Morphological Differentiation of Human Choriocarcinoma Cells Induced by Methotrexate

Susan J. Friedman and Philip Skehan

ABSTRACT

BeWo is a malignant human choriocarcinoma line derived from a methotrexate-resistant tumor. Under normal conditions, two BeWo phenotypes coexist in culture. The predominant form, 96 to 99% of the total population, consists of cytotrophoblast-like (CTL) cells that are proliferative, mononucleate, and moderate in size. The remaining cells are syncytiotrophoblast-like (STL), being giant, nonproliferative, and multinucleated. Treatment of BeWo cultures with 1 μM methotrexate causes a total inhibition of cell division but does not stop nuclear division or DNA or protein synthesis. Consequently, there arises a population of giant, multinucleated, nonproliferative cells that is morphologically indistinguishable from the naturally occurring STL cells. CTL BeWo cells possess prominent surface ruffles, contain moderate numbers of microvilli and filopodia, contain a prominent network of interlacing filament bundles, and are connected by fascia adherens and desmosomes. STL BeWo cells are 3 to 10 times larger than CTL's; have reduced ruffles, filopodia, cytoplasmic filament bundles, and desmosome and fascia adherens junctions; but possess increased microvilli, Golgi apparatus, smooth and dilated endoplasmic reticulum, and a variety of vesicles and granules. The methotrexate induction of STL cells is reversible upon drug removal. A comparison of our observations with literature data for the normal in utero trophoblast indicates that CTL BeWo cells are ultrastructurally similar to cells of the cytotrophoblast, while STL BeWo cells are ultrastructurally similar to the normal syncytiotrophoblast. Our findings indicate that the BeWo line provides an excellent culture model system for investigating the nontoxic, cytodifferentiative changes that chemotherapeutic agents induce in human tumor cells and raise the possibility that the BeWo line may also prove a valuable culture model system for the investigation of mammalian trophoblast development.

INTRODUCTION

Following uterine implantation, the mammalian trophoblast differentiates into 2 layers which later form the fetal placenta (28). The inner of these layers is the cytotrophoblast, the syncytiotrophoblast, is a syncytium of nonproliferative, relatively undifferentiated cells of which are proliferative, and moderate in size. The outer layer, or syncytiotrophoblast, is a syncytium of nonproliferative, multinucleated giant cells (27, 31) which are specialized for the production of placental hormones (5, 14, 21). The production of placental hormones (5, 14, 21), and CA-23985. A portion of this work was presented at the American Society of Cell Biology Meeting, San Diego, Calif. 1977 (7).

Choriocarcinomas are gestational tumors of the trophoblast. BeWo is a malignant human choriocarcinoma line derived from a methotrexate-resistant tumor (9, 18). BeWo cells in culture, like the normal cytotrophoblast, produce only small amounts of PALP and HCG. Methotrexate, however, greatly stimulates the BeWo production of both PALP and HCG (26). Elevated PALP and HCG levels are both characteristics of the differentiated syncytiotrophoblast but not of the comparatively undifferentiated cytotrophoblast (5, 14, 21).

In this paper, we report that BeWo cultures possess many of the same ultrastructural features that have been reported for the undifferentiated in utero cytotrophoblast. Moreover, following methotrexate 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.

MATERIALS AND METHODS

Cell Culture. Human BeWo choriocarcinoma cells were cultured at 37° as monolayers in growth medium consisting of RPMI 1640 plus 10% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.). Twenty-four hr before the addition of methotrexate, confluent monolayers were dissociated with 0.25% trypsin in NKT and subcultured at a density of 5 to 6 × 10⁵ cells/25-cm Falcon tissue culture flasks in 10 ml of growth medium. Medium was changed daily on all cultures in order to avoid depletion artifacts. Methotrexate was purchased from Lederle Laboratories, Pearl River, N. Y., and used at a final concentration of 1 μM. Culture protein was assayed by the Lowry method of Oyama and Eagle (17), and DNA was assayed by the diphenylamine method (4). Cell number was determined by means of a hemocytometer following trypsin dissociation.

