DEVELOPMENTAL BIOLOGY 130, 693-702 (1988)

The Human Villous Cytotrophoblast: Interactions with Extracellular Matrix Proteins, Endocrine Function, and Cytoplasmic Differentiation in the Absence of Syncytium Formation

LEE-CHUAN KAO,* STEPHEN CALTABIANO,† SAMUEL WU, JEROME F. STRAUSS III,*,† AND HARVEY J. KLIMAN†,[1]

Departments of Obstetrics and Gynecology, and †Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Accepted August 18, 1988

Human syncytiotrophoblasts are derived from villous cytotrophoblasts by cell fusion. Coincident with this morphologic transformation, trophoblasts acquire specific endocrine functions, including elaboration of chorionic gonadotropin (hCG). We wondered if syncytia formation was a prerequisite for biochemical differentiation or simply was one part of the differentiation program. By growing purified human cytotrophoblasts under serum-free conditions and manipulating the culture surface, we were able to disassociate morphologic from biochemical differentiation. We have shown previously (*Endocrinology* 1986, 118:1567) that human cytotrophoblasts grown in the presence of fetal calf serum flatten out, aggregate, and form functional syncytiotrophoblasts *in vitro* over 24-96 hr. Here we demonstrate that when grown in the absence of serum, the cells do not undergo these morphologic changes, but remain as individual spherical cells. If the culture surface was precoated with fibronectin or a variety of collagens, but not albumin, the cells regained their ability to flatten, aggregate, and form syncytia. Attachment to and syncytia formation on fibronectin was blocked by the addition of the R-G-D-S-containing peptide, Gly-Arg-Gly-Asp-Ser-Pro. Attachment to and syncytia formation on type I collagen was blocked by anti-human fibronectin $F(ab')_2$ fragments, while association with type IV collagen was not affected by this antibody, suggesting that fibronectin mediates trophoblast association with type I collagen, but not type IV. Although syncytia formation did not occur when cytotrophoblasts were cultured under serum-free conditions in the absence of ECM proteins, biochemical differentiation was not affected. These cells secreted hCG at the same rate under serum-free conditions whether they were plated on plastic only—which prevented syncytia formation—or fibronectin, laminin or, type IV collagen—which allowed syncytia formation to occur. Furthermore, cytoplasmic differentiation in the absence of syncytia formation was confirmed by performing transmission electron microscopy on cytotrophoblasts grown under serum-free conditions in the presence of 8-bromo-cAMP. We conclude that syncytia formation is not a prerequisite for biochemical differentiation, but simply part of the trophoblast differentiation program.

INTRODUCTION

Human villous cytotrophoblasts form syncytial trophoblasts through a process of cell fusion (Boyd and Hamilton, 1970; Kliman *et al.*, 1986). There has been speculation that biochemical differentiation, such as the ability to secrete chorionic gonadotropin (hCG)[2] (Pierce and Midgley, 1963, Morrish *et al.*, 1987) and placental lactogen (Hoshina *et al.*, 1983, 1985), is tied to syncytia formation. We have shown previously that cultured human cytotrophoblasts differentiate biochemically and morphologically in a time-dependent fashion (Kliman *et al.*, 1986; Feinman *et al.*, 1986; Kliman *et al.*, 1987). In the presence of fetal calf serum (FCS), the mononuclear cytotrophoblasts flatten out onto the culture surface, aggregate, and form syncytia over a 24-to 96-hr period. Simultaneously with these morphological changes, the trophoblasts secrete increasing amounts of hCG into the culture media. This *in vitro* system did not allow us to dissociate the morphologic and biochemical changes. Here we demonstrate that by culturing cytotrophoblasts under serum-free conditions and manipulating the culture surface by precoating with a variety of extracellular matrix proteins, morphologic and biochemical differentiation can be uncoupled. In addition, we have gained insight into the specificity of associations between cultured cytotrophoblasts and components of the extracellular matrix.

MATERIALS AND METHODS

Preparation and Culture of Human Cytotrophoblasts

Human cytotrophoblasts were purified from normal term placentae obtained following spontaneous vaginal delivery or uncomplicated Cesarean section using the

[1] To whom correspondence should be addressed at Department of Pathology, 6 Founders, 3400 Spruce St., Philadelphia, PA 19104.

[2] Abbreviations used: hCG, human chorionic gonadotropin; FCS, fetal calf serum; DMEM-H-G, Dulbecco's modified Eagle's medium containing 25 m*M* Hepes and 25 m*M* glucose; RER, rough endoplasmic reticulum.

0012-1606/88 $3.00

method of Kliman *et al.* (1986). Briefly, 30 g of soft, villous tissue was subjected to three trypsin/DNase digestions. The resultant cell suspensions were pooled, placed on a discontinuous Percoll gradient (5 to 70% Percoll in 5% steps), and centrifuged at 1200*g* for 20 min. A greater than 95% pure population of cytotrophoblasts resulted as a band at a density of 1.05 to 1.06 g/ml. The cytotrophoblasts were diluted to a final concentration of 10^6 cells/ml with Dulbecco's modified Eagles' medium containing 25 m*M* Hepes and 25 m*M* glucose (DMEM-H-G) with glutamine (4 m*M*) and gentamicin (50 μg/ml) added, and, in serum-supplemented media, 1 to 20% (v/v) heat-inactivated fetal calf serum. 8-Bromo-cAMP (1.5 m*M*, Sigma Chemical Co., St. Louis, MO) was added to some media. One ml of the cell suspension was plated into 16-mm Nunclon multidishes (Nunc, Roskilde, Denmark) for biochemical studies, while 0.2 to 1.0 ml was plated onto 22-mm^2 coverslips (No. 1 or $1\frac{1}{2}$) placed in 35-mm Nunclon multidishes or into four-chamber Lab-Tek (Miles Laboratories, Mishawaka, IN) culture slides for histologic studies. For electron microscopy, 10 ml of the cell suspension was cultured in 80 cm^2 Nunclon flasks in the absence of serum, with or without added 8-bromo-cAMP. The cells were incubated at 37°C in humidified 5% CO_2/95% air with media changes every 24 hr.

