Kinetics of Growth and Ectopic Production of Human Chorionic Gonadotropin by an Ovarian Cystadenocarcinoma Cell Line Maintained in Vitro

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ABSTRACT

An established ovarian papillary cystadenocarcinoma cell line, designated 163, released immunoreactive human chorionic gonadotropin (HCG) in vitro into the culture medium. The maximal rate of this ectopic activity (10 to 15 ng/day/10^6 cells) occurred at the onset of the logarithmic phase of cell growth and greatly exceeded the intracellular HCG content of cells approaching confluency (0.037 ng/10^6 cells). The HCG production was not detected during the plateau phase of growth.

The rate of cellular proliferation and HCG release depended upon the frequency of media change in a manner suggesting that the two processes are interrelated and may be affected by such environmental factors as cell density, nutrient availability, and the accumulation of waste products. The addition of purified HCG to the cultures had no appreciable effect on either the cell growth or the accumulation of HCG.

The release of HCG into the medium was greatly stimulated in the presence of sodium butyrate at concentrations from 1 to 10 mM, despite the fact that these concentrations of butyrate resulted in a marked decrease in the cell number/culture in comparison with control media. Equivalent amounts of sodium acetate had no effect on either cell growth or the release of HCG.

INTRODUCTION

Large quantities of HCG are secreted by the normal placenta and various trophoblastic tumors. Relatively smaller amounts of HCG are also found in the serum of approximately 16% of patients with cancers that do not contain trophoblastic elements (6, 13, 25, 26, 30). In this instance the HCG is considered to result from ectopic hormone synthesis by the tumors. The ectopic production of HCG has been confirmed by the observation of HCG release in vitro by 3 established cell lines derived from bronchogenic and uterine cervical carcinomas (8, 12, 22, 23). Moreover, various clones of these cell lines displayed specialization toward the production of either the complete hormone or 1 of its subunits (15-17, 28). The efficiency of HCG production by these cells in vitro appeared to depend on environmental factors such as pH, cell population density (15), and the chemical composition of the media (8, 12, 17).

This report deals with the kinetic aspects of the HCG release in vitro by an established cell line derived from a papillary cystadenocarcinoma of the ovary (9). The effects of culture conditions on the process are described. A partial characterization of the HCG secreted by these cells has been presented elsewhere (4).

MATERIALS AND METHODS

Cell Line 163. The establishment of cell line 163 has been described in detail elsewhere (9). The cells were obtained from ascitic fluid collected in June 1972 from a 45-year-old woman who, 1 year earlier, had been found to have Stage III papillary cystadenocarcinoma of the ovary. No trophoblastic elements have been found in multiple histological sections of the tumor. Cells that had been passaged 40 to 80 times were used in these studies. Cell line 163 has a glucose-6-phosphate dehydrogenase isoenzyme with the type B electrophoretic mobility distinguishing these cells from HeLa cells (10, 19).

Cell Culture Conditions. Cell line 163 has been grown in Ham's F-10 medium (Flow Laboratories, Rockville, Md.) supplemented with 20% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 /ug/ml), and kanamycin (50 /g/ml) in plastic tissue culture flasks (Falcon Plastics Co., Oxnard, Calif.) at 37° in a humidified atmosphere of 5% CO2 and 95% air.

Confluent cultures were dispersed using 0.1% trypsin in 0.02% EDTA and 0.9% NaCl solution at pH 7.2 to 7.4 (all reagents were from Flow Laboratories). Cell monolayers were first preincubated for 15 to 30 min at 37° in the EDTA solution without trypsin to ensure a good cell separation. Subsequent trypsin treatment resulted in cell detachment within 5 to 10 min. The cell suspension was then mixed with an equal volume of the complete medium and centrifuged for 5 min at 150 × g. The pelleted cells were washed once more with the complete medium and counted in a hemocytometer. Cell viability was assessed using the trypan blue exclusion technique, and the cell batches with viability lower than 95% were not used in these experiments. At least 100 but usually 200 to 500 cells were counted in each batch. For the experiments described below, the cells were inoculated at a density of 0.5 × 10^6 cells in 5 ml of medium per flask (25-sq cm growth area).

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$ The abbreviation used is: HCG, human chorionic gonadotropin.