Light Microscopy. Control and treated cultures were observed daily by phase-contrast microscopy and photographed on Kodak Panatomic X-35 film with a Nikon Model M35-S camera attached to a Nikon inverted phase-contrast microscope.

Transmission Electron Microscopy. Cells in plastic tissue culture flasks were washed with Puck's Saline G, fixed with 3% glutaraldehyde buffered with 0.2 M cacodylate buffer (pH 7.2) for 1 hr, rinsed five times with cacodylate buffer, postfixed with 1% osmium for 1 hr, rinsed with distilled water, and then stained in 2% uranyl acetate for 1 hr, dehydrated in ethanol, and embedded in Epon-Araldite. Cells layers were silver-sectioned on a Porter-Blum ultramicrotome, poststained with uranyl acetate and lead citrate, and examined with a Zeiss EM 9S-2 electron microscope.

The abbreviations used are: PALP, placental alkaline phosphatase; HCG, human chorionic gonadotropin; NKT, 1 mM Tris-138 mM NaCl-5.3 mM KCl, pH 7.2, at room temperature; CPD, critical point drying apparatus; CTL, cytotrophoblast-like; STL, syncytiotrophoblast-like; RER, rough endoplasmic reticulum; MTX-STL, STL BeWo cells induced by methotrexate; DHFR, dihydrofolate reductase.

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Scanning Electron Microscopy. For scanning electron microscopy, cells grown on 35-mm plastic tissue culture dishes were washed with Puck's Saline G and then fixed for 15 min with 1.5% glutaraldehyde in 50 mm cacodylate buffer (pH 7.2). These cultures were washed with distilled water and then dehydrated in a graded series of increasing ethanol concentrations. Specimens were dried in a Sorvall CPD, coated with gold, and examined with a Cambridge S4 stereoscan microscope. To avoid partial melting of the plastic and to minimize its warping, CPD pressure was not allowed to exceed 1200 psi, and temperature was kept below 42°. To minimize agitation, the introduction of CO\textsubscript{2} to the CPD chamber and the elevation of temperature and pressure above the critical point were done very slowly. When these precautions were observed, samples on plastic substrata maintained surface morphology as well as samples on glass coverslips.

Adhesion. Cell adhesiveness to the substratum was measured by ease of detachment with a solution of 0.25% trypsin plus 1 mM EDTA in NKT buffer. Cells were plated in 35-mm dishes at various densities in growth medium on Day 0. On Day 1, medium was changed, and cultures were incubated either with or without 1 μM methotrexate for an additional 4 days with daily feedings. On Day 5, cultures were replated, again with or without methotrexate, and incubated for another 24 hr. At the end of this time, cultures were washed with NKT and then incubated at room temperature with 0.25% trypsin plus 1 mM EDTA. The time necessary for total spontaneous detachment of cells from the dish was measured.

RESULTS AND DISCUSSION

Morphological Phenotypes. Two very different morphological phenotypes of BeWo cells coexist under normal culture conditions (Figs. 1 and 4; Chart 1). The majority of cells are moderate in size, mononucleate, proliferative, and generally CTL in phase-contrast appearance, but a few are giant, multinucleated, and STL.

In the presence of 1 μM methotrexate, BeWo cells undergo a striking morphological transformation from a predominantly CTL to a predominantly STL phenotype. Proliferation ceases (Chart 2), and the cells both enlarge and multinucleate (Figs. 2, 3, and 6). These changes are similar to those which have been reported to occur in utero when the syncytiotrophoblast differentiates from cells of the cytotrophoblast (6, 15, 19, 20, 27, 31).

CTL BeWo Cells. CTL BeWo cells are compact, of moderate size, and possess a single ovoid nucleus (Figs. 1 and 4). They are extensively ruffled on their dorsal and lateral surfaces, possess moderate numbers of microvilli and filopodia, but only rarely generate lamellipodia (Figs. 4, 5, and 7).