Extracellular Matrix Proteins

Fibronectin (Collaborative Research, Bedford, MA) was dissolved in DMEM-H-G with 50 μg/ml gentamicin to a final concentration of 50 μg/ml. Laminim (Collaborative Research) was dissolved in DMEM-H-G with 50 μg/ml gentamicin to a final concentration of 20 μg/ml. Collagens (Sigma) were dissolved in 0.15% NaCl (w/v) with 50 μg/ml gentamicin to a final concentration of 80 or 100 μg/ml. Bovine serum albumin, fraction 5 (Miles Scientific, Naperville, IL) was dissolved in DH_2O to a final concentration of 80 μg/ml. All solutions were filtered with a 0.2-μm syringe filter, applied to either cover glasses or plastic culture surfaces until covered, allowed to stand overnight in a humidified incubator at 37°C, aspirated, and dried in a culture hood for 1–2 hr prior to use.

Antibodies and R-G-D-S Peptide

Affinity-purified $F(ab')_2$ fragment goat anti-human fibronectin (Accurate Chemical and Scientific Corp., Westbury, NY) was diluted to 20 μg/ml into each well from a 1.1 mg/ml stock at the time of cell plating. Chromatography-purified goat IgG $F(ab')_2$ fragment (Accurate) was used as a control at 20 μg/ml as described above for anti-fibronectin fragments. An R-G-D-S-containing hexapeptide, Gly-Arg-Gly-Asp-Ser-Pro (G-R-G-D-S-P), generously provided by Dr. Henry T. Keutman, Endocrine Unit, Massachusetts General Hospital, was used at a final concentration of 1 m*M*.

Histology and Immunohistochemistry

Cultured cells were grown on coverslips, fixed, stained, and photographed as previously described (Kliman *et al.*, 1986). The percentage of nuclei in syncytia was determined counting 500 nuclei in sequential high power fields and determining whether they were in single isolated cells, single cells of an aggregate (two or more cells attached to each other or single cells attached to syncytia), or syncytia (defined as a cell with two or more nuclei within one obvious cytoplasmic border). The total number of nuclei in syncytia was divided by 500.

Transmission Electron Microscopy

Following 24 hr of culture, the trophoblasts were washed once with warm DMEM-H-G, and then fixed with half-strength Karnovsky's fixative (Karnovsky, 1965) for 15 min at room temperature, followed by incubation overnight at 4°C. The fixed cells were removed from the flasks by adding approximately 100 precleaned 3-mm glass beads to the tissue culture flasks, agitating gently for 1 min, and then aspirating off the cell suspension. The cells were then pelleted, transferred to 0.1 *M* cacodylate buffer, pH 7.4, postfixed with 1% osmium for 1 hr, dehydrated in graded ethanols and propylene oxide, and embedded in Epon. Silver sections were stained with uranyl acetate and lead citrate and examined at 75 kV with a Hitachi 600 electron microscope (Hitachi Corp., Tokyo).

Analytical Methods

hCG was quantitated using reagents obtained from Serono (hCG MAIA clone; Braintree, MA). The assay was calibrated to the First International Reference Preparation.

RESULTS

Effect of Fetal Calf Serum on Trophoblast Syncytium Formation

When purified human cytotrophoblasts were cultured on plastic or glass in the presence of 20% FCS (Fig. 1a), they flattened out, aggregated, and formed syncytial trophoblasts over a 96-hr period as previously described (Kliman *et al.*, 1986, 1987). If the concentration of FCS was reduced to 1%, there was little appreciable difference in the morphology after 96 hr. But, if the cells were cultured in serum-free medium, the majority of the cells did not flatten out, form aggregates,