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Within each experimental series single batches of cells were used by pooling cells from sister flasks derived from 1 monolayer at the preceding passage. Media within each experiment were prepared from the same batches of commercial solutions. Stock solutions of the chemicals to be tested were prepared at 5 times the desired final concentration in the same media and were used by combining 1-ml aliquots with 4 ml of the standard complete medium inside the flask.

**Cell Counts in Monolayers.** Cells in the monolayers were counted *in situ* after fixation with methanol and staining with Wright's stain. Cell counts were performed with an inverted phase-contrast microscope fitted with a calibrated rectangular grid inserted into 1 of the eyepieces, which subdivided the observation field into 36 squares. The mean number of cells per culture flask (25 sq cm) was based on the cells counted in 5 distant fields at a 100- or 200-fold magnification, depending on cell density. The results are expressed as the mean of cell numbers observed in 2 or more replicate cultures. Where S.D.'s of the cell numbers are given, at least 3 cultures were examined. Cells washed away during fixation and staining were disregarded, since tests with intact monolayers indicated that their number represented approximately 1 to 2% of the total cell number, well within the range of variation of cell counts from field to field. This method of cell counting was chosen because cell line 163 cannot be readily and uniformly dispersed from a monolayer with the use of trypsin and/or EDTA. When applied to monolayers of cells that disperse easily in trypsin (1), this method of cell counting gives results comparable to those obtained by hemocytometer counts of cell suspensions. Although such a method of cell counting is subject to error due to an uneven distribution of the cells throughout the growth surface, it eliminates the substantial error caused by cell loss occurring during the process of trypsination. This error was particularly noticeable in cultures treated with toxic agents such as butyrate and in those subjected to media starvation.

**Culture Medium Collection.** After various periods of incubation, the medium was centrifuged for 5 min at 150 × g and then for 10 min at 1000 × g, decanted, and stored in tightly capped plastic tubes at −18°. Such storage for up to 6 months did not alter the reactivity of HCG in the β-HCG radioimmunoassay. Before the assay the samples were coded and randomized. All the samples from a given experimental series were assayed within a single radioimmunoassay run.

**Intracellular HCG.** The replicate cultures of cell line 163 were grown in large (75-sq cm) culture flasks for 7 days with media change on Days 2, 4, and 6. Confluent monolayers were rinsed 3 times with fresh Ham's medium without serum and once with 0.02% EDTA solution in 0.9% NaCl solution. Cells were dispersed with trypsin as above, pooled, divided into 2 equal batches, and centrifuged at 150 × g, followed by 2 additional washes with 50 ml of the complete medium and recentrifugation. The cell number in each batch was determined using a hemocytometer, and the cells were centrifuged once more and suspended in complete medium in a final volume of 1.5 ml each. Cells from 1 batch were disrupted by rapid freezing and thawing 3 times with the use of a dry-ice methanol bath alternating with a 37° water bath. The second cell batch was sonically disrupted twice for 15 sec with a 1-min intermission with the use of a Model W 185 Sonifier cell disruptor (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) at 100 watts. During sonication and the 1-min break, the cell suspension was cooled in ice. Subsequent phase-contrast microscopic examination of both suspensions in the presence of trypan blue did not reveal intact, viable cells. Both samples were centrifuged for 20 min at 10,000 × g at 2°, and the supernatant fluids were stored at −18° until assayed for HCG.

**Radioimmunoassay of HCG.** The β-HCG radioimmunooassay described in detail elsewhere (29) was used throughout these studies. The urinary HCG reference preparation, CR 117, was provided by the Hormone Distribution Officer, National Institute of Arthritis, Metabolism and Digestive Diseases, NIH, Bethesda, Md. The standard curves for the radioimmunoassay were made in fresh Ham's F-10 medium. There was no immunoreactive HCG present in the medium when assayed against standards prepared in 0.01 n phosphate-buffered saline, pH 7.8, nor was there interference with the double antibody radioimmunoassay reaction by the medium. The lower limit of sensitivity in this assay is 0.2 to 0.5 ng of HCG per ml. Neither free α nor free β subunits of HCG were detected in the tissue culture medium (4). Since the material released by cell line 163 into the medium behaved like native HCG in the β-HCG radioimmunoassay, the rat Leydig cell radioreceptor assay, and the rat ventral prostate bioassay (4), it will be subsequently referred to as HCG.