In thin section, CTL BeWo's are largely conventional in morphology. They contain moderate numbers of mitochondria, smooth and rough endoplasmic reticulum, Golgi apparatus, and assorted small vesicles and granules (Figs. 9 and 10). Although RER is common, the majority of ribosomes are cytoplasmic rather than membrane bound. Most of the RER is of conventional laminar morphology, but greatly dilated RER cisternae filled with an amorphous material of undetermined nature are occasionally observed (Figs. 9 and 10). One of the most prominent cytoplasmic features of CTL BeWo is an interlacing network of thick bundles of filaments (Fig. 9). These bundles are common throughout the cytoplasm but are particularly prominent near the nucleus, where they appear to form a nuclear filament sheath similar to that described for other types of cells (22).

In control cultures, CTL BeWo cells grow as clusters. Within these clusters cells pile up into small multilayered domes well before global confluency is reached. Cell borders remain distinct, and adjacent CTL cells are joined to one another at multiple discrete foci by fascia adherens and desmosomal junctions (Fig. 10).

Control STL BeWo Cells. The naturally occurring STL BeWo cells of control cultures are considerably flatter and occupy 3 to 10 times more substratum surface area than do the CTL's (Figs. 1 and 4). The STL cells possess numerous short, stubby microvilli and have broad lamellopodial expanse. Their surfaces are largely devoid of ruffles, and the few which do occur are restricted to small areas of the peripheral lamella. Naturally occurring STL BeWo cells are frequently multinucleated. The nuclei are generally enlarged and multilobed, and they frequently possess prominent intranuclear filamentous bundles which are visible by light microscopy (Fig. 1).

The percentage of cells possessing the STL phenotype within control BeWo cultures varies with population density (Chart 1). At near-confluency, about 1.4% of the cells in a BeWo culture
are enlarged and flattened, while about 0.3% are also multinucleated. As population density declines, both phenotypic traits increase in frequency. At very sparse densities, up to 4% of the cells become enlarged and flattened, while 2.5% become multinucleated.

**MTX-STL Cells.** In the presence of sublethal concentrations of methotrexate (1 μM), BeWo cultures undergo a striking transformation from a predominantly CTL to a predominantly STL morphology (Figs. 2, 3, 6, and 8). This transformation begins about 24 hr after the addition of methotrexate to the culture medium and reaches completion on about Day 4 of treatment, when 80 to 90% of the cells possess the STL phenotype (Fig. 3).

Addition of methotrexate to a BeWo culture produces a rapid inhibition of cell proliferation (Chart 2). No net increase in cell number occurs within the cultures after drug addition. We observed no increase in cell debris during methotrexate incubation, and the plating efficiency of methotrexate-treated BeWo cells was high, generally in excess of 90%. Thus, the inhibition of proliferation by methotrexate appears to result from a blockade of cell division rather than from an increase in the rate of cell death.

While cell division appears to cease in methotrexate-treated BeWo cultures, cell growth does not (Chart 3). DNA synthesis continues for several days after methotrexate addition, while net protein synthesis continues for at least 1 week and begins to slow only as confluency is approached. After 5 days of incubation in 1 μM methotrexate, culture DNA increased about 65% while culture protein increased about 700% (Chart 3A). During this same period, there was no increase in cell number. As a result, the average DNA content per cell increased from 20 to 33 pg/cell, while the average protein content increased from 169 to 1163 pg/cell. Thus, the apparent methotrexate-induced cellular enlargement observed microscopically represents a true formation of giant cells and is not merely an illusion resulting from an enhancement of cell flattening. Further, methotrexate uncouples cell division from other aspects of the growth process. Although the drug inhibits cytokinesis almost immediately, it permits a continuation of DNA synthesis, protein synthesis, and nuclear division.

MTX-STL cells are morphologically indistinguishable from the naturally occurring STL cells of control cultures at both the light and scanning electron microscope levels (Figs. 1 to 4 and 6).