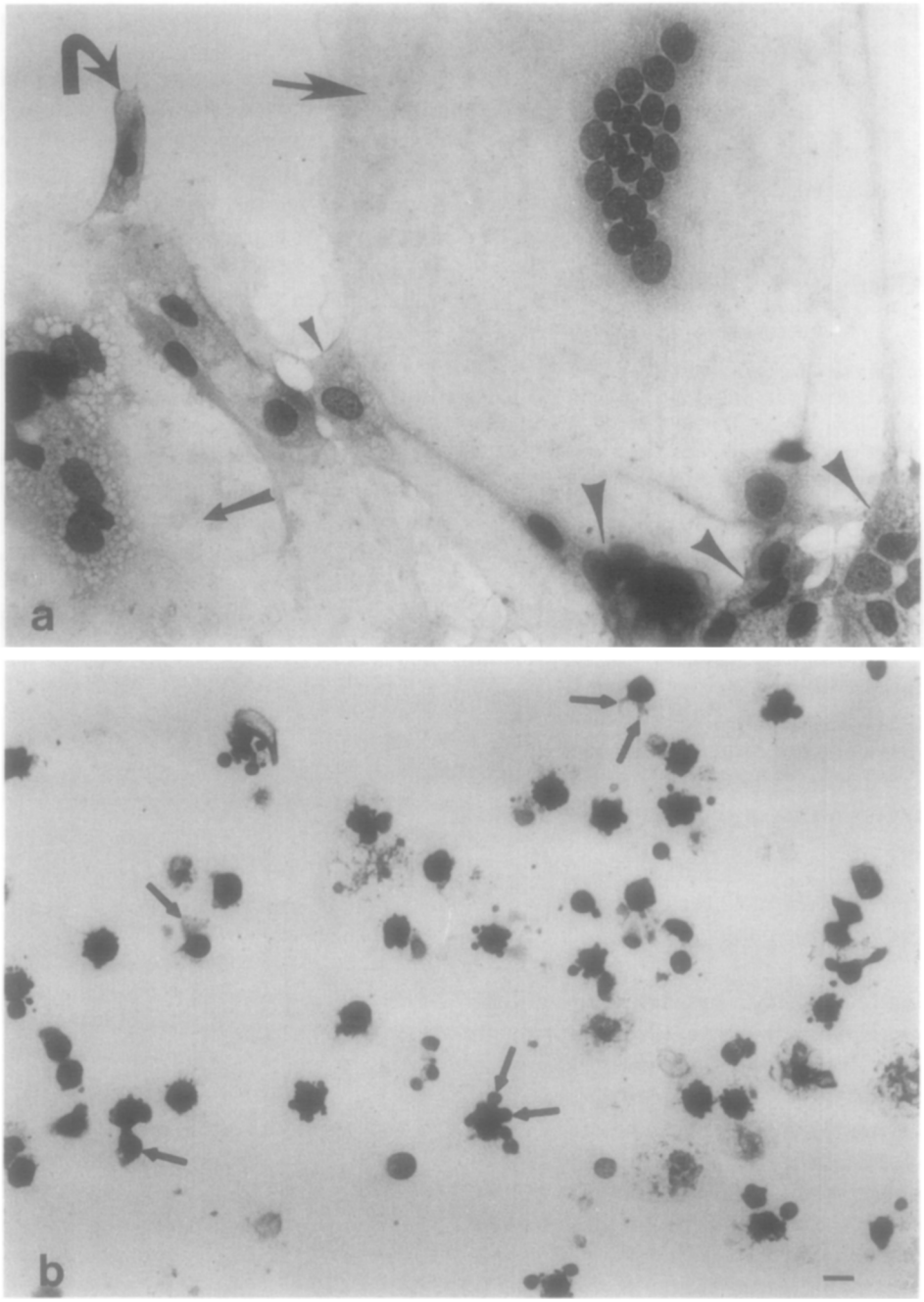


FIG. 1. Effect of FCS on cultured cytotrophoblasts. Cytotrophoblasts were cultured in the presence of 20% (a) or 0% (b) FCS on coverslips as described under Materials and Methods. (a) After 96 hr in culture, the majority of the trophoblasts have flattened out, aggregated, and formed syncytia. Note large multinucleated syncytiotrophoblast (large arrow). Surrounding this syncytia is a single cell (small arrow head), aggregates of trophoblasts (large arrow heads), and a smaller syncytia (small arrow). Note single trophoblast making initial contact with edge of smaller syncytiotrophoblast (curved arrow). (b) In the absence of FCS, the trophoblasts remained spherical and individual—and consequently very dark staining—with little evidence of flattening, aggregation, or syncytia formation. Note that some cells are beginning to extend cytoplasmic material onto the coverslip (arrows). A few cells do flatten out and make contact with neighboring cells, leading to small syncytiotrophoblasts. Both micrographs are at the same magnification; the bar represents 10 μm.

or syncytia (Fig. 1b). Quantitative analysis demonstrated that as many as 83% of the nuclei were in syncytia in serum-supplemented media (Fig. 2), while only 2.5% of the trophoblast nuclei were found in syncytial structures at 96 hr of culture under serum-free conditions. The syncytia formed under serum-free conditions were generally small (20–30 μm in diameter) and had only two to four nuclei per cell, while the syncytia

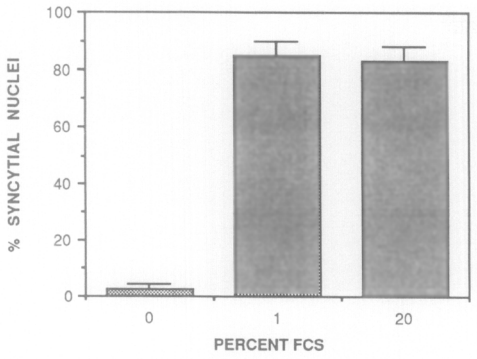


FIG. 2. Syncytia formation as a function of FCS concentration. Cytotrophoblasts were cultured in media supplemented with the indicated concentrations of FCS for 96 hr. The percentage of nuclei in syncytia was determined as described under Materials and Methods. The results represent the means ± SEM from four separate experiments.

formed in the presence of FCS were large (up to 100 μm in diameter) and contained as many as 25 nuclei per cell. Since syncytiotrophoblasts *in vitro* form by fusion of cytotrophoblasts (Kliman *et al.*, 1986), these results imply that only a few cells are able to make contact and fuse under serum-free conditions, while many cells participate in syncytia formation in the presence of serum.

Effects of Pretreatment of Glass Coverslips with Extracellular Matrix Components

We next investigated whether purified extracellular matrix proteins could support syncytia formation in serum-free medium. Precoating glass coverslips with fibronectin or a variety of collagens permitted cytotrophoblasts to form syncytia in serum-free media (Fig. 3). Although culture for 96 hr in the presence of FCS produced the greatest degree of syncytial formation (83%), approximately 50% of the trophoblast nuclei were found in syncytia when the culture surface was precoated with fibronectin; placental collagens types I, IV, and V; calf skin collagen type I; and rat tail collagen. Approximately 33% of the trophoblast nuclei were found in syncytia when the culture surface was precoated with placental collagens types III and V. In contrast, without serum or precoating, only 2.5% of the trophoblast nuclei were found in syncytia at the end of the 96 hr of culture period. Precoating with bovine serum albumin yielded results similar to no precoating. This result suggests that fibronectin and collagens do not support syncytia formation by, for example, neutralization of negative charges on the coverslip, but mediate flattening, aggregation, and syncytia formation by a more specific mechanism.

R-G-D-S-Specific Binding of Trophoblasts to Fibronectin

We next investigated the specificity of trophoblast binding to fibronectin. When human trophoblasts were cultured on fibronectin pretreated coverslips in serum-free media, they flattened out, aggregated, and formed syncytia (Fig. 4a), in a fashion similar to that of cells grown in the presence of FCS (Fig. 1). When the R-G-D-S-containing hexapeptide, G-R-G-D-S-P, was added at the time of plating, virtually all adhesion to the fibronectin-coated coverslip was eliminated (Fig. 4b). In order to verify that the inhibition of fibronectin adhesion was specific, we performed the same experiment using type IV collagen precoated coverslips. In the presence of the hexapeptide, human trophoblasts attached, flattened, aggregated, and formed syncytia on the type IV collagen surface in the presence of the hexapeptide (Fig. 4c). These results suggest that human trophoblasts have a specific, R-G-D-S-sensitive receptor for fibronectin, but that they also express binding sites or molecules which allow them to adhere to collagen.

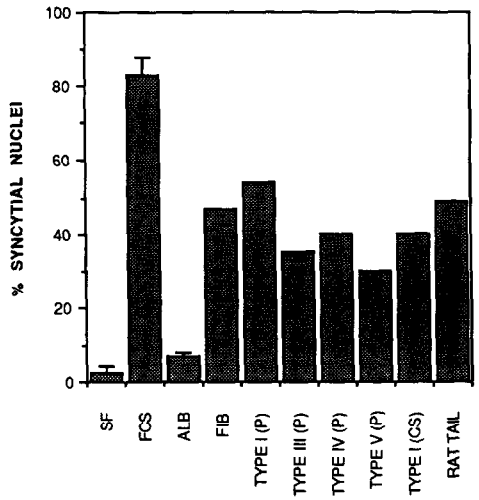


FIG. 3. Effects of coverslip pretreatment on trophoblast syncytium formation. Cytotrophoblasts were cultured under serum-free conditions for 96 hr as described under Materials and Methods. Coverslips were pretreated with the following: albumin (ALB); fibronectin (FIB); Type I, III, IV, and V collagens from placenta (Type I (P), Type III (P), Type IV (P), Type V(P); Type I collagen from calf skin (Type I (CS)); and rat tail collagen (RT). In addition, the results of control cultures grown without serum (SF) or with fetal calf serum (FCS) with no coverslip pretreatment are shown in the first two bars. Data presented for the serum-free, fetal calf serum and albumin treatments are means ± SEM from three to five separate experiments. Data for the fibronectin and collagen treatment groups are from a single experiment in which these treatments were examined simultaneously with the same cytotrophoblast preparation. Similar results were obtained with fibronectin and placental type I and IV collagens from at least five other experiments.

