The Stimulation by Methotrexate of Human Chorionic Gonadotropin and Placental Alkaline Phosphatase in Cultured Choriocarcinoma Cells

K. V. Speeg, Jr.,' Jane Clifford Azizkhan, and Kurt Stromberg

Gene Regulation Section, Laboratory of Molecular Biology, Division of Cancer Biology and Diagnosis, National Cancer Institute, Bethesda, Maryland 20014

SUMMARY

Treatment of the BeWo line of choriocarcinoma cells with methotrexate in doses that inhibit DNA synthesis causes a tenfold increase in synthesis of human chorionic gonadotropin and a threefold increase in activity of placental alkaline phosphatase. No concomitant increase in lactic dehydrogenase activity occurs under these conditions. This effect of methotrexate can be blocked by simultaneous addition of thymidine or folinic acid, neither of which alone increases human chorionic gonadotropin synthesis or placental alkaline phosphatase activity in BeWo cells.

INTRODUCTION

The fall in HCG1 during the course of chemotherapy for gestational choriocarcinoma is 1 of the oldest and, perhaps, the most specific example of using a biochemical marker as an index of tumor cell destruction (1). As such it represents a significant achievement in chemotherapy by offering an assessment of clinical progress and an end point of chemotherapy. It was observed, however, that early in the course of chemotherapy with MTX and/or actinomycin D, a transient rise occurred in the serum level of HCG (1, 18). This has been attributed to release of HCG from lysed choriocarcinoma cells (1). Hussa et al. (18) demonstrated that actinomycin D added to a continuous line of choriocarcinoma cells in culture (BeWo line) resulted in increased amounts of immunoprecipitable HCG in the cells themselves and in their cell culture media. However, there was far more HCG than could be accounted for on the basis of cell lysis alone. Very few details are known about the synthesis, storage, and release of HCG by either the trophoblast cells of normal placenta or choriocarcinoma cells. However, because no intracellular storage form of HCG has been detected, it is unlikely that normal trophoblast or choriocarcinoma cells store any appreciable amount of HCG. Moreover, the mechanism of increased HCG synthesis in choriocarcinoma cells following exposure to actinomycin D remains unknown.

Alkaline phosphatase has also gained prominence as a biochemical marker of malignant cells. The form that has been most clearly associated with cancer is the placental isozyme of alkaline phosphatase. This isozyme is heat stable and has been referred to as the Ragan enzyme when found in pathological circumstances. Thus far, whenever found in any appreciable titer in nonpregnant states, placental alkaline phosphatase expression indicates a pathological condition (7, 20, 21, 28, 34, 36). Whether placental alkaline phosphatase is a normal constituent of choriocarcinoma because of its trophoblastic origin or whether it represents a derepressed function as in other types of cancer is at present unknown. Placental alkaline phosphatase has been studied in a number of human cell culture lines, and its activity can be increased by glucocorticoids (5, 12, 13, 29), substrates (6), butyrate (10), and hyperosmolarity (12, 13, 30). The increase in placental alkaline phosphatase activity by hyperosmolarity is particularly intriguing, since hyperosmolarity can also inhibit DNA synthesis (38).

This paper examines the effect of MTX upon HCG production and placental alkaline phosphatase activity in the BeWo line of choriocarcinoma cells and demonstrates that MTX, in doses that inhibit DNA synthesis, stimulates both HCG synthesis and placental alkaline phosphatase activity.