**Statistical Methods.** Student's paired t test was used to compare the cell numbers and the HCG concentrations in the experimental and control cultures. A statistically significant difference was considered to be present at $p < 0.05$. A modification of the statistical methods of Rodbard (24) was used for dose interpolation for the radioimmunoassay data.

**RESULTS**

**Growth and HCG Production in Control Media.** Monolayers of cell line 163 could be maintained in culture for a considerable time provided that fresh medium was given every 2 days (fed cultures). The cell density in such cultures often reached 10⁷ cells/25-sq cm growth area. The cell growth initially proceeded in a logarithmic fashion, but after approximately 6 days it became progressively slower (Chart 1). The presence of HCG in the culture medium was first detected on the second day after cell seeding. The total daily output of HCG per 25-sq cm flask increased slightly during the first 6 days of culture. However, since the cell growth during that period was considerably faster, the HCG production calculated on a "per cell" basis decreased rapidly. This decrease was roughly linear on a semilogarithmic plot of the rate of HCG production versus time (Chart 1, circles), as long as HCG could be detected. The total HCG production/culture/day was maximal at the time when the cells attained confluence. The subsequent increase in the cell number was much slower and resulted in a progressive crowding of the monolayer without the formation of multiple layers. Several dense monolayer cultures were maintained for up to 4 months, during which time no...
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Effects of Exogenous HCG. The addition of 4 ng of purified urinary HCG to each 5 ml of medium did not alter the growth of the cells of line 163, as judged by the cell number/flask observed on Day 11 for the nonfed cultures and on Day 21 for the regularly fed ones. The amount of HCG released by these cells into the medium was similar regardless of whether exogenous HCG was present. In the nonfed control cultures, 16.75 ng of HCG (S.D., 1.1 ng) were observed on Day 11, compared with 21.56 ng (S.D., 0.6 ng) in those which received 4 ng of urinary HCG at the time of inoculation. These values are not significantly different after correcting for the amount of exogenous HCG. Similarly, in the cultures in which the medium was changed additional HCG production could be detected.

Cells that were treated identically to those above but that did not receive fresh media every 2 days initially grew and produced HCG at approximately the same rate as the fed cultures. However, the subsequent rate of decrease in these 2 processes was much higher (Chart 2). The maximal cell number/culture reached only 2 to 3 x 10^6 compared with 10^7 in the refed cultures. In the absence of media change, gross morphological changes were observed in the monolayer after approximately 6 days in culture. This was followed by cell detachment and death. Despite these morphological changes the total amount of HCG in such cultures remained consistent at the maximal level, indicating that no degradation of the immunoreactive HCG took place.

Production of HCG by the starved but not confluent cultures could be restored by the addition of fresh medium, provided that a portion of the monolayer remained viable (Chart 3). This is in contrast to the effect of the repeated addition of fresh media to the fully confluent cultures (Chart 1), which prevented cell death but did not restore HCG production.

Intracellular HCG. Serial dilutions of the supernatants from the sonicated or frozen and thawed cells were parallel to the HCG reference preparation in the β-HCG radioimmunnoassay. The amount of intracellular HCG determined after the destruction of trypsinized cells by sonication (0.033 ng/10^6 cells) and freeze-thawing (0.040 ng/10^6 cells) corresponds to approximately 0.3% of the daily HCG output at the peak of the productive period (Day 1) and to less than 2% of the HCG output on Day 7, at which time the intracellular HCG was determined.

Chart 1. Cell growth, HCG accumulation, and HCG production rate in cultures of cell line 163 in which a complete media change was performed at the times indicated by the arrows. Each point represents an average of the results from 2 replicate flasks. Actual amounts of HCG observed at the end of each consecutive time period: calculated total HCG output by the cultures from the time of inoculation.

Chart 2. Cell growth, HCG accumulation, and HCG production rate in cultures of cell line 163 maintained without media change. Each point represents an average of the results from 2 replicate flasks.

Chart 3. Accumulation of HCG in cultures of cell line 163 in which the start of media change was delayed. No feeding throughout the 13 days after seeding; media first changed on the seventh day of culture and changed every other day thereafter; media first changed on the fifth day of culture and changed every other day thereafter. Each point represents the mean of 3 replicate cultures.
every 2 days beginning with Day 1, the corrected values for the total HCG released over a 21-day period into the control medium (23.17 ng; S.D., 1.3 ng) and the HCG-containing medium (23.66 ng; S.D., 3.0 ng) were not significantly different from each other.

