INTRODUCTION

Choriocarcinoma, a highly malignant tumor arising from the trophoblast, comprises a heterogeneous population of cells including cytotrophoblasts, intermediate trophoblasts, and syncytiotrophoblasts. In order to investigate trophoblast differentiation, we used centrifugal elutriation to separate cells from the JAr choriocarcinoma cell line according to their size and to further show that the resultant cell populations differ in their stage of differentiation. Two % of the cell population consists of large, multinuclear cells, which display the highest level of choric gonadotropin (CG) mRNAs. The increase in the CGβ mRNA with cell size is a consequence of the transcriptional mechanism, since agents which induce differentiation in JAR cells, i.e., methotrexate, increase the level of CGα and CGβ transcripts, cause a shift in cell size, and result in the formation of multinuclear cells. The multinuclear cells in the JAR population arise, at least partly, from kariokinesis without cytokinesis.

ABSTRACT

Choriocarcinoma, a highly malignant tumor arising from the trophoblast, comprises a heterogeneous population of cells that are apparently in different stages of differentiation. The native choriocarcinoma is a mixture of cytotrophoblasts, intermediate trophoblasts, and syncytiotrophoblasts (1) and lacks the presence of choriocarcin grams. Such tumors produce high amounts of CG2 and some synthesize hPL or pregnancy-specific β1-glycoprotein (1). In situ hybridization studies of sections from placenta and trophoblast tumors suggest that expression of these placentals proteins is linked to particular stages of trophoblast differentiation (2-4). Previous reports suggested that both in vivo and cultured choriocarcinoma cells apparently undergo a cell fusion and differentiate into CG-synthesizing cells (1, 5).

Cell lines derived from choriocarcinoma such as JEG-3, BeWo, and JAR were intensively used in order to investigate trophoblast differentiation. Although factors causing cytotrophoblast stem cells either to divide or to terminally differentiate are not known, adenosine derivatives (5, 6), MTX (7, 8), and cell fusion and differentiate into CG-synthesizing cells (1, 5).

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Here we show that, by using centrifugal elutriation, one can fractionate and isolate trophoblast cells according to their stage of differentiation. We follow the biological behavior of each separate fraction in culture, in order to study the mechanism of the differentiation pathway in choriocarcinoma.

MATERIALS AND METHODS

Cell Culture. The JAR choriocarcinoma cell line was maintained in Medium 199 containing 10% fetal calf serum, 25 mm HEPES (pH 7.4), penicillin (180 units/ml), streptomycin (100 μg/ml), and amphotericin B (0.2 μg/ml). Four × 10⁴ cells/cm² were plated in polystyrene culture dishes (Nunc). The medium was changed every 24 h. Every 3 days the cells were trypsinized with 0.05% trypsin-EDTA solution (Beit Hasekem) for 5 min and again plated at the same initial density or as described.

For JAR-Kpn, the CGβ CAT construct was stably integrated in the JAR cells now designated JAR-Kpn, containing the 5' region of the CGβ gene extending to the KpnI site (10). This corresponds to a distance of 3.5 kilobases from the mRNA Cap site.

Centrifugal Elutriation. Two to 2.5 × 10⁶ JAR cells were concentrated into 10 ml of Medium 199 containing 25 mm HEPES (pH 7.4) and loaded into a Beckman J2-21M elutriator rotor (Beckman, Palo Alto, CA), using a standard chamber and a Masterflex peristaltic pump (Cole-Parmer Instruments, Chicago, IL). Constant rotor speed was maintained at 2000 rpm. The elutriation buffer was HBSS containing 2% newborn calf serum at 4°C. The cells were loaded at a flow rate of 10 ml/min. The first 150 ml served as a washing step and were discarded. The flow rate was increased from 15 to 65 ml/min, in 5-ml steps, and 11 fractions (100 ml each) were collected. At the last flow rate, the centrifuge was stopped. The fractions were spun at 2000 rpm, and the pellets were resuspended in incubation medium. The first fraction (at a flow rate of 15 ml/min) from JAR cells contained mostly cell debris and was discarded.

Cell Size Determination. Cells from each of the fractions were suspended in HBSS, and their number and size distribution were determined using a Coulter Counter (Coulter Electronics, Harpenden, Herts, England).