MATERIALS AND METHODS

Media and Chemicals. Waymouth's 752/1 medium and Gey's balanced salt solution were purchased from Grand Island Biological Co., Grand Island, N. Y. Calcium- and magnesium-free Earle's balanced salt solution was prepared by the NIH media unit. Fetal bovine serum was purchased from Colorado Serum Company Laboratories, Denver, Colo., and from North American Biologicals, Inc., Miami, Fla. Newborn calf serum was from Grand Island Biological Co. Lyophilized trypsin (250 units/mg) was purchased from Worthington Biochemical Corp., Freehold, N. J. EDTA was from Schwarz/Mann, Orangeburg, N. Y. MTX (sodium salt) was from Laderla Laboratories, Division of American Cyanamid Co., Pearl River, N. Y. N-5-Formyltetrahydrofolate (folic acid) was from Grand Island Biological Co. Thymidine, hydroxyurea, cytosine arabinoside, cycloheximide, actinomycin D, disodium p-nitrophenyl phosphate, p-nitrophenol standard, 2-amino-2-methyl-1-propenal buffer, pyruvate, and NADH were from Sigma Chemical Co., St. Louis, Mo. 125I-labeled HCG (90 to 100 μCi/μg), L-[4,5-3H]leucine (60 Ci/m mole), [6-3H]uridine (24.2 Ci/μmol).
mmole), [5-^H]uridine (26.2 Ci/mmol), and [methyl-^4C]thymidine (57.1 mCi/mmol) were purchased from New England Nuclear, Boston, Mass. Rabbit anti-human chorionic gonadotropin antibody and goat anti-rabbit gamma globulin antibody were purchased from Cappel Laboratories, Inc., Downington, Pa.

**Cells and Culture Technique.** BeWo choriocarcinoma cells were obtained from the American Type Culture Collection, Rockville, Md. (Catalog CCL 98). Cells were grown in Falcon plastic flasks in medium containing 50% Waymouth's 752/1 medium, 40% Gey's balanced salt solution, and 10% bovine serum. Stock cultures were grown in the presence of penicillin (100 units/ml) and streptomycin (100 μg/ml). BeWo has previously been grown in 10% newborn calf serum (18, 19, 32), but we found that fetal bovine serum gave much better growth with retention of hormonal properties (shortened population-doubling time, better plating efficiency, and the added advantage of successful subculture up to 1:10 when grown in fetal bovine serum rather than 1:2 or 1:3 as recommended for growth in newborn calf serum). Therefore, this report utilizes cells maintained in fetal bovine serum. Cultures were maintained at 37° in a nonhumidified atmosphere of 5% CO2-95% air. Cells were subcultured with 0.05% trypsin-0.02% EDTA in calcium- and magnesium-free Earle's balanced salt solution. For the experiments to be described, cells were subcultured to give monolayer cultures approximately 20 to 30% confluent. Initially, the cells received medium containing fetal bovine serum for approximately 24 hr. The cultures were then switched to serum-free medium (55% Waymouth's 752/1 medium and 45% Gey's balanced salt solution), and medium was changed every 24 hr. Inhibitors or other agents were then added daily in serum-free medium. The medium of treated cultures was collected daily, centrifuged to remove cell debris, and then stored at —50° until HCG was assayed. For assay of protein, placental alkaline phosphatase, or cellular HCG, the cell layer of appropriate cultures was washed with phosphate-buffered 0.9% NaCl solution, pH 7.1, to remove debris and unattached cells. The cell sheet was then harvested by scraping with a rubber policeman and rewarshed with phosphate-buffered 0.9% NaCl solution by centrifugation. The cells were resuspended in a small volume of phosphate-buffered 0.9% NaCl solution, disrupted by sonic disruption, and stored at —50° until assayed for protein, placental alkaline phosphatase, and cellular HCG.

**Assays.** Alkaline phosphatase (EC 3.1.3.1) was measured by the method of Lowry (25) using p-nitrophenyl phosphate as substrate at pH 10.3. BeWo cells contain 2 types of alkaline phosphatase (manuscript in preparation). One of these is the typical placental isozyme (placental alkaline phosphatase) which is stable at 65°. The alkaline phosphatase activity reported here is that which remains after heating the enzyme preparation for 5 min at 65°. One unit of placental alkaline phosphatase is defined as that activity which catalyzes the formation of 1 nmole of p-nitrophenol per min at 37°. MTX added to the enzyme assay was found to have no effect on activity. Lactic dehydrogenase (EC 1.1.1.27) was measured in the BeWo cell sonic extract by the method of Reeves and Fimognari (33), which follows the oxidation of NADH to NAD at 340 nm as lactate is formed from pyruvate. All enzyme assays were performed in duplicate after optimal conditions were established for linearity in respect to incubation time and enzyme concentration. Protein was measured by the method of Lowry et al. (26) using bovine serum albumin as a standard. HCG titers were measured in duplicate by radioimmunoassay (31). The assay was standardized with a standard purchased from Serono Laboratories, Inc., Boston, Mass., which had been radioimmunologically calibrated against the 2nd International Standard of HCG. There was no HCG detected in freshly prepared medium. MTX did not interfere with the radioimmunoassay. Increasing aliquots of BeWo cellular sonic extract or media in which BeWo had grown gave parallel displacement curves with known HCG.