**Effects of Sodium Butyrate.** Addition of varying concentrations of sodium butyrate (1 to 10 mM) to the culture medium stimulated the HCG production by cell line 163 throughout a period of 11 days when compared to similar cultures receiving the same molar concentrations of sodium acetate (Chart 4; Table 1). Sodium acetate had no effect on HCG production in comparison with media controls (Chart 4). Cell growth was not affected by the presence of sodium acetate during 11 days in culture, whereas 4 and 10 mM butyrate produced a significant decrease in cell numbers/flask (Chart 5). A statistically insignificant effect was also seen with the 0.2 and 1.0 mM butyrate. Similar, but less drastic, concentration-dependent effects of the butyrate on cell growth were also seen after 5 days in culture (not shown).

The rate of HCG production on a per cell basis was greatly increased in the presence of sodium butyrate relative to the media controls and the acetate-containing cultures. For 2 experimental series a ratio of the average production rates over a given 2-day incubation period in the presence of the butyrate and acetate was calculated (Table 1). Such a ratio increased from approximately 1.2 on Days 1 to 3 to over 100 on Days 9 to 11. This effect was concentration dependent and appeared to intensify with the time of culture following the first 3 days after inoculation. The variability of the HCG production rate in the cultures treated with the higher butyrate concentrations (Table 1) most probably reflects the inherent error in the cell-counting methodology when the absolute number of cells is reduced below $5 \times 10^5$ cells/flask. Addition of sodium butyrate to fully confluent, healthy cultures of cell line 163 had no significant effect on their production of HCG.

**DISCUSSION**

The presence of HCG in the serum of patients with...
nontrophoblastic neoplasms of diverse tissue origin has been described by several authors (6, 13, 25, 26, 30). However, immunoreactive HCG-like material has also been reported in the sera of patients with benign disorders (30) and in normal human tissues (5, 7, 31). Therefore, ectopic HCG production by malignant tumors cannot be established with certainty without the demonstration of HCG elaboration by the isolated tumor cells. Such ectopic production of HCG or its subunits in vitro has been previously demonstrated for a hepatoblastoma grown in a short-term culture (3), cell line ChaGo derived from a bronchogenic carcinoma (23), and cell lines HeLa (12) and CaSki (22), both from squamous cell carcinomas of the uterine cervix.

Although ovarian epithelial cancers are among the tumors most frequently associated with ectopic HCG production (25, 30), there have been no previously reported examples of in vitro HCG release by an ovarian carcinoma cell line. Cell line 163, used in this study, was initiated from the ascitic fluid of a patient with a papillary cystadenocarcinoma of the ovary. For elimination of the possible presence of trophoblastic elements in the neoplasm, the original sections from the primary tumor were reexamined. No trophoblastic cells were identified. The cells have been maintained in culture for 5 years and passaged over 100 times. The cells have retained their epithelioid morphology in monolayer culture and can be readily distinguished from the other cell lines derived from gynecological cancers that have been grown in this laboratory.

Several recent reports have indicated that a large number of established tumor cell lines have been contaminated or replaced by HeLa cells (11, 19, 20). Although HeLa cells have never been knowingly grown in this laboratory, it was important to eliminate such contamination of cell line 163 from consideration, since HeLa cell clones secrete HCG or its subunits in vitro (8, 12, 16, 17). Electrophoretic analysis of the glucose-6-phosphate dehydrogenase of cell line 163 revealed the presence of the type B isoenzyme, usually considered to be a sufficient basis for excluding HeLa cell contamination (10).

The time course of the cell growth and HCG release into the medium in cell line 163 cultures (Charts 1 and 2) suggests that the 2 processes are interrelated since HCG release was observed only when a net increase in the cell number took place. Cessation of growth was accompanied by a fall in the HCG production rate to a level below detection. The pattern of cell growth and HCG release is similar to that observed in the ChaGo cell line (23). The mechanisms responsible for halting cell growth and HCG production by cell line 163 remain unknown, but it is apparent that an inhibitory effect of the accumulated HCG or deprivation of essential nutrients are not directly involved, since the addition of fresh medium to the confluent, nonproductive cultures did not bring about a renewed HCG release or cell growth. Moreover, exogenous HCG, added in an amount typical for stagnant cultures, had no apparent effect on the amount of HCG elaborated by the vigorously growing ones.