Flow Cytometry. Five × 10⁴ cells from each of the fractions after elutriation were prepared for flow cytometric DNA analysis (11). Determination of the DNA content of the nuclei in each fraction was evaluated by FACS 440 cell sorter (Becton Dickinson, Sunnyvale, CA).

RNA Isolation and Northern Blot Hybridization. Total cellular RNA was isolated from the cells in each fraction immediately after elutriation by the guanidinium-thiocyanate/cesium-chloride method (12). Ten μg of each RNA sample were separated by 1% agarose-formaldehyde gel electrophoresis and transferred to a Hybond-N nylon filter (Amersham, England). The blots were hybridized with specific cDNA probes at 42°C in 50% formamide, 5X SSPE (sodium chloride, sodium phosphate, EDTA) 5X Denhardt’s solution, 0.1% SDS, and 0.1 mg/ml of herring testis DNA. The probes used for hybridization were CGα (13), CGβ (14), and hPL (15). The blots were washed twice in 0.1x standard saline citrate (0.15 M NaCl/0.05 M sodium citrate):0.1% SDS at 65°C and exposed to AGFA Curix film at -80°C.

Determination of DNA Synthesis. JAR cells (10⁴) were cultured in 12-well dishes in 1 ml of Medium 199 containing HEPES, fetal calf serum, and antibiotics as described above.

To determine incorporation of [3H]thymidine into DNA, 10 μl of [methyl-3H]thymidine (1 μCi/μl; 81 Ci/mmol; ICN Biomedicals, Irvine, CA) were added to the culture medium in each well. After 24 h of labeling, the medium was removed, the cells were washed twice with HBSS, 1 ml of distilled water was added, and the cells were scraped from the plate and frozen. After thawing, aliquots of the lysate were taken for protein determination by the Bio-Rad protein assay dye reagent (Bio-Rad Laboratories GmbH, Munich, Germany; Catalogue No. 500-0006). [3H]Thymidine incorporation was determined by tri-chloroacetic acid precipitation (16).
RESULTS

Using centrifugal elutriation we attempted to separate and isolate the various intermediate cells which were in different stages of the differentiation pathway. Cells were incubated for 72 h, trypsinized, and separated according to their sedimentation coefficient by centrifugal elutriation. We have separated 11 fractions and measured the physical, morphological features and the gene expression of each cell population. Fraction 1 contained mostly cell debris and was not analyzed. The percentage size distribution of cells is shown in Fig. 1A. The sum of the cells in Fractions 2 to 6 contained more than 80% of the whole cell population. The other fractions contained 1 to 5% each.

Centrifugal elutriation is used to obtain cell cycle stage-specific populations (17-19), by virtue of the increase in cell size as cells progress from G₀-G₁ to G₂-M. The DNA content histograms obtained from flow cytometric analysis for the cell fractions are shown in Fig. 1B. The nuclei in the elutriated fractions display increasing DNA content; indeed, JAr cells are exponentially growing cells. Cells in different stages of the cell cycle are represented in Fractions 2 to 6 From G₀-G₁ in Fraction 2 (2 times the haploid DNA content, 2n) through S in Fractions 3 and 4 to G₂-M in Fractions 5 and 6 (4n). In successive nuclei fractions, more than 4n DNA was detected and at least 6 to 8n in Fractions 9 to 11. From the flow cytometric analysis (Fig. 1B), the percentage of nuclei containing more than 4n DNA in each fraction was calculated (Fig. 1C). In Fractions 2 to 4, less than 1% of the nuclei contained more than 4n DNA, though from Fraction 5, there is a gradual increase in the percentage of nuclei with more than 4n DNA, reaching up to 43% in Fraction 11. Using the method applied for flow cytometric analysis, the DNA content in the nuclei and not in the intact cells was determined, as the cells were treated with trypsin, RNase, and detergent (11). The polyploid nuclei phenomenon (i.e., nuclei containing 8n) can be explained in two ways: either the nucleus contains 8n DNA; or there exist 4 tightly connected nuclei, each containing 2n DNA and not separated by the treatment.