**Incorporation of Radioactive Precursors.** Synthesis of DNA, RNA, and protein was estimated by incorporation of labeled thymidine, uridine, or leucine into macromolecules by a modification of the method of Breen and De Vellis (4). For labeling cellular DNA and RNA, BeWo cells maintained in serum-free media were pulsed with 1 μCi of [methyl-^14C]thymidine and 1 μCi of [5-^3H]uridine for 3 hr. The cell layer was then washed 3 times with 5 ml of 0.9% NaCl solution containing 4 mM uridine and 17 μM thymidine. The cells were then harvested by scraping with a rubber policeman in a 3-ml volume of fresh, cold 5% trichloroacetic acid and by centrifuging at 1000 × g for 10 min at 4°. The supernatant was discarded. The pellet was washed twice with a 2-ml volume of cold 5% trichloroacetic acid and once with a 2-ml volume of 100% ethanol. The pellet was dissolved in 1.5 ml of 0.1 M KOH and heated at 100° for 30 min to hydrolyze RNA. The mixture was cooled, and 5 μl perchloric acid were added to a final volume of 2 ml. The precipitate containing DNA was collected by centrifugation and saved. Aliquots of the supernatant were counted in Aquasol in a liquid scintillation spectrometer. The DNA content was determined from the absorbance of the supernatant at 260 nm. The pellet containing DNA was washed twice with cold 1 n perchloric acid. To the pellet was then added 2 ml of 0.25 n NaOH, and this was heated at 100° for 10 min. The solubilized DNA was cooled to room temperature, and the DNA content was determined from the absorbance at 260 nm. Radioactivity was measured by liquid scintillation spectrometry in Aquasol. This method gave 95% separation of DNA and RNA based on separation of ^3H and ^14C radioactive activity. The above method was used to assess the inhibition by actinomycin D. The method was also used to assess the inhibition by MTX, except that [6-^3H]uridine was incorporated into DNA and RNA instead of thymidine and [5-^3H]uridine. Labeling of cellular protein was by the method of Breen and De Vellis (4).

**RESULTS**

**Stimulation of Placental Alkaline Phosphatase by MTX.** Chart 1 illustrates the effect of adding 1 μM MTX for 24 hr to choriocarcinoma cells (BeWo) grown in defined medium in cell culture. Cell growth is arrested after the addition of 1 μM MTX, and this effect persists for approximately 2 days after removal of MTX (Chart 1E). Cell growth, as measured
by increase in cell protein (14), then resumes at the same rate as in the control flasks. There is an increase in total cellular placental alkaline phosphatase activity in the MTX-treated flasks in comparison to untreated control flasks (Chart 1A). In Chart 1B, the placental alkaline phosphatase activity per mg cell protein (placental alkaline phosphatase specific activity) is seen to have a lag period of approximately 1 day followed by a more rapid increase for 2 days. This is then followed by a return in specific activity of placental alkaline phosphatase to levels approaching those of the control flasks. The average (± S.E.) increase of specific activity of placental alkaline phosphatase observed 72 hr after addition of 1 μM MTX was 295 ± 35% (range, 220 to 410% increase).

As shown in Chart 1, C and D, HCG production and secretion into the medium in untreated control flasks decline to a baseline level after subculture and removal of serum. If BeWo cells are treated with 1 μM MTX, the amount of HCG released into the medium rises after a 1-day lag period and persists for about 2 days after removal of MTX. The level then falls toward that of untreated cells. These changes in HCG found in the media are also reflected by cellular HCG (Chart 1F), except that the cellular HCG per mg cell protein is 50- to 100-fold less than that found in the media. The average amount (± S.E.) of stimulation of HCG synthesis seen with 1 μM MTX 72 hr after addition of the drug was 980 ± 250% (range, 360 to 2300% increase). The changes in placental alkaline phosphatase activity or HCG content correlate closely with the inhibition of cell growth caused by a 24-hr exposure to MTX and the resumption of cell growth 2 to 3 days after the removal of MTX, as shown in Chart 1E.

