Differential Modulation of Human Chorionic Gonadotropin Production by Methotrexate in Normal and Malignant Placental Cultures and Its Increase by Dibutyryl Cyclic Adenosine Monophosphate and/or Actinomycin D in Normal Cultures

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ABSTRACT

The influence of methotrexate (MTX), dibutyryl cyclic AMP, and actinomycin D on production of human chorionic gonadotropin (HCG) in normal first trimester human placental organ cultures was compared. Actinomycin D (10^{-6} to 10^{-4} M) elevated HCG production by as much as 3.5-fold in normal placenta, and a 2-fold increase in HCG levels was obtained by treatment with dibutyryl cyclic AMP (1 mM) and theophylline (1 mM). The combination of dibutyryl cyclic AMP (1 mM) plus theophylline (1 mM) and actinomycin D (10^{-4} M) additively enhanced HCG production by 4.5-fold. In contrast, HCG levels in normal placental organ cultures were unaffected by MTX (10^{-4} to 10^{-5} M) despite several differing treatment regimens. The JAr line of human choriocarcinoma cells, on the other hand, exhibited an 8-fold increase in HCG levels following MTX exposure (10^{-4} M).

Incorporation of selected radiolabeled precursors of the de novo and salvage pathways of DNA synthesis was evaluated to assess potential metabolic alterations underlying the differential HCG response of these cultures to MTX. Deoxycytidine incorporation into DNA was decreased similarly in both normal and malignant placenta following MTX exposure. However, deoxycytidine incorporation was inhibited by MTX in normal placental cultures but was elevated by as much as 4-fold in JAr cultures exposed to MTX. Thymidine incorporation into DNA was increased in both groups in the presence of MTX; however, thymidine incorporation was more profoundly stimulated (5-fold) in normal placenta than in JAr cultures (2.5-fold). These data indicate dissimilar utilization of the de novo and salvage pathways of DNA synthesis by these cultures which may explain their differential responsiveness to MTX.

INTRODUCTION

Choriocarcinoma is a cancer of trophoblastic origin which secretes HCG (1, 2), a useful biochemical marker for assessing the success of chemotherapy (3). Patients with choriocarcinoma who undergo MTX or AMD therapy reportedly exhibit transient rises in HCG (3, 4), followed by a rapid decline with continued treatment. Cultured human choriocarcinoma cells also exhibit a rise in HCG content followed by a decline when treated with a variety of compounds including the chemotherapeutic agents MTX (5–8) and AMD (9, 10), as well as dbcAMP (11–13). In contrast, we are unaware of any reports describing similar elevations of HCG levels in patients receiving therapy for benign trophoblastic conditions, such as ectopic pregnancies, or by normal trophoblast in vitro.

The paradoxical clinical findings in which MTX and AMD enhanced HCG secretion in malignant but not in benign trophoblast encouraged us to determine whether this differential HCG response to MTX could be reproduced in vitro. To date, no direct in vitro comparisons havereported the response of HCG levels of normal and malignant trophoblast to methotrexate. In this report, we describe the results of studies comparing HCG responsiveness to MTX, AMD, and dbcAMP in normal human placental organ cultures. In addition, we contrast HCG production and incorporation of radiolabeled precursors of DNA synthesis in both normal and malignant placental cultures in response to MTX. These results, taken together with our previous finding that epidermal growth factor stimulates HCG production in JAr but not in placental cultures (14), suggests that HCG production in response to exposure to certain metabolic agents is differentially modulated in normal and malignant placental cells.

MATERIALS AND METHODS

Cell and Organ Culture. Organ cultures of human first trimester (8–12 weeks) placenta were prepared as previously described (15). JAr choriocarcinoma cells (16) were a kind gift from Dr. Roland Pattillo, Department of Obstetrics and Gynecology, Medical College of Wisconsin (Milwaukee, WI). Stock cultures of JAr cells were subcultured as previously described (5). All cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. All experiments were performed in triplicate and data are the means of duplicate or triplicate replicates.