Phase microscopy of JAr cells immediately after elutriation revealed a gradual increase in cell size in the different cell populations accompanied by an increase in the number of nuclei per cell (Fig. 2). Most of the cells in Fractions 2 to 7 are mononuclear (Fig. 2, A and B), but a profound increase in the percentage of multinuclear cells is revealed in Fractions 8 to 11. At least 50% of the cells in Fraction 11 are multinuclear (between 2 and 6 nuclei per cell; Fig. 2C). Phase microscopy of cells from Fraction 11, 96 h after plating (Fig. 2D), revealed at least 6 nuclei tightly connected. This phenomenon was observed in the majority of the cells in Fraction 11. Among unfractienced cells plated for 96 h, few cells with the multinuclei appearance can be seen (not shown). Cells from Fraction 11 were compared after 24 or 96 h of incubation, and an increase in the
Fig. 3. Percentage distribution of reelutriated JAr cells. Cells were separated using centrifugal elutriation, and each fraction was plated separately. After 72 h the cells were trypsinized, reelutriated, and counted. The percentage distributions of the reelutriated cells from Fraction 5 (A) and Fraction 11 (B) were determined.

number of nuclei per cell was observed.

We compared multinuclear JAr cells to multinuclear human placental syncytiotrophoblasts. Trophoblasts isolated from term placenta were separated using centrifugal elutriation. Fraction 11 contained multinuclear syncytiotrophoblasts. After flow cytometric analysis of syncytiotrophoblasts, only 2n nuclei were observed (not shown), indicating that each nucleus in the syncytiotrophoblast cell is separate from the other.

We addressed the question of whether the cells in Fraction 11 are in a terminal stage of differentiation or can reenter the cell cycle. Cells from Fractions 5 and 11 were cultured separately and, after 96 h, were trypsinized and reelutriated. The percentage of cells in each fraction was determined (Fig. 3). While the distribution of reelutriated cells from Fraction 5 (Fig. 3A) resembled the distribution of cells elutriated at time zero (Fig. 1A), most of the cells in Fraction 11 have not changed in size and were reisolated in the same fraction (i.e., Fraction 11; Fig. 3B). We conclude that the cells in Fraction 11 are not dividing and have exited the cell cycle.

DNA synthesis in the different fractions of JAr cells (as determined by measuring [$^3$H]thymidine incorporation into DNA/µg of protein) was the same in all the cell fractions when measured between 0 and 24 or 72 and 96 h after plating (data not shown). Despite the fact that cells in Fraction 11 are not dividing, their nuclei maintain an active incorporation of thymidine into DNA to the same extent as do the cells in the other fractions.

The expression of CG and other pregnancy-specific proteins is linked to particular stages of trophoblast differentiation (2, 20). CG is a placental specific protein and an established differentiation marker of trophoblast cells (20–22). We followed the expression of the CG genes in the different elutriated cells. RNA was isolated from the different elutriation fractions and hybridized with CGα and CGβ probes (Fig. 4). CGα (Fig. 4A) and CGβ (Fig. 4B) mRNAs were not detected in Fractions 2 to 4, but gradually increased in RNA from Fractions 5 to 10 reaching their highest level in Fraction 11.

Although the total population of JAr cells does not seem to express hPL, this does not exclude the possibility that only a small fraction of the total cell population does express this gene. We, therefore, isolated RNA from the different elutriation fractions and hybridized it to the hPL probe. None of the elutriated fractions expressed hPL mRNA.

In order to investigate whether the increase of CGβ mRNA level as a function of the cell differentiation state is a consequence of the transcriptional mechanism, we cultured JAr cells stably transfected with a plasmid containing the CAT reporter gene linked to the promoter of the CGβ gene, designated JAr-Kpn (10). After 72 h of incubation the cells were subjected to centrifugal elutriation. The CAT activity was measured in the cell extracts from each fraction (Fig. 5A). The spots corresponding to the acetylated chloramphenicol were excised and counted (Fig. 5B). A gradual increase in the promoter activation in the different cell fractions is seen from Fractions 2 to 10, with a sharp increase in Fraction 11 (5-fold as compared with Fraction 2).