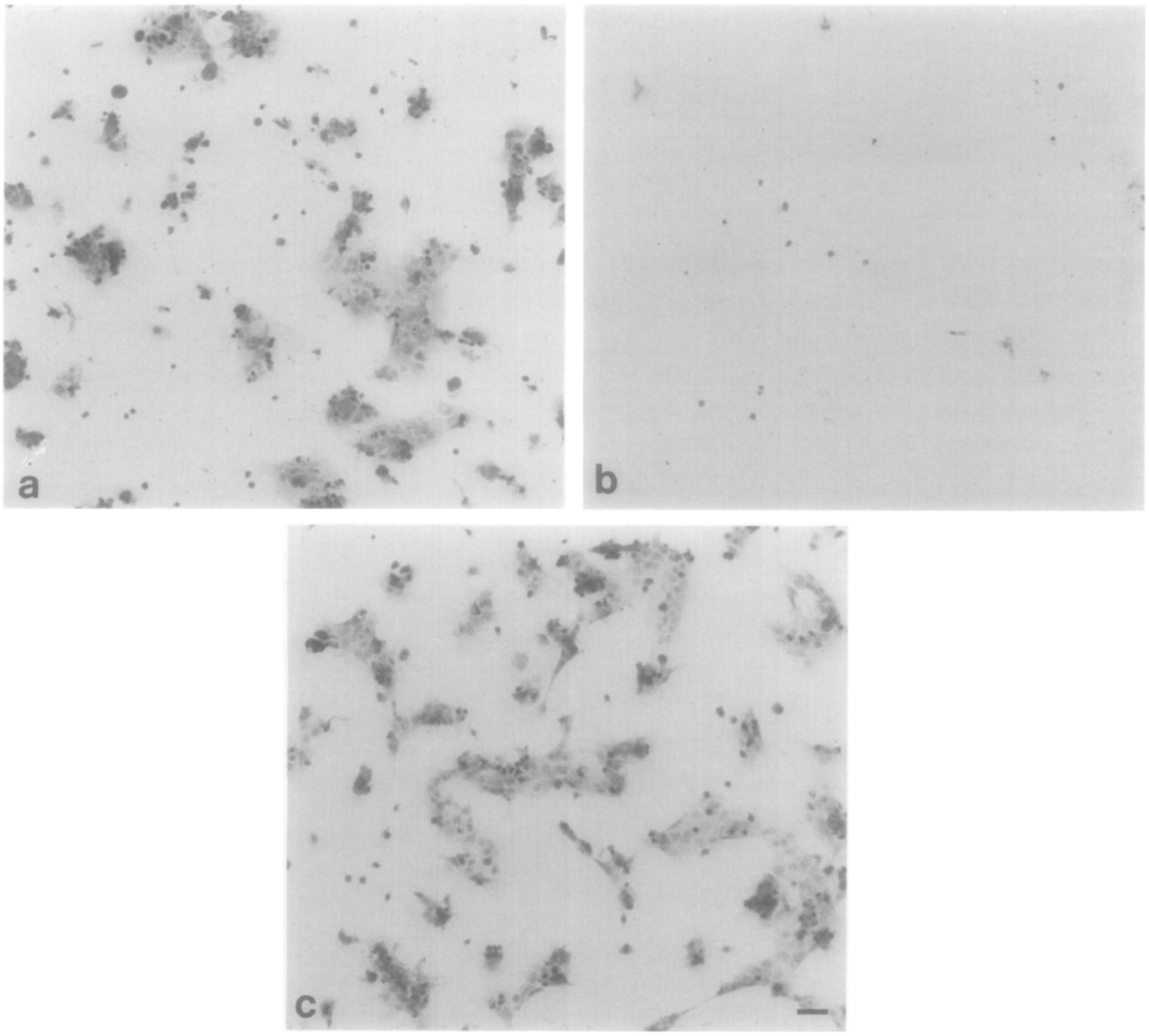
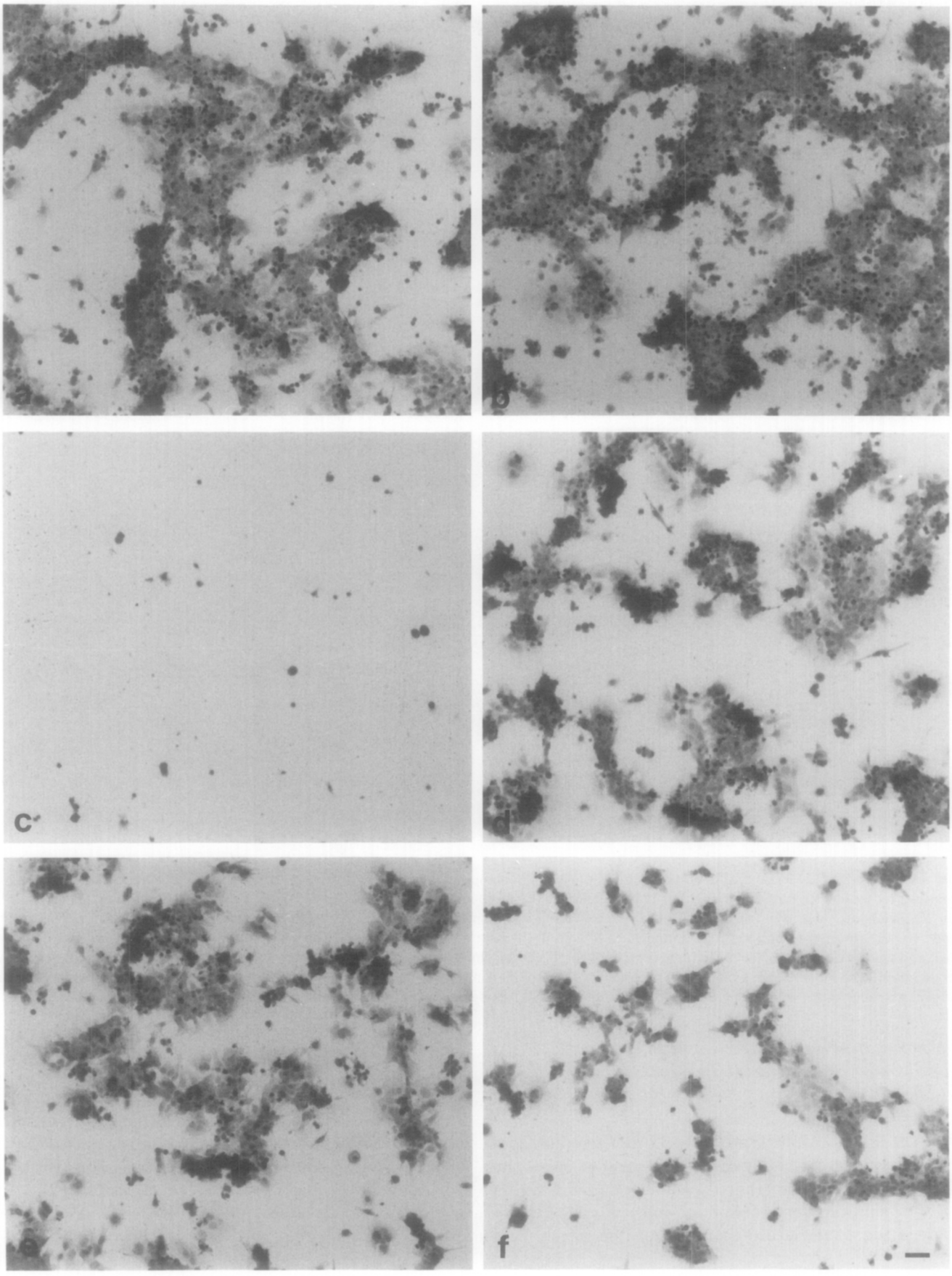