Effect of MTX on DNA, RNA, and Protein Synthesis. The effect of various concentrations of MTX on DNA, RNA, and protein synthesis in BeWo cells grown without serum is shown in Table 1. DNA synthesis was inhibited 90 to 95% with either 0.1 or 1 μM MTX. In contrast, 0.01 μM MTX had essentially no effect on DNA synthesis. None of these concentrations of MTX inhibited RNA synthesis even after 72 hr in the presence of the drug. MTX inhibited protein synthesis to a moderate extent. Cycloheximide (3.5 μM) is included as a reference for inhibition of protein synthesis. Note that this concentration of cycloheximide inhibited protein synthesis approximately 85% initially, but, subsequently, its effect diminished. Actinomycin D (5 nM) inhibited RNA synthesis approximately 60% and reduced DNA synthesis somewhat more.

Dose-Response Relationship of Stimulation by MTX. The change in specific activity of placental alkaline phosphatase and HCG with increasing doses of MTX is shown in Chart 2. Exposure of BeWo cells to 0.01 μM MTX over a 72-hr period results in placental alkaline phosphatase activity similar to that of untreated cells (Chart 2A). However, MTX doses between 0.1 and 10 μM cause an increase in placental alkaline phosphatase activity that becomes more dose related late in the experiment. There is no response in HCG production with 0.01 μM MTX, but an increase occurs with doses between 0.1 and 10 μM (Chart 2B). In contrast to the change in placental alkaline phosphatase activity, the increase in HCG production appears to be dose related earlier in the experiment. These results agree well with those presented in Table 1, where 0.01 μM MTX caused no inhibition of DNA or RNA synthesis, but higher doses resulted in significant inhibition of DNA synthesis. It should be pointed out that the “response” depicted here is clearly a complex one involving transport of MTX into the cell, inhibition of dihydrofolate reductase, a probable accumulation of unbound MTX, a decrease in available thymidine, an inhibition of DNA synthesis (3, 8, 11, 15, 16, 37), and, ultimately, an increase in HCG and placental alkaline phosphatase specific activity by an unknown mechanism(s). Superimposed on this is a possible attempt by the cell to synthesize new dihydrofolate reductase and reverse the inhibition of DNA synthesis caused by a lack of thymidine (11, 15, 37).

Lack of Stimulation of Lactic Dehydrogenase. To ascertain whether MTX might be increasing other choriocarcinoma functions in a nonspecific way, the cellular lactic
Stimulation of HCG and Alkaline Phosphatase by MTX

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DNA synthesis (cpm/ug DNA expressed as % control ± S.E.)</th>
<th>RNA synthesis (cpm/mg protein expressed as % control ± S.E.)</th>
<th>Protein synthesis (cpm/mg protein expressed as % control ± S.E.)</th>
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</thead>
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<td>None (control)</td>
<td>100 ± 7.8</td>
<td>100 ± 7.0</td>
<td>100 ± 6.8</td>
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<tr>
<td>MTX 0.01 µM</td>
<td>70.0 ± 5.0</td>
<td>78.4 ± 6.3</td>
<td>78.4 ± 6.3</td>
</tr>
<tr>
<td>MTX 0.01 µM</td>
<td>70.0 ± 5.0</td>
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<tr>
<td>MTX 1.0 µM</td>
<td>14.9 ± 0.8</td>
<td>215 ± 4.0</td>
<td>215 ± 4.0</td>
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<tr>
<td>MTX 3.5 µM</td>
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<td>5.0</td>
<td>5.0</td>
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<tr>
<td>Actinomycin D 5 mM</td>
<td>71.1 ± 2.2</td>
<td>215 ± 4.0</td>
<td>215 ± 4.0</td>
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</tbody>
</table>

Chart 2. Dose-response stimulation of BeWo placental alkaline phosphatase and HCG by MTX. Cells were subcultured on Day 0 and fed serum-free medium on each subsequent day. On Day 3, MTX was added in serum-free medium for the 72 hr indicated . At the indicated times, cells were collected for assay of specific activity of placental alkaline phosphatase (PAP) (A) or assay of the amount of HCG secreted into the culture medium per 24 hr expressed per mg cellular protein (B). Doses of MTX: none ( ), 10 nM ( ), 0.1 µM ( ), 1 µM ( ), 10 µM ( ).

