulfate-polyacrylamide gels. This antiserum recognizes both free CGα subunit and CG dimer, which is indicated by co-precipitation of the CG subunit. While JAr cells synthesized primarily CG dimer (lane 1), term cytотrophoblasts synthesized little, if any, detectable CG dimer, even after 72 h in culture (lane 3). Co-culturing JAr and cytотrophoblasts enhanced CG dimer production 10- and 40-fold over the levels seen in JAr and cytотrophoblasts, respectively (lane 4). The increase of CG in the co-cultures was not a result of an increase of total protein synthesis, since [35S]cysteine incorporation into total proteins of the individual and co-cultures was the same. These data show that interaction of the two cell types greatly stimulated CG production compared with the additive effect of the individual cell types. Comparable stimulation was seen in co-cultures containing cytотrophoblasts derived from first trimester tissue (data not shown).

To address if stimulated CG production was unique to the JAr line, we analyzed co-cultures of another choriocarcinoma line, JEG. The ratio of these cells to the cytотrophoblasts was also 1:10. CG synthesis was increased over 10-fold in the co-cultures (lane 5) compared with the JEG cells (lane 2). Addition of comparable numbers of non-trophoblast cells such as human fibroblasts or Chinese hamster ovary (not shown) and HeLa to cytотrophoblasts in a 1:10 ratio had no effect (Fig. 2). Thus, stimulation of CG production in co-cultures was restricted to choriocarcinoma cells.

To determine if the induction of CG synthesized by the mixed culture occurred in the cytотrophoblasts or the JAr cells, or both, we co-cultured cytотrophoblasts with a stable line of JAr cells containing the CAT reporter gene linked to the promoter of the CGβ gene (15). If transcription of the
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FIG. 2. Synthesis of CG in co-cultures containing cytotrophoblasts (Cyto) and HeLa cells. Approximately $5 \times 10^5$ JAr (lane 1) or HeLa (lane 2) cells were incubated either individually, together (lane 6), or with $5 \times 10^5$ cytotrophoblasts (lanes 4 and 5) in 0.5 ml of Medium 199 minus cysteine as described in Fig. 1.

FIG. 3. Expression of the CAT gene in co-cultures comprised of cytotrophoblasts (Cyto) plus JAr cells containing CAT linked to the promoter region of the CGβ gene. Twenty μg of cell extract protein was incubated with 0.05 μCi of [3H]chloramphenicol. The stably integrated CGβ CAT construct in the JAr cells contains the 5' region of the CGβ5 gene extending to the KpnI site (15). This corresponds to a distance of 3.5 kilobases from the CAP site. Where indicated, cells were treated for 72 h with 50 μM 8-bromo-cAMP. The arrow denotes the position of acetylated chloramphenicol. The spots corresponding to the acetylated chloramphenicol were excised and counted. JAr-Kpn, 454 ± 20; cytotrophoblasts, 133 ± 30; JAr-Kpn + cytotrophoblasts, 501 ± 70; JAr-Kpn + cAMP, 4432 ± 400; cytotrophoblasts + cAMP, 149 ± 40; JAr-Kpn + cytotrophoblasts + cAMP, 4829 ± 400. These data were obtained from five independent experiments.

endogenous JAr CGβ gene was activated, the CGβ5 promoter linked to CAT should behave similarly, resulting in increased CAT activity in the JAr component of the mixed culture (Fig. 3). The CAT activity in co-cultures (lane 3) was no greater than that in the JAr-Kpn cells alone (lane 4). (The relatively low level of noninduced CAT activity was presumably due to the observation that only a fraction of the JAr cell population expresses the CGβ gene, as discussed in Ref. 15.) To test that the CG promoter was still responsive to stimulation in the mixed culture, 50 μM cAMP was added to JAr, cytotrophoblasts, and co-cultures. Cyclic AMP is a potent activator of CG biosynthesis in both JAr cells and cytotrophoblasts (17–19). The nucleotide increased CAT activity about 10-fold both in JAr-Kpn cells (lane 4) as shown previously (16, 21) and in the mixed culture (compare lane 6 with lane 3). As expected, individual cultures of cytotrophoblasts exhibited no CAT activity (lanes 2 and 5). These data show that in the co-cultures CG promoter linked to CAT was still responsive. However, while the Kpn-CAT construct may lack element(s) that are required for the induction of the host β gene seen in the co-cultures, the data suggest the cytotrophoblasts are the source for the enhanced synthesis of hCG.