MTX-STL cells have distinct cell borders which are interwoven by membrane infoldings and microvilli (Figs. 6 and 11). Both fascia adherens and desmosomal junctions are noticeably less frequent in STL cells than in CTL, and both cell-cell and cell-substratum adhesions are reduced considerably (Figs. 11 and 12; Table 1). By comparison with the CTL’s, MTX-STL cells possess more abundant vesicles, granules, smooth and rough endoplasmic reticulum, and Golgi apparatus (Figs. 11 and 14). The RER cisternae are more often dilated than laminar and are filled with an amorphous material (Fig. 13). Mitochondria are considerably more elongated than in CTL cells (Fig. 13), and coated vesicles, which are not common in CTL’s, are more frequently observed in MTX-STL’s (Fig. 14). Cytoplasmic filament bundles are less common in STL than in CTL cells, and when present they are located primarily in the subplasmalemmal and cell contact regions rather than around the nucleus.

**Subculture of MTX-STL Cells.** When STL BeWo cultures prepared by 4 days of incubation with 1 μM methotrexate were subcultured at sparse density in the continued presence of the drug, there occurred a stimulation of nuclear division. Although average nuclear size decreased, the number of nuclei per cell rose considerably (Fig. 15). In extreme cases, up to 20 nuclei/cell were observed. The number of nucleoli per cell also increased. Otherwise, there was little change in the morphology of MTX-STL cells subcultured in the continued presence of methotrexate.

When MTX-STL cells were subcultured at sparse density and the methotrexate replaced with fresh, drug-free medium, there occurred a morphological reversion to the smaller, mononucleated CTL phenotype. The giant, multinucleated cells began to disappear about 24 hr after drug removal and were replaced by clusters of CTL cells over the next several days. During this transition, there was no significant increase in cell debris within the cultures. However, enlarged, multinucleated cells were commonly observed in a configuration similar to that of cytokinesis (Fig. 16). This observation suggests the possibility that a single STL cell may be able to produce multiple daughter cells by dividing several times in the absence of nuclear division. We intend to examine this possibility by time lapse cinematography.

**Table 1**

*Difference in CTL and STL cell adhesiveness*

<table>
<thead>
<tr>
<th>Cell density (% of control)</th>
<th>Trypsin-EDTA incubation (min) required for total detachment of cells from the plastic flask</th>
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<td>5</td>
<td>Control cultures</td>
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<tr>
<td>25</td>
<td>12</td>
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<td>50</td>
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**Chart 3.** The effect of methotrexate upon BeWo cell growth and division. Cultures were seeded at 6.5 x 10^4 cells/T-25 flask in the presence of 1 μM methotrexate. After cell attachment on Day 0, and at 24-h intervals thereafter, cell number (N), culture protein (P), and DNA content were measured. Left, increase in cell number totally blocked immediately by methotrexate. By contrast, DNA continued to increase through Day 4, while protein continued to increase throughout the experiment. Right, cell enlargement involved an increase in the average cellular content of both DNA and protein.


BeWo as an in Vitro Model System. The morphological characteristics which we have described in this paper for CTL and MTX-STL BeWo cells are compared in Table 2 with those reported in the literature for the in utero trophoblast (6, 27, 31). Morphologically, CTL BeWo cells bear a close resemblance to the cells of the in utero cytotrophoblast. When incubated with methotrexate, however, they transform into the STL phenotype and acquire most of the differentiated morphological characteristics of the in utero syncytiotrophoblast.

The similarity between CTL and STL BeWo cells and their in utero trophoblast counterparts extends to the biochemical level as well. In utero, the cytotrophoblast has low levels of both HCG (5, 14) and PALP (21), while its cellular membranes are rich in glycoproteins containing galactosyl residues (16). The in utero syncytiotrophoblast, by contrast, has elevated HCG and PALP levels but contains membrane glycoproteins that are galactose poor (5, 14, 16, 21).

Speeg et al. (26) have demonstrated that the treatment of BeWo cultures with methotrexate induces a considerable increase in PALP activity and HCG production. That the methotrexate induction of the STL phenotype might also be associated with a reduction in surface membrane galactosyl residues is suggested by the finding from our laboratory that 2 lectins with high affinities for galactosyl and N-acetylgalactosyl residues, peanut agglutinin and soybean agglutinin, are considered less effective at agglutinating STL BeWo cells than CTL's (25).