The peak HCG production rate by cell line 163 is 15 ng/day/10⁶ cells, which is approximately 10 times greater than that estimated for the ChaGo cell line (23). By comparison, there have been no previously reported examinations of established tumor cell lines have been contaminated or replaced by HeLa cells (11, 19, 20). Although HeLa cells have never been knowingly grown in this laboratory, it was important to eliminate such contamination of cell line 163 from consideration, since HeLa cell clones secrete HCG or its subunits in vitro (8, 12, 16, 17). Electrophoretic analysis of the glucose-6-phosphate dehydrogenase of cell line 163 revealed the presence of the type B isoenzyme, usually considered to be a sufficient basis for excluding HeLa cell contamination (10).

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The peak HCG production rate by cell line 163 is 15 ng/day/10⁶ cells, which is approximately 10 times greater than that estimated for the ChaGo cell line (23). By comparison, 3 gestational choriocarcinoma cell lines, JEG-3 (15), Jar (27), and BeWo (21), and the SCH cell line, derived from a gastric choriocarcinoma (14), release approximately 120, 400, 1000, and 130 ng of HCG per day per 10⁶ cells, respectively, during the maximal production period. The rates of in vitro production of HCG by these cell lines reflect the disparity between eutopic and ectopic HCG secretion by neoplasms in vivo; patients with trophoblastic neoplasms generally have much greater quantities of immunoreactive HCG in their circulation than do those patients with nontrophoblastic neoplasms (2).

The in vitro HCG production by cell line 163 is stimulated by the addition of sodium butyrate to the growth medium. Similar observations have been made by other investigators in studies on HeLa and ChaGo cell lines (8, 12, 17). Although the stimulation index for cell line 163 computed on a per cell basis (Table 1) appears considerable when comparing cultures containing butyrate to the controls, the absolute rate of HCG production/cell does not exceed twice the peak rate determined for the cultures grown in the standard media (Charts 1 and 2). The effect of the butyrate on HCG production may be secondary to its toxicity toward the cells. By eliminating a considerable portion of the cells within the monolayer, the butyrate may produce a situation of no cell-to-cell contact, similar to that encountered in the very early phases of growth. Since HCG production is maximal during the rapid growth phase, a return to this stage, induced by butyrate, may result in a resumption of HCG production. This hypothesis is supported by the observation that the effect of the butyrate on HCG production by cell line 163 is negligible when the processes are proceeding at a peak rate (Days 1 to 3). Also, butyrate has no effect on HCG production by cell line 163 when tested over a period of 2 days in healthy, confluent cultures. During this short period the toxic effects of butyrate are not yet morphologically evident.

Recent studies by Chou et al. (8) have demonstrated that sodium butyrate in a concentration of 2 mm suppresses the production of HCG and the α subunit of HCG from 3 trophoblastic cell lines (JEG-3, Reid, and BeWo) while stimulating the production of this hormone and its α subunit by 3 nontrophoblastic cell lines (HeLa CCL2, HeLa S₁, and ChaGo). This differential effect of butyrate on HCG production and the quantitative differences in HCG production between trophoblastic and nontrophoblastic cell lines suggest that a fundamental difference exists in the control mechanism for production and/or secretion of HCG in cells derived from trophoblastic tissue that is normally capable of HCG production and in those that have acquired this ability through neoplastic transformation.

The data presented herein indicate that environmental factors such as cell density, nutrient availability, and accumulation of waste products may alter growth dynamics and HCG release. Under favorable culture conditions, these 2 processes appear to be closely related and concordant. However, they may be dissociated under special circumstances, such as following the addition of toxic levels of butyrate. Analogous observations have been made in patients with trophoblastic and nontrophoblastic tumors. In patients with trophoblastic tumors, the serum or urinary
HCG titer generally reflects the tumor burden (2). Although a similar situation may be found in patients with nontrophoblastic neoplasms associated with HCG production, several examples of a dichotomy between cell growth and serum levels of HCG have been noted during therapy (3, 18, 25). The evaluation of the effects of chemotherapeutic agents on these parameters in cell line 163 and other HCG-producing tumors maintained in vitro may elucidate the mechanism underlying this dichotomy.

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