Following MTX treatment, the majority of cells within a
BeWo culture transform morphologically, acquiring many of the same ultrastructural characteristics that have been reported for the differentiated in utero syncytiotrophoblast (7). Following 72 and 120 h of incubation in the presence of 1 μM MTX, JAr cells were subjected to centrifugal elutriation, and the percentage distribution of the cell fractions was determined (Fig. 6). MTX treatment induced a shift in cell size in the majority of cells in culture toward the size of cells in Fraction 11. This observation is also based on phase microscopy (not shown). While the majority of the cells in the untreated cultures are collected in Fractions 2 to 5 (Fig. 1A), most of the cells in cultures treated for 72 h with MTX are collected in Fractions 5 to 8 (Fig. 6A) and in Fractions 8 to 11 in cultures treated for 120 h (Fig. 6B). MTX did not change thymidine incorporation into DNA (not shown) as was previously reported (7, 8).

Arbiser et al. (8) reported an induction of synthesis of the mRNA encoding CGα and CGβ by MTX in BeWo and JEG-3 cell lines. They have also shown that hydroxyurea induces the activation of the CGα promoter. Similarly incubation of JAr-Kpn cells for 72 h with MTX resulted in a 5-fold increase in the activity of the CAT reporter gene driven by the CGβ3 promoter, compared to control (Fig. 7).

In order to elucidate whether the induced expression of CGα by MTX is linked to an increase in cell size, cells were grown for 96 h in the presence of MTX. After incubation, cells were trypsinized and separated using centrifugal elutriation. RNA was isolated from the different elutriation fractions and hybridized with the CGα probe (Fig. 8). The level of CGα mRNA in Fractions 5 to 11 in the MTX-treated cells (Fig. 8B) was higher than the control (Fig. 8A), though no gradual increase of CGα mRNA is observed with increasing cell size. We suggest that MTX activates the CGα promoter regardless of the change in cell size. Similar results were seen with CGβ mRNA (not shown).

DISCUSSION

During differentiation, pluripotent stem cells give rise to committed precursor cells which lose their capacity to divide as they reach the terminal stages. As differentiation progresses, cell division is reduced and eventually lost. Cancer cells fail to differentiate normally, though many tumors grown in tissue culture can be induced to differentiate (23).

The trophoblast differentiation pathway includes morphological intermediate or transitional cells, with distinctive biochemical and cytological characteristics, which are the direct precursors of syncytial trophoblasts. The above observations imply that multiple intermediates are formed during the trophoblast differentiation pathway (2, 6). Normal placentae, native choriocarcinoma, and choriocarcinoma cell lines comprise a heterogeneous population of intermediate cells in different stages of differentiation, the isolation of which facilitates the elucidation of the differentiation pathway. Isolation of such cells has previously not been described.

Centrifugal elutriation is currently a widely used preparative cell separation technique, that under optimal conditions can separate cells that differ only slightly in their size or their sedimentation velocity. This is the method of choice for the separation of cells according to their cell cycle stage (17-19).

Here we report on the separation of JAr cells according to their cell size and show that the various cell populations differ in their stage of differentiation. Based on morphological and biochemical characteristics observed in the different cell fractions obtained by centrifugal elutriation, we propose that JAr cells are dividing cells, but very few exit the cell cycle and differentiate. Those cells that do differentiate express both CGα and CGβ, the expressions of which are closely coupled. The JAr differentiation includes an increase in cell size accompanied with polyploidy. However, further differentiation is limited, and hPL synthesis is not seen. The polynuclear cells (Fraction 11) are in a terminal stage of JAr differentiation, as these cells exit the cell cycle and express the highest level of CGα and CGβ, as was evident in the level of CGα and CGβ protein (5).
and mRNA. Furthermore, the increase in the CGβ mRNA with cell size is a consequence of an increase in the rate of transcription as shown through the CAT reporter gene. Agents which induce differentiation in choriocarcinoma cell lines, i.e., MTX (7, 8) and hydroxyurea (8), increased the CGα and CGβ transcripts level and also caused a shift in cell size and the formation of multinuclear cells.

The multinuclear cells may arise from either cell fusion (as was shown for placent al cytotrophoblasts) or from karyokinesis which is not followed by cytokinesis. We suggest that the multinuclear cells observed in JAr cultures are formed by karyokinesis without cytokinesis, since active DNA synthesis is measured despite the fact that they have differentiated and little if any cell division is seen. Further investigation in native choriocarcinoma can provide the answer to whether the multinuclear cells are formed by the same mechanism as was described here for JAr cells.

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**Differentiation of Choriocarcinoma Cell Line (JAr)**

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