Thus, the rapid cytodifferentiative changes induced by methotrexate, and their rapid reversal following drug removal, make the methotrexate-BeWo system an excellent in vitro model for investigating the nontoxic, cytodifferentiative changes induced in human tumor cells by chemotherapeutic agents. Moreover, the many parallels between BeWo cells in culture and the in utero trophoblast raise the possibility that the BeWo line may prove to be a valuable culture model system for the investigation of mammalian trophoblast development.

Mechanisms of Methotrexate Action. Our results indicate that the inhibition of DNA synthesis is not necessary for the induction by methotrexate of morphological cytodifferentiation (Chart 3). Cellular DNA increased from 20 to 33 pg/cell during a 5-day incubation period with 1 μM methotrexate, providing growth medium was changed daily to avoid medium depletion, and it was during this same period that morphological transformation occurred. These results differ from those of other investigators, who have reported that 0.1 to 1.0 μM methotrexate inhibits DNA synthesis (10, 26). This discrepancy may well arise from methodological differences in culture technique. In our studies, cultures were maintained in complete growth medium and fed at 24-hr intervals. In the other studies, cultures either were not fed after plating (10) or were maintained in serum-free medium (26). Both of these latter conditions might be expected to inhibit DNA synthesis independently of methotrexate through an insufficiency of the nutrients or serum factors required for DNA synthesis.

It has been postulated that the inhibition of DNA synthesis is necessary for the stimulation by methotrexate of PALP and HCG production in the JAR and BeWo choriocarcinoma lines (1, 26). However, in the BeWo line, the stimulation of PALP and HCG occurred only in a methotrexate concentration range that was supramaximal for the inhibition of DNA synthesis. Since this observation could be interpreted to mean that methotrexate inhibits DNA synthesis by a different mechanism than the way it stimulates PALP and HCG production, the hypothesis that the inhibition of DNA synthesis is required for the stimulation of HCG and PALP production remains unproven at the present time.

The pharmacology of methotrexate is complex. Although it is widely assumed that methotrexate exerts its effects by inhibiting the enzyme DHFR, there is considerable evidence in the literature that this is not always true (2, 3, 13). Both thymidylate synthetase and the transformylase enzymes of purine biosynthesis, for example are also targets of methotrexate inhibition (3, 23). Even in those instances where methotrexate may act by inhibiting the production of reduced folate coenzymes from dihydrofolate, it is unlikely that all of the reduced folate coenzymes are inhibited to the same degree as the total pool of tetrahydrofolate, since certain of the coenzymes can be spared at the expense of others (30).

It has been reported for several systems that methotrexate levels sufficient to inhibit DHFR activity maximally do not necessarily result in the inhibition of DNA synthesis (11, 12). In the papers by Goldman et al. (8, 29, 30), they have shown that in Ehrlich ascites and L-cells the incorporation of deoxyuridine into DNA is not significantly affected even when greater than 99% of the high-affinity DHFR sites were occupied by methotrexate. Nor is the incorporation of [3H]formate into RNA, DNA, or protein seriously impaired until the exchangeable pool of intracellular methotrexate accumulates to concentrations in excess of 0.2 μM. In the absence of such an exchangeable pool, the production of reduced folate coenzymes from dihydrofolate continues unabated (30).

The inhibition of DNA synthesis is by no means an invariable consequence of methotrexate treatment. In some systems, cell division is more sensitive to inhibition by methotrexate than is DNA synthesis (24). Indeed, the finding that the blockade of methotrexate inhibits DNA synthesis by a different mechanism than the way it stimulates PALP and HCG production, the hypothesis that the inhibition of DNA synthesis is required for the stimulation of HCG and PALP production remains unproven at the present time.
cell division by methotrexate can be competitively inhibited by 
$N^\delta$-formyItetrahydrofolic acid suggests that a folate-binding site
may be essential to the process of cell division (2).

Thus, it is necessary to consider the possibility that the
effects of methotrexate upon BeWo cells may depend either
upon the differential sensitivity of a number of different target
sites or upon the differential depletion of the several different
reduced folate cofactors. Given the large number of morpho-
logical, biochemical, and growth effects of methotrexate upon
BeWo cells, and the