FIG. 4. Effect of R-G-D hexapeptide on trophoblast plating. Cytotrophoblasts were cultured in serum-free DMEM-H-G for 24 hr on glass coverslips precoated with fibronectin (a, b) or type IV collagen (c) without (a) or with (b, c) 1 m*M* Gly-Arg-Gly-Asp-Ser-Pro added as described under Materials and Methods. (a) Although the trophoblasts have been cultured under serum-free conditions, the fibronectin precoating has allowed the cells to flatten out, aggregate, and form syncytia. (b) In the presence of the R-G-D-containing hexapeptide, very few cells have plated, and those on the glass coverslip have remained round (except for an occasional macrophage). (c) The trophoblasts have flattened and aggregated on type IV collagen, even in the presence of the R-G-D-containing hexapeptide—suggesting that the trophoblasts adhere to the collagen by a mechanism other than an R-G-D receptor site. All micrographs are at the same magnification. The bar represents 10 μm.

Trophoblast Binding to Types I and IV Collagens: Involvement of Fibronectin in Binding to Type I Collagen

Figures 3 and 4c illustrate that human trophoblasts bind to a variety of collagens. These results raise the following question: Are there specific collagen binding sites on the human trophoblast, or do these cells bind to collagens via other proteins, such as fibronectin and laminin?

Utilizing specific anti-human fibronectin ($F(ab')_2$ fragments, we attempted to determine if trophoblasts bind to type I and IV collagens directly or indirectly through fibronectin. When trophoblasts were cultured on type I collagen pretreated coverslips, they adhered, flattened, and formed aggregates within 24 hr (Fig. 5a). Normal goat serum $F(ab')_2$ fragments added at the time of plating had no effect on the cultures (Fig. 5b). If, on the other hand, goat anti-human fibronectin $F(ab')_2$ fragments were added at the same concentration at the


c
d
f

time of plating, attachment of trophoblasts was virtually eliminated. In addition, the few cells that did attach did not flatten out or aggregate, but remained spherical—similar to the results under serum-free conditions with no precoating of the culture surface (Fig. 5c). Since no fibronectin was added to these cultures, but the anti-human fibronectin $F(ab')_2$ fragments still prevented attachment of trophoblasts to type I collagen, we verified that this particular antibody did not cross-react with type I collagen. Ouchterlony double-immunodiffusion analysis of the anti-human fibronectin $F(ab')_2$ fragments against type I collagen revealed no detectable line of reaction, while a strong line of reaction was seen against fibronectin.

Type IV collagen supported attachment, aggregation, and syncytia formation (Fig. 5d) and the presence of normal goat serum $F(ab')_2$ fragments had no effect on these interactions (Fig. 5e). In contrast to the results shown in Fig. 5c for type I collagen, the presence of goat anti-human fibronectin $F(ab')_2$ fragments had no effect on the interaction of the cytotrophoblasts with type IV collagen (Fig. 5f). Thus, anti-fibronectin $F(ab')_2$ only interfered with trophoblast binding to type I collagen, not type IV collagen. These results suggest that trophoblasts bind to type I collagen via cell-associated fibronectin, while they either bind to type IV collagen directly or through some other intermediate protein, such as laminim. These results are consistent with emerging evidence which indicates that fibronectin as an intermediate between cells and type I collagen (Akiyama and Yamada, 1987).

Stimulation of hCG Secretion in the Absence of Syncytia Formation

We have demonstrated that under serum-free conditions without precoating or with type I collagen precoating in the presence of anti-fibronectin $F(ab')_2$ that cytotrophoblasts do not flatten out, aggregate, or form syncytia. One might ask: Are these cells viable? Are they biochemically active? In order to answer these questions, we performed biochemical and electron microscopic studies on human trophoblasts cultured under

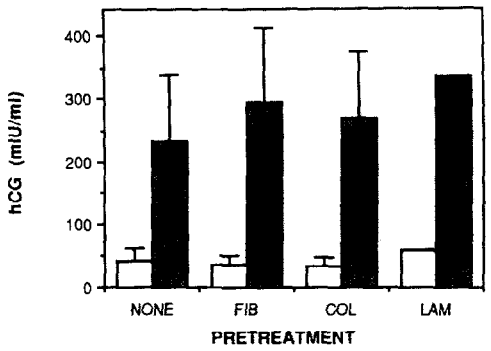


FIG. 6. hCG secretion as a function of culture surface and 8-bromo-cAMP. Cytotrophoblasts were cultured in plastic wells without precoating (NONE) or within wells precoated with fibronectin (FIB), type IV collage (COL), or laminin (LAM) in media without (light bars) or with (dark bars) 1.5 m*M* 8-bromo-cAMP for 24 hr as described under Materials and Methods. Values presented are the means ± SEM of four separate experiments (except for the laminin data, which represent the mean of two separate experiments).

serum-free conditions with and without pretreatment of the culture surfaces.