hPL is expressed in the syncytiotrophoblast in vivo (see Refs. 5 and 9, and references therein) and is not detected in JAr or JEG choriocarcinoma cell lines. Since hPL production occurs in highly differentiated trophoblasts, we examined its expression in the co-cultures. Immunoprecipitation of secreted 35S-labeled protein from the choriocarcinoma cells and co-cultures showed that the amount of hPL synthesized in the co-cultures was five (n = 7) times greater than in cytotrophoblasts. As expected, no synthesis was observed in JAr cells alone (Fig. 4, lane 1), and thus the data imply that the

FIG. 4. Synthesis of hPL in JAr cells, cytotrophoblasts (Cyto), and co-culture. Culturing, labeling, and immunoprecipitation was performed as described in the legend to Fig. 1. The region of the gel containing hPL was excised and counted. Synthesis of hPL in co-cultures was 5.4 (±0.9) greater than that seen in cytotrophoblasts. The experiment was performed seven times.

3 I. Boime, unpublished observations.
increased synthesis of hPL occurred in the cytotrophoblasts. However, we cannot exclude the possibility that a component in the cytotrophoblasts activates the hPL gene in the JAr cells. Based on these data and the above experiment, we conclude that JAr cells enhance the expression of differentiation markers in the cytotrophoblasts.

Expression of CG in co-cultures was analyzed by immunohistochemical staining. CGβ subunit-specific antisera together with the Vectastain ABC peroxidase kit were used to stain the cells (Fig. 5). Only a few JAr cells stained (panel A, arrow). The cytotrophoblast cells, which begin to fuse after 24–48 h, formed multinucleated islands at 96 h, which stained markedly for CGα (data not shown) but faintly for CGβ (panel B, arrow). CGβ staining is much more pronounced (primarily in multinucleated structures) when cytotrophoblasts are incubated for 120 h. Staining for CGβ was much greater in the co-cultures (panel C) than in JAr or cytotrophoblasts. In

Fig. 5. Immunocytochemistry of JAr cells, cytotrophoblasts (Cyto), and co-cultures. The cells were grown for 96 h, fixed, and stained using a rabbit polyclonal antiserum against CGβ diluted 1:1600. Diaminobenzidine was used as chromogen, and staining is seen here as darker areas (magnification, ×10). Each is a representative staining pattern of the entire field. Panel A, JAr cells; panel B, cytotrophoblasts; panel C, JAr + cytotrophoblast cells.

Fig. 6. Determination of the optimal ratio of cytotrophoblasts and JAr cells for maximal CG synthesis. A constant amount of cytotrophoblasts (5 × 10^6) was co-plated with a decreasing amount of JAr cells. Lanes 1 and 2 show the amount of CG synthesized in the individual cultures of JAr cells and cytotrophoblasts, respectively. Lanes 3–7 correspond to the following ratios of JAr to cytotrophoblasts: 1:5, 1:10, 1:20, 1:50, 1:100.

Fig. 7. Time course of CGα (A) and CG dimer (B) synthesis by JAr and JEG choriocarcinoma cell lines, cytotrophoblasts, and co-cultures (JAr-C and JEG-C). Protein in the medium was immunoprecipitated with either CGα-specific (A) or CGβ-specific antisera (B). The bands corresponding to α and β subunits of CG were excised and counted. Synthesis of dimer is expressed as the sum of counts/min in the CG α + β regions.
FIG. 8. Schematic diagram of the differentiation pathway followed by cytotrophoblasts in culture.