Normal placental organ cultures and JAr cells were maintained in the following media as required by the particular protocol: DMEM with glucose (1000 mg/liter), WGM (5), or a customized CMRL 1066 (GIBCO formulation 78-0584, free of thymidine and folate but with hypoxanthine at 25 mg/liter). All media were supplemented with 1-glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 µg/ml), Fungizone (2.5 µg/ml), and neomycin (100 µg/ml). Additionally, during the first 24 h of culture, JAr cells were plated into medium that contained 5% fetal bovine serum (North American Biological Co., Miami, FL). After the initial 24 h of culture, fresh serum-free or serum-containing medium with or without MTX, AMD, or dbcAMP (Sigma Chemical Co., St. Louis, MO) was added as described below. Because serum contains salvage pathway metabolites (17) and dTdT (18), which inhibit MTX mediated HCG production (5, 7), we used serum-free medium for all assessments of MTX effects on HCG content of test cultures. Medium was changed daily for all cell cultures. Placental cultures were maintained in serum-free culture unless specified otherwise. Organ cultures maintained on customized CMRL, but not DMEM, were supplemented with insulin (1 µg/ml), hydrocortisone (0.1 µg/ml), and retinyl acetate (0.1 µg/ml), also from Sigma.

Received 10/25/85; revised 6/27/86, 9/24/86; accepted 10/2/86.

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4 The abbreviations used are: HCG, human chorionic gonadotropin; MTX, methotrexate; AMD, actinomycin D; dTdT, thymidine; dTdR, deoxythymidine; dCyd, deoxycytidine; DMEM, Dulbecco’s minimal essential medium; dbcAMP, dibutyryl cyclic AMP; WGM, Waymouth-Gey’s medium; HAT, 200 µM hypoxanthine, 400 µM aminopterin, 16 µM thymidine.
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were obtained from Sigma. Although individual placentas differ from each other in HCG content (14, 15) and [H]dThd incorporation (14), all placentas showed the same qualitative, if not quantitative, response to any particular agent. Thus, all data depicted for any particular experiment are from a single representative placenta chosen as that showing the average response. All data points represent the mean of duplicate or triplicate cultures. Three placentas were assessed for each experiment.

Measurement of HCG. Supernatants were collected daily or every 48 h for quantitation of HCG by double antibody radioimmunoassay, as previously described (7). Cultures were prepared in either duplicate or triplicate and HCG assays were performed using two sample dilutions in duplicate. HCG content of replicate samples varied by less than 5%. To eliminate any possibility of artificially induced differences in data as a result of interassay variation, all samples from a single placenta or from JAr cultures in a single experiment were measured within the same assay.

Incorporation of Radioactively Labeled Precursors into DNA and Measurement of Protein Concentration. To assess precursor incorporation in JAr cultures, isotope was applied 3 h prior to harvest. We selectively used methyl-[H]-thymidine (dThd: 1 μCi/ml; specific activity, 40-60 Ci/mmol); [5-3H]deoxyctydine (dCyd: 1 μCi/ml; specific activity, 40-60 Ci/mmol); or [6-3H]deoxyuridine (dUrd: 1 μCi/ml; specific activity, 15-30 Ci/mmol) obtained from New England Nuclear (Boston, MA). Because MTX blocks de novo DNA synthesis (19), we assessed [3H]dUrd incorporation to monitor rates of DNA synthesis in the presence of this compound. Prior to harvest, cultures were washed three times with Dulbecco's phosphate-buffered saline. Cells were then scraped into 2 ml distilled water and sonicated for 10 s in a Sonifier Cell Disrupter, Model W185D (Heat Systems-Ultrasonics, Inc., Plainview, NY) set at 100 W. Since placental cultures incorporate [H]dThd at a low rate (14), they were labeled with [H] precursors at a concentration of 2 μCi/ml for 24- or 48-h periods. At the termination of the incubations, placental tissue was rinsed in Dulbecco's phosphate-buffered saline, blotted free of medium on absorbent paper, weighed, and sonicated for 15 s in 3 ml water at a setting of 100 W. Radioisotope incorporation into placenta or JAr cell DNA was measured by standard acid precipitation techniques. Sample protein content was determined by the procedure of Lowry et al. (20) using bovine serum albumin as standard. Because JAr cells often grow as syncytia, data are expressed per mg protein rather than per cell or per μg DNA. All samples from a single experiment were quantitated in duplicate within the same assay in order to eliminate interassay variation.