We had shown previously that 8-bromo-cAMP stimulates hCG secretion by cultured human trophoblasts in the presence of serum (Feinman *et al.*, 1986). We wondered if trophoblasts could be stimulated to secrete hCG under serum-free conditions where (1) they could not form syncytia or (2) they could syncytialize because they were plated on fibronectin, laminin, or type IV collagen-treated surfaces. Figure 6 illustrates the results of several such experiments. Trophoblasts were cultured over a 24-hr period under serum-free conditions without pretreatment or in serum-free medium on surfaces coated with fibronectin, type IV collagen, or laminin. In addition, half of the cultures were treated with 1.5 m*M* 8-bromo-cAMP. These data establish that trophoblasts secrete hCG in a qualitatively similar fashion under serum-free conditions whether cultured in the absence or presence of extracellular matrix proteins. Furthermore, these results indicate that trophoblasts which do not flatten down on the culture surface and do not subsequently form syncytia because they are grown under serum-free conditions (see Fig. 1b) still

FIG. 5. Effect of anti-fibronectin $F(ab')_2$ on trophoblast adherence to type I and IV collagens. Cytotrophoblasts were cultured in serum-free DMEM-H-G for 24 hr on coverslips precoated with type I collagen (a–c) or type IV collagen (d–f) as described under Materials and Methods. $F(ab')_2$ fragments were added to a final concentration of 50 μg/ml for the full incubation period as follows: controls (a, d), normal rabbit serum $F(ab')_2$ (b, e), anti-fibronectin $F(ab')_2$ (c, f). (a) The trophoblasts have flattened and aggregated on type I collagen. (b) The addition of normal rabbit serum $F(ab')_2$ has had no effect. (c) The anti-fibronectin antibodies have prevented many cells from adhering and those that are present have remained spherical without evidence of aggregation. This effect is not due to nonspecific binding of the antibody to the substrate since the anti-fibronectin $F(ab')_2$ did not react against type I collagen by Ouchterlony double-diffusion analysis. (d) The trophoblasts have flattened out and aggregated on type IV collagen. (e) The addition of normal rabbit serum $F(ab')_2$ has had no effect. (f) Unlike (c), the anti-fibronectin $F(ab')_2$ does not prevent flattening and aggregation of the trophoblasts grown on type IV collagen. This suggests that trophoblasts are able to bind to type IV collagen through a fibronectin-independent mechanism—possibly via laminim. All micrographs are at the same magnification. The bar represents 10 μm.

express endocrine activities associated with the syncytial trophoblast.

Cytoplasmic Differentiation in the Absence of Syncytial Formation

To explore further the concept of biochemical differentiation of mononuclear cytotrophoblasts, we performed transmission electron microscopy on trophoblasts which were cultured under conditions that did not allow for syncytia formation, but that did permit stimulated hCG secretion. Cytotrophoblasts were cultured without serum in plastic flasks which were not precoated (thus, the cells could not flatten out and remained spherical), with or without 1.5 m*M* 8-bromo-cAMP, and were processed for transmission electron microscopy. Examination of cells grown in the absence of the cyclic nucleotide analog revealed spherical cells with diameters of ~13 μm (Fig. 7a). These cells had a rather simple cytoplasm, but had microvilli and coated pits—features typical of trophoblasts—and contained scattered irregular segments of rough endoplasmic reticulum (RER), large mitochondria, occasional lipid droplets, and small golgi apparatus (Fig. 7c). In contrast, trophoblasts grown in the presence of 8-bromo-cAMP (Fig. 7b) were still spherical but were larger (~20 μm), contained many stacks of dilated RER, had larger golgi, had well developed perinuclear intermediate filament bundles, and had more (but smaller) mitochondria (Fig. 7d), features consistent with syncytiotrophoblasts (Boyd and Hamilton, 1970). These changes suggest a global increase in the secretory machinery of the trophoblast as a result of 8-bromo-cAMP stimulation, a finding consistent with the biochemical studies illustrated in Fig. 6.

DISCUSSION

One goal of this work was to develop a serum-free culture system which could be used to investigate the factors which regulate the endocrine functions of the human trophoblast. In the process of achieving that goal, we discovered that in the absence of serum, purified human cytotrophoblasts do not flatten out, aggregate, or form syncytia—as is seen in the presence of serum (Figs. 1 and 2). We thought it likely that the fibronectin in the serum played a role in the adhesion of these cells to the culture surface. We verified that fibronectin—as well as a variety of collagens—could, to a variable extent, replace the adhesion factors in serum (Fig. 3). These results raised the question: do human trophoblasts have a receptor for fibronectin?

We answered this question by plating trophoblasts onto fibronectin in the absence or presence of an R-G-D-S-containing hexapeptide, G-R-G-D-S-P, (Fig. 4), which is known to block fibronectin-receptor-specific cell attachment to fibronectin (Yamada *et al.*, 1985). This experiment demonstrated virtually complete inhibition of adhesion of the trophoblasts to fibronectin in the presence of the hexapeptide. Interestingly, the few cells that did adhere in the presence of the hexapeptide remained spherical. This suggests that human trophoblasts bind to fibronectin through an R-G-D-S-specific receptor (Akiyama and Yamada, 1987), and further, that interaction with fibronectin may facilitate spreading of these cells. Further experiments with type I and IV collagens (Fig. 5) suggested that these cells bind to type I collagen via fibronectin and that in contrast, association to type IV collagen does not involve fibronectin. Cytotrophoblasts synthesize and secrete fibronectin (Ulloa-Aguirre *et al.*, 1987) and the inability of cytotrophoblasts to flatten out and form syncytia in serum-free media in the absence of an exogenous matrix component suggests that the fibronectin produced by the cells is either insufficient in quantity or not the appropriate type to promote normal morphologic differentiation *in vitro*. The latter possibility is supported by biochemical studies on human placental fibronectin. Zhu and co-workers (Zhu *et al.*, 1984; Zhu and Laine, 1985) and Isemura *et al.* (1984) have determined that human placental fibronectin and serum fibronectin contain different carbohydrates. Zhu and Laine (1985) have demonstrated that placental fibronectin has high molecular weight polylactosamine carbohydrate and that this additional carbohydrate imparts to the placental fibronectin a decreased binding affinity to gelatin. Furthermore, these workers (Zhu and Laine, 1987) have shown that placental fibronectin acquires increasing amounts of these carbohydrates as gestation proceeds, raising the possibility that this change may play a role in placental separation from the uterus. These biochemical studies, therefore, may explain why human cytotrophoblasts cultured under serum-free conditions without exogenous matrix do not flatten in spite of the fact that they produce fibronectin.