1. Pre CGα stage
2. CGα
3. CGα
4. CGα
5. CGα

In placenta, cytotrophoblasts can divide and proceed through a multistep differentiation resulting in the synthesis of CG, hPL, and other placenta-specific proteins. Based on several studies examining the morphological and biochemical changes of trophoblast in culture and in vivo (5-8, 20), we propose that differentiation of cytotrophoblast cells is associated with the following steps (Fig. 8). Stem cells continually pass through the cell cycle and divide, or enter the G0 phase and are committed to differentiate (step 1). At this point, cytotrophoblast cells, which are still at the single cell stage, express CGα. At a subsequent stage (step 2) while undergoing morphological differentiation, expression of the CGβ gene is initiated. Multinucleated cells are then formed (step 3), leading to maximal production of CGα and CGβ subunits and the appearance of hPL. Because hPL is expressed exclusively in the syncytiotrophoblast of the placental villus, a structure not seen in the culture, it is unclear if an additional step(a) is required for maximum expression of hPL (step 4).

Choriocarcinoma cells and isolated cytotrophoblasts are in different stages of differentiation, since neither population has all the cell types seen in vivo. JAr cells divide, but very few differentiate, and there is no formation of the multinucleated islands seen in trophoblast tumors in vivo and in normal placenta (Fig. 5A). Those JAr cells that do differentiate express both CGα and CGβ (17, 18). However, further differentiation is limited, and hPL synthesis is not seen. Cytotrophoblasts isolated from normal placenta are committed to differentiate, and very few divide. These cells fuse and form multinucleated islands. Differentiation results in expression of CGα and then CGβ, but this is less tightly coupled than that seen in JAr cells; a greater excess of CGα compared with CGβ is observed in the cultured cytotrophoblasts (6, 17). Differentiating cytotrophoblast cells can synthesize hPL, which suggests that they are capable of further differentiation than the JAr intermediates.

Despite the limitations of using individual JAr and cultured cytotrophoblasts as models for cytotrophoblast differentiation in vivo, they do have in common the ability to fuse and differentiate. This was an essential component for the rationale of the experiments presented. The interaction of the choriocarcinoma cells and the cytotrophoblasts is shown by several experiments. First, expression of CG subunits and hPL by the co-cultured cells was much higher than that seen in the individual cultures, and the interaction was specific for trophoblast-derived cells. Second, because differentiating cytotrophoblasts synthesize hPL while JAr cells cannot, this implies that the source of increased hormone production in the co-cultures is the cytotrophoblasts. Although the rate of cytotrophoblast differentiation appears unchanged by the added choriocarcinoma cells, more cytotrophoblasts are recruited to intermediate cells producing CGβ and hPL. If the initial fusion (step 3) is rate-limiting, the choriocarcinoma cells may activate the cytotrophoblasts at this point. However, since synthesis of the β subunit was also increased, an earlier step in the pathway may be stimulated.

The nature of the JAr-cytotrophoblast interaction is not clear. Do JAr cells produce a factor as a result of fusion with a cytotrophoblast, or does a heterokaryon form when the two cell types fuse, thereby generating a component that enhances cytotrophoblast differentiation? The component is apparently not present in the conditioned medium of the co-cultures; addition of this media to either individual cultures of JAr cells or cytotrophoblasts did not alter synthesis of CG (data not shown). The interaction is presumably not related to an intrinsic feature of the CGα and CGβ subunits, since HeLa cells, which have the capacity to synthesize these proteins (16, 17, 19), did not display the interaction seen with the choriocarcinoma cell lines. HeLa cells (referred to as ectopic CG producers) unlike choriocarcinoma cells (eupiotic CG producers) are devoid of multinucleated cells. Thus the capacity to undergo cell-cell interaction is a prerequisite for the effect discussed here.

The morphological features of the mixed cultures, multinucleated islands surrounded by dividing stem cells, are often seen in sections of choriocarcinoma in patients (10-13). These tumors contain a mixture of cytotrophoblasts and intermediate trophoblasts which produce CG. Since gestational choriocarcinoma is a neoplasm derived from the trophoblast epithe-
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lium, the co-cultures seen here may be similar to the in vivo interaction between transformed cytotrophoblasts and normal cells. Thus not only do the interactions reported here suggest complementary intermediates in trophoblast differentiation, but they could also serve as a model for choriocarcinoma in vivo.

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