Dehydrogenase was measured at the same time-points and MTX concentrations. The lactic dehydrogenase specific activity was found to be unchanged in control and MTX-treated cells.

**Use of Other Inhibitors.** Attempts at stimulation of the level of HCG production and placental alkaline phosphatase activity with other inhibitors of DNA synthesis (35) are shown in Chart 3. Cytosine arabinoside at 1 µM caused a 2-fold increase in placental alkaline phosphatase activity and a 4-fold increase in HCG production. A less dramatic stimulation of HCG synthesis and, probably, no stimulation of placental alkaline phosphatase activity occurred following exposure to 1 µM hydroxyurea. Further study would be required to demonstrate that either of these drugs is capable of inhibiting DNA synthesis in BeWo cells, specifically, and what might be the optimal conditions for stimulation.

**Effect of Thymidine and Folinic Acid.** BeWo cells were found to grow well in hypoxanthine-aminopterin-thymidine medium. This presumably reflects the presence in these cells of thymidine kinase and hypoxanthine-guanine phosphoribosyltransferase (23, 24). Because Waymouth's 752/1 medium is relatively rich in hypoxanthine, the increase in HCG synthesis and placental alkaline phosphatase activity after exposure to MTX was considered to be secondary to deprivation of thymidine and subsequent inhibition of DNA synthesis. Because MTX is a potent inhibitor of dihydrofolate reductase, which is essential for biosynthesis of thymidine, several mixing experiments with MTX, thymidine, and folinic acid were performed. Chart 4 illustrates that MTX alone results in an increase in HCG and placental alkaline phosphatase. However, thymidine alone or the simultaneous addition of MTX and excess thymidine did not result in
Chart 3. Effect of other inhibitors on HCG and placental alkaline phosphatase in BeWo cells. Cells were subcultured on Day 0 and fed serum-free medium on each subsequent day. On Day 3, 1 μM hydroxyurea (A, C) or 1 μM cytosine arabinoside (B, D) was added for the indicated time ( ). At appropriate times, cells treated with inhibitor ( ) or cells grown without inhibitor ( ) were harvested for placental alkaline phosphatase specific activity or the amount of HCG secreted into the culture medium per 24 hr expressed per mg cellular protein.

an increase in either HCG synthesis or placental alkaline phosphatase specific activity. Moreover, as shown in Chart 5, the increase in both HCG synthesis and specific activity of placental alkaline phosphatase can be prevented by the addition of 0.1 mM folinic acid at the time of addition of 1 μM MTX. The folinic acid acts as a 1-carbon donor toward the biosynthesis of thymidine and is not dependent upon dihydrofolate reductase activity (15).

DISCUSSION

The demonstration that MTX stimulates HCG synthesis and placental alkaline phosphatase activity and that the stimulation is related to inhibition of DNA synthesis is based on the following data: (a) the addition of MTX results in an increase in the amount of cellular HCG and HCG secreted into the cell culture medium as measured by radioimmunoassay, as well as an increase in the specific activity of cellular placental alkaline phosphatase; (b) it is those doses of MTX that can be shown to inhibit DNA synthesis but that do not inhibit RNA synthesis that stimulated HCG synthesis and placental alkaline phosphatase activity [the inhibition of DNA synthesis by the doses of MTX reported here is in good agreement with that found by Hussa and Patillo using BeWo cells (17)]; (c) drugs (thymidine and folinic acid) that circumvent the action of MTX on DNA synthesis prevent the stimulation of HCG and placental alkaline phosphatase. The effect may be a specific one because lactic dehydrogenase does not increase with doses of MTX which inhibit DNA synthesis.