Our experiments revealed that we could modulate morphologic differentiation—that is, progression of mononuclear cytotrophoblasts to multinuclear syncytial trophoblasts—by modifying the surface on which the cells were cultured. We wondered whether cytotrophoblasts cultured in the absence of serum without the precoating of the surface (thus constraining them to remain as single cells) could still differentiate biochemically (i.e., could still be stimulated to secrete hCG) in spite of their inability to form syncytial structures. The results presented in Fig. 6 clearly demonstrate that trophoblasts grown in the absence of serum can secrete hCG and that 8-bromo-cAMP augments this secretory activity whether they are placed on uncoated surfaces or surfaces coated with fibronectin, type IV collagen, or laminin. Thus, trophoblasts which remain as single

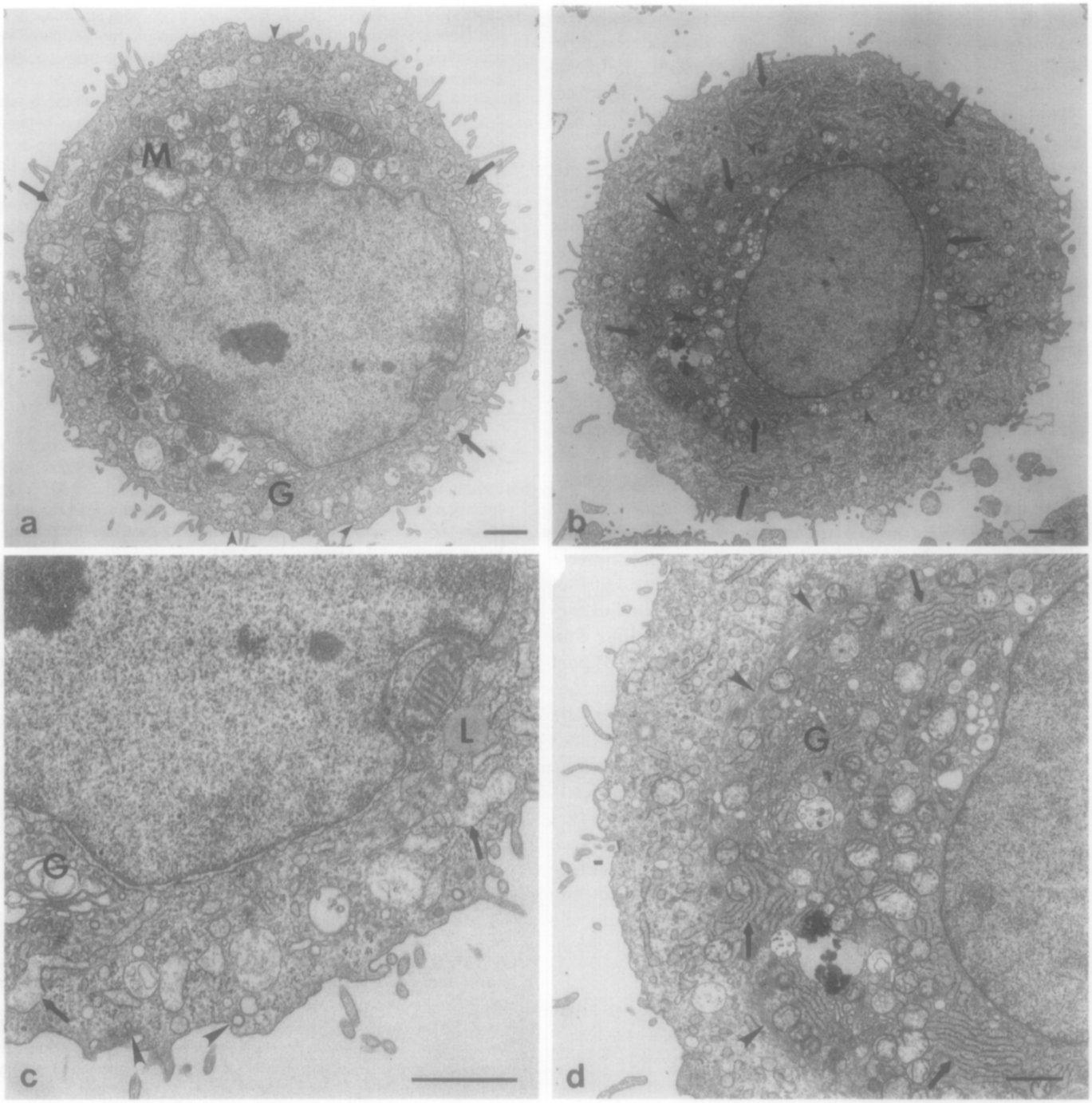


FIG. 7. Transmission electron microscopy of cytotrophoblasts cultured in serum-free media. Cytotrophoblasts were cultured in serum-free media for 24 hr without (a, c) or with (b, d) 1.5 m*M* 8-bromo-cAMP and processed as described under Materials and Methods. (a) Cross section of spherical control cytotrophoblast exhibiting microvilli, coated pits (arrow heads), golgi (G), mitochondria, numerous free polyribosomes, and short stacks of irregular RER (arrows). (b) Cross-section of treated cytotrophoblast exhibiting many RER stacks (small arrows), golgi apparatus (large arrow), bundles of perinuclear intermediate filaments (small arrow heads), and many small mitochondria (large arrow heads). (c) Enlargement from (a) showing golgi apparatus (G), coated pits (arrow heads), short stacks of irregular RER (arrows), lipid droplet (L), and numerous free polyribosomes. (d) Enlargement from (b) showing active golgi apparatus with numerous small vesicles (G), many stacks of RER (arrows), and intermediate filament bundles (arrow heads). Bars represent 1 μm.

mononuclear cells can express endocrine functions of the syncytial trophoblast.