Statistical Methods. Significance of observed differences between control and treated cultures at single time point observations was assessed by one-way analysis of variance by least squares difference for multiple comparisons and by the Student's t test for paired comparisons (21). Comparative effects of methotrexate on normal and malignant cultures were examined by two-factor analysis of variance (22). Data were judged significant if P < 0.05.

RESULTS

Effects of MTX on Growth and HCG Production of JAr and Placental Cells. When cultured in WGM, the customary medium for JAr cells (5), placental organ cultures rapidly degenerate as judged by morphological observation and declining HCG output. It was, thus, important to determine if JAr cells could be cultured in the customized CMRL 1066 or DMEM used to maintain placental organ cultures. JAr cells, plated at 2.5 x 10⁶ cells/60-mm dish, grew equally well in WGM, CMRL 1066, or DMEM. HCG production by JAr cells also was readily and equally enhanced by MTX in all three media. In a representative determination (Fig. 1) we obtained an 8-fold increase in HCG production by JAr cells cultured in customized CMRL containing MTX (10⁻⁵ M). We have shown that DMEM medium could be used to culture human placenta successfully (14). For current studies, it was essential to ascertain whether placental organ cultures maintained on serum-free, customized CMRL (folate free, with hypoxanthine in place of thymidine) medium remained viable and responsive to environmental stimuli. We compared [H]dThd incorporation into DNA and HCG production of placental cultures maintained on serum-free customized CMRL (with or without exogenous radioinert thymidine) or on serum-free unmodified CMRL 1066 medium. Both media permitted similar HCG levels and [H]dThd incorporation into cell DNA; addition of thymidine to both doubled HCG production and halved the incorporation of [H]dThd into DNA (data not shown). These data, thus, demonstrated that customized CMRL could be used under serum-free conditions to evaluate the effect of MTX on placental organ cultures.

When placental organ cultures were exposed to MTX (10⁻⁵ to 10⁻⁴ M) in serum-free DMEM or customized CMRL for 24 or 48 h (Fig. 2), at the start of culture or after 1 or 2 days of serum-free culture, MTX at 10⁻⁵, 10⁻⁴, or 10⁻³ M decreased HCG content in all placentas. The differences were not statistically significant. However, MTX at 10⁻³ M either had no effect or caused a slight but statistically insignificant stimulation (<40%; 20% at most in the placenta shown) in HCG production when compared to untreated cultures. [H]dUrd incorporation into DNA was inhibited by MTX in a dose-dependent manner and was statistically significant from the controls at 10⁻⁵ to 10⁻³ M.

Comparison of Radiolabeled DNA Precursor Incorporation into DNA of JAr Cells and Normal Placenta. Earlier reports...
from this laboratory have suggested a relationship between inhibition of DNA synthesis and stimulation of HCG production (5, 7). In an effort to explain the differential stimulation by MTX of HCG levels in normal and malignant placenta, we examined normal and malignant trophoblast cultures to determine whether or not they differed in their utilization of the DNA synthetic pathways.

We first examined whether placental organ cultures possessed active de novo and salvage DNA synthetic pathways by culturing them in HAT medium prepared with customized CMRL. Cells lacking either pathway are killed in the presence of HAT medium (23). The normal kinetics of HCG production was significantly enhanced 3.5-fold and 2-fold, respectively (Fig. 3). We then studied the incorporation of [3H]dUrd, [3H]dThd, and [3H]dCyd into DNA of placental organ cultures maintained in serum-free customized CMRL. Cultures were exposed during the initial 48 h in culture to MTX at concentrations of 10^{-5}, 10^{-6}, 10^{-7}, or 10^{-8} M. Supernatant fluids were collected every 48 h for measurement of HCG levels by radioimmunoassay. Cultures were labeled during the first 48 h in culture with [3H]dUrd (2 μCi/ml). The levels of HCG shown were derived from day 4 in culture when HCG levels were at their highest. Points, means of triplicate cultures from a single, representative placenta; bars, SD. Data from a single representative placenta each are depicted for each [3H] precursor examined.

Effects of AMD and dbcAMP on Placental HCG Production. When placental organ cultures contained AMD at either 10^{-7} or 10^{-8} M for 24 h at the initiation of culture, HCG content was significationally enhanced 3.5-fold and 2-fold, respectively (Fig. 4). When AMD concentration was 10^{-6} M, placental culture HCG content was unchanged (Fig. 4). When normal placenta was cultured in regular CMRL medium with fetal bovine serum (5%), AMD (10^{-6} M), and/or dbcAMP (1 mM) with theophylline (1 mM) during days 2 to 4 in culture, we observed that AMD, dbcAMP plus theophylline, and unexpectedly, the combination of these agents elevated placental HCG production significantly by 2- to 4.5-fold (Fig. 5).

DISCUSSION

MTX (5-8), dbcAMP (11-13, 25), and AMD (9, 10) stimulate the production of HCG in cultured human choriocarcinoma cells. Rises in HCG levels are also initially observed in patients with choriocarcinoma following chemotherapy. The significance of these rises is unclear, but drug-induced cell lysis appears unlikely to be the cause as choriocarcinoma cells store very little HCG (26). We have now examined the ability of these agents to modulate HCG content of normal first trimester human placental organ cultures. Our results reveal that normal placental HCG levels rise in response to dbcAMP or AMD but not to MTX. These results, coupled with our earlier work on epidermal growth factor (14), suggest dissimilar regulation of HCG production in normal and malignant trophoblast in response to metabolic agents.

Why HCG levels rise in response to MTX in choriocarcinoma but not in normal placental cultures is both uncertain and intriguing. Both culture groups remained viable under all media depicted and unaffected at 10^{-8} M. In JAr cultures, significant elevations (not exceeding 2.5-fold) in [3H]dThd incorporation into DNA were also noted at MTX concentrations of 10^{-5} and 10^{-6} M but unaffected at 10^{-7} and 10^{-8} M. Thus, in summary, [3H]dCyd and [3H]dThd but not [3H]dUrd incorporation patterns into DNA differed between JAr and placental cultures under the influence of MTX.
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Fig. 4. Stimulation of HCG secretion in normal placental organ cultures by AMD. Cultures maintained in DMEM supplemented with fetal bovine serum (5%) were exposed to AMD at concentrations of (•) control; (O) $10^{-6}$ M; (A) $10^{-7}$ M; and (•) $10^{-8}$ M during the initial 24 h of culture. Supernatant fluids were collected daily for the measurement of HCG production. Points, means of 2 cultures from a single, representative placenta; bars, SD. AMD in box, exposure period of the cultures to AMD.

Fig. 5. Stimulation of HCG secretion by normal placental organ cultures exposed to AMD at a concentration of $10^{-6}$ M and/or dbcAMP and theophylline, both at a concentration of 1 mM. Cultures were maintained in CMRL 1066 with fetal bovine serum (5%) and exposed to AMD or dbcAMP during days 2 to 4 in culture as follows: (•) control; (O) AMD; (A) dbcAMP and theophylline; (B) AMD, dbcAMP, and theophylline. Points, means of 2 cultures from a single, representative placenta; bars, SD. AMD in box, exposure period of the cultures to AMD.

conditions tested, as judged by $[^3]$H]dThd incorporation into DNA and by HCG production. Both JAr (7) and normal placental cultures survived in HAT (23) medium and thus possessed both the salvage and de novo pathways of DNA synthesis. The presence of other heterogeneous cell types in the placental organ cultures probably did not alter the trophoblastic response to MTX; normal human trophoblast monolayers also show a similar lack of HCG responsiveness to MTX (27).

Unlike JAr cells, noncycling, differentiated HCG-secreting syncytiotrophoblasts might be unresponsive to MTX which exerts its most profound effect during S phase (28). Furthermore, if only a few susceptible trophoblasts responded to MTX, the already high levels of HCG present in normal placental cultures (15) could mask any slight increases in HCG output. The small but statistically insignificant rises in HCG levels occasionally noted in placental cultures treated with MTX ($10^{-6}$ M) would be consistent with this explanation.

Previous reports from this laboratory demonstrated a correlation between inhibition of DNA synthesis and elevation of HCG production by choriocarcinoma cells (5, 7). Similar increases in products associated with a more differentiated state have been reported in other systems (29) following inhibition of DNA synthesis. Furthermore, it has been well recognized that many normal cells and resting cells (30, 31) prefer the salvage pathways of DNA synthesis, while many tumor cells favor the de novo pathways (32). We thus examined in further detail a role for DNA synthesis in the dissimilar HCG production induced by MTX in JAr and normal placental cultures.

We have noted striking dissimilarities in the incorporation of $[^3]$H]dThd and $[^3]$H]dCyd into DNA of normal and malignant trophoblast in the presence of MTX. These differences are particularly evident between MTX concentrations of $10^{-7}$ M and $10^{-6}$ M or between $10^{-8}$ M and $10^{-7}$ M. These observations may be significant, because the concentration at which MTX induces maximal HCG production in JAr cells is $10^{-7}$ M (7). Because we did not measure nucleotide pool sizes, we cannot say whether the heightened incorporation of $[^3]$H]dThd into placental DNA, in the presence of MTX, represents greater activity of placental DNA salvage pathway enzymes and/or greater apparent incorporation of radiolabeled nucleotides into diminished nucleotide pools. The incorporation of $[^3]$H]dUrd is the same in normal and malignant trophoblast cells, which suggests that utilization of the salvage pathways of DNA synthesis differ but not the de novo pathways. Sekiya et al. (6) noted that choriocarcinoma cells unresponsive to MTX-induced HCG stimulation demonstrated impaired cellular MTX uptake and increased levels of intracellular dihydrofolate reductase, a key enzyme in the de novo pathways of DNA synthesis (18, 33). We did not examine and cannot exclude a role for dihydrofolate reductase in our observed differences in MTX sensitivity. However, the marked inhibition of $[^3]$H]dUrd incorporation into DNA of both normal and malignant placental cultures argues against differential impairment of MTX uptake by our cultures. Thus, differential reliance on the DNA synthetic pathways by JAr and placental cultures may account for the inability of MTX to stimulate HCG production in placental cultures. Similar differences in DNA synthetic pathways might contribute to the MTX resistance observed in some choriocarcinomas (3). Transient rises in HCG levels following MTX therapy in choriocarcinoma patients might serve as a predictor of tumors most susceptible to MTX treatment.

In contrast to MTX, AMD stimulates HCG production by normal placental cultures. AMD effectively acts on choriocarcinoma through its inhibition of RNA polymerase (34). Non-
cycling HCG-secreting cells of the placenta might be selectively responsive to AMD but not to MTX, whereas JAR cells, because of their continual division, would be susceptible to both.

Hussa et al. (9) reported that AMD enhanced HCG secretion in the BeWo line of choriocarcinoma cells. They proposed that AMD raised HCG levels posttranscriptionally through "superinduction" in which longer-lived mRNA templates remain active for protein synthesis despite the presence of inhibitors such as AMD (35, 36). To test the possibility that this mechanism might account for the stimulation of HCG production by AMD in normal placental cultures, organ cultures were treated with dbcAMP plus theophylline, AMD, or the combination of both. dbcAMP and theophylline are known stimulators of HCG production in choriocarcinoma (11–13, 25) and in normal placental organ (15), explant (37), and cell cultures (27). The combination of AMD with dbcAMP plus theophylline resulted in an additive effect on HCG production compared with either alone. This observed effect apparently requires lengthy exposure to these drugs because short-term treatment of choriocarcinoma cells with AMD alone (5, 25) and with dbcAMP plus theophylline (25) for 8 h or less results in an inhibition of HCG stimulation by dbcAMP. The additive effect of AMD and dbcAMP on HCG production compared to either alone is consistent with but does not prove superinduction or other posttranscriptional mechanisms as the manner in which AMD stimulates HCG production in normal placenta. Actual measurements of mRNA levels (38, 39) and HCG synthesis (38, 40) would be needed to document this sort of mechanism.

In conclusion, our paired culture model of normal and malignant trophoblast offers a means to examine a product of differentiated gene function, HCG, which appears to be differentially modulated in these cultures.

REFERENCES

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