The assumption of an actual increase in the synthesis of

Chart 4. Effect of thymidine on stimulation of BeWo HCG and placental alkaline phosphatase by MTX. Cells were subcultured on Day 0 and fed serum-free medium on each subsequent day. On Day 3, 1 μM MTX ( ), 1 μM MTX plus 0.1 mM thymidine ( ), 0.1 mM thymidine ( ), or no addition ( ) in serum-free medium was added for the indicated time ( ). At appropriate times, cells were harvested for placental alkaline phosphatase (PAP) specific activity (top) or the amount of HCG secreted into the culture medium per 24 hr expressed per mg cellular protein (bottom).

Chart 5. Effect of folinic acid on stimulation of BeWo HCG and placental alkaline phosphatase by MTX. Cells were subcultured on Day 0 and fed serum-free medium on each subsequent day. On Day 3, 1 μM MTX ( ), 1 μM MTX plus 0.1 mM folinic acid ( ), or no addition ( ) in serum-free medium was added for the indicated time ( ). At appropriate times, cells were harvested for placental alkaline phosphatase (PAP) specific activity (top) or the amount of HCG secreted into the culture medium per 24 hr expressed per mg cellular protein (bottom).
HCG has been made because of the measured increase by competition radioimmunoassay and lack of evidence for a mechanism of destroying HCG in BeWo cells. Confirmation of an increase in newly synthesized HCG after BeWo cells are exposed to MTX is ultimately required by another method. Induction of placental alkaline phosphatase cannot be assumed, since measurement is made of enzyme activity and not of enzyme content. Whether the activity increases secondary to increased synthesis, decreased degradation, or due to enzyme modification is at present unknown and requires further study.

After prolonged exposure to 1 μM MTX, BeWo cells are eventually killed. Therefore, it might be argued that MTX eliminates a subpopulation of BeWo cells which is both more sensitive to the drug and also one which produces less HCG and a lower specific activity of placental alkaline phosphatase. The result would be a factitious increase in specific activity of both after MTX treatment. The data in Chart 1 demonstrate that selective cell destruction by MTX is unlikely. When the total activity of placental alkaline phosphatase or content of HCG are plotted as in Chart 1, A and C (uncorrected for the amount of cellular protein), both functions are significantly elevated over control cultures. The increase in HCG is much more than can be accounted for on the basis of cell lysis.

Wiltshaw and Moloney (40) have previously reported that leukocyte alkaline phosphatase activity from normal human controls, patients with chronic myelogenous leukemia, and patients with pyogenic infections can be stimulated with both aminopterin and amethopterin (MTX). MTX gave better stimulation than aminopterin. The stimulation of alkaline phosphatase by both drugs could be blocked by folinic acid if added with the inhibitors. Glutamine synthetase activity in chick embryonic neural retina has been reported to be stimulated by cytosine arabinoside, and the increase in stimulation than aminopterin. The stimulation of alkaline phosphatase activity could be blocked by prior addition of actinomycin D by the fact that actinomycin D inhibits RNA synthesis also. Because HCG can be increased by MTX (which specifically inhibits DNA synthesis) and can also be increased by actinomycin D (which inhibits both DNA and RNA synthesis), it would appear that caution should be exercised in assuming that changes occurring secondary to addition of actinomycin D to a system are due to inhibition of RNA synthesis alone.

While the previously mentioned transient increase in serum HCG of patients undergoing chemotherapy for gestational choriocarcinoma probably does reflect to some extent lysis of malignant cells, it is clear that cell lysis need not be the only explanation. Although the use of HCG has been a reliable marker during chemotherapy of choriocarcinoma, it should be clear that a broader understanding of the factors modulating the HCG level, or the level of any tumor marker under various circumstances, is critical to the proper interpretation of the marker level. The reliability of HCG as a marker may be due more to the fact that all choriocarcinomas make HCG (thus far no human choriocarcinoma). There is, however, no evidence as yet to indicate that these phenomena are related (13).
cinoma that lacks HCG synthesis has been well documented) rather than to a steady level of synthesis of HCG by the choriocarcinoma cells.

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