To confirm this finding, we performed transmission electron microscopy on cytotrophoblasts grown without serum or precoating, with or without added 8-bromo-cAMP, to evaluate whether the cells when stimulated acquire the necessary machinery for synthesis and secretion of hormones. Cytotrophoblasts grown in serum-

free media exhibited relatively simple fine structural features after 24 hr of culture. They contained microvilli and coated pits, occasional lipid droplets, and scattered short segments of RER—consistent with cytotrophoblast ultrastructure (Boyd and Hamilton, 1970; Kliman *et al.*, 1986). Cells cultured in the presence of 8-bromo-cAMP remained spherical and mononuclear, but they were larger, contained stacks of dilated RER, had well-developed golgi, and contained greater numbers of mitochondria—features typical of the syncytiotrophoblast—all confirming their increase in biosynthetic capacity. Therefore, we have demonstrated that these cells differentiated both biochemically and structurally in culture in the absence of syncytium formation.

It is generally accepted that the cytotrophoblast is the undifferentiated stem cell of the villous trophoblast and that the syncytial trophoblast is the fully differentiated, end-stage trophoblast (Pierce and Midgley, 1963; Boyd and Hamilton, 1970). Some have suggested that biochemical differentiation can occur only after syncytial formation (Morrish *et al.*, 1987). Our work contradicts this notion. We believe that the trophoblasts of the human chorionic villi form a continuum of differentiated cells, some of which are mononuclear and others mutlinuclear. In addition, our results support the hypothesis that syncytia formation is not a prerequisite for biochemical differentiation. Therefore, the formation of the syncytiotrophoblast is not the trigger for biochemical differentiation, but is only one of the consequences of the differentiation program. This scheme parallels very closely the biology of myoblast differentiation (Holtzer *et al.*, 1980). The serum-free system described here, and its ability to disassociate morphologic from biochemical differentiation in the human trophoblast, should permit us to elucidate the mechanisms of trophoblast regulation and maturation.

We gratefully acknowledge Justina Minda for assistance in the electron microscopy and Julia Haimowitz for the preparation of the manuscript. This work was supported by USPHS Grants DK-07314 (L.C.K.), HD-06274 (J.F.S.), and HD-00715 (H.J.K.).

REFERENCES

AKIYAMA, S. K., and YAMADA, K. M. (1987). Fibronectin. *Adv. Enzymol.* **59**, 1–57.

BOYD, J. D., and HAMILTON, W. J. (1970). "The Human Placenta," pp. 167–174. MacMillan, London.

FEINMAN, M. A., KLIMAN, H. J., CALTABIANO, S., and STRAUSS, J. F., III. (1986). 8-Bromo-3′5′-adenosine monophosphate stimulates the endocrine activity of human cytotrophoblasts in culture. *J. Clin. Endocrinol. Metal.* **63**, 1211–1217.

HOLTZER, B., CROOP, J., TOYAMA, Y., BENNETT, G. H., FELLINI, S., and WEST, C. (1980). Differences in differentiation programs between presumptive myoblasts and their daughters, the definitive myoblast and myotubes. *In* "Plasticity of Muscle" (D. Pete, Ed.), p. 133. de Gruyter, New York.

HOSHINA, M., BOOTHBY, M., HUSSA, R., PATTILLO, R., CAMEL, H. M., and BOIME, I. (1985). Linkage of human chorionic gonadotrophin and placental lactogen biosynthesis to trophoblast differentiation and tumorigenesis. *Placenta* **6**, 163–172.

HOSHINA, M., HUSSA, R., PATTILLO, R., and BOIME, I. (1983). Cytologic distribution of chorionic gonadotropin subunit and placental lactogen messenger RNA in neoplasms derived from human placenta. *J. Cell Biol.* **97**, 1200–1206.

ISEMURA, M., YAMAGUCHI, Y., MUNAKATA, H., AIKAWA, J., KAN, M., YAMANE, I., and YOSIZAWA, Z. (1984). Isolation and characterization of human placenta fibronectin. *J. Biochem.* **96**, 163–169.

KARNOVSKY, M. (1965). A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. *J. Cell Biol.* **27**, 127A.

KLIMAN, H. J., FEINMAN, M. A., and STRAUSS, J. F., III. (1987). Differentiation of human cytotrophoblasts into syncytiotrophoblasts in culture. *In* "Trophoblast Research" (R. Miller and H. Thied, Eds.), Vol. 2, pp. 407–421. Plenum, New York.

KLIMAN, H. J., NESTLER, J. E., SERMASI, E., SANGER, J. M., and STRAUSS, J. F., III. (1986). Purification, characterization and in vitro differentiation of cytotrophoblasts from human term placentae. *Endocrinology* **118**, 1567–1582.

MORRISH, D. W., BHARDWAJ, D., DABBAGH, L. K., MARUSYK, H., and SIY, O. (1987). Epidermal growth factor induces differentiation and secretion of human chorionic gonadotropin and placental lactogen in normal human placenta. *J. Clin. Endocrinol. Metab.* **65**, 1282–1290.

PIERCE, G. B., JR., and MIDGLEY, A. R., JR. (1963). The origin and function of human syncytiotrophoblastic giant cells. *Amer. J. Pathol.* **43**, 153–173.

ULLOA-AGUIRRE, A., AUGUST, A. M., GOLOS, T. G., KAO, L.-C., SUKURAGI, N., KLIMAN, H. J., and STRAUSS, J. F., III. (1987). 8-Bromo-3′5′ adenosine monophosphate regulates expression of chorionic gonadotropin and fibronectin in human cytotrophoblasts. *J. Clin. Endocrinol. Metab.* **64**, 1002–1009.

YAMADA, K. M., AKIYAMA, S. K., HASEGAWA, T., HASEGAWA, E., HUMPHRIES, M. J., KENNEDY, D. W., NAGATA, K., URUSHIHARA, U., OLDEN, K., and CHEN, W.-T. (1985). Recent advances in research on fibronectin and other cell attachment proteins. *J. Cell Biochem.* **28**, 79–97.

ZHU, B. C.-R., FISHER, S. F., PANDE, H., CALAYCAY, J., SHIVELY, J. E., and LAINE, R. A. (1984). Human placental (fetal) fibronectin, increased glycosylation and higher protease resistance than plasma fibronectin. *J. Biol. Chem.* **259**, 3962–3970.

ZHU, B. C., and LAINE, R. A. (1985). Polylactosamine glycosylation on human fetal placental fibronectin weakens the binding affinity of fibronectin to gelatin. *J. Biol. Chem.* **260**, 4041–4045.

ZHU, B. C., and LAINE, R. A. (1987). Developmental study of human fetal placental fibronectin: Alterations in carbohydrates of tissue fibronectin during gestation. *Arch. Biochem. Biophys.* **252**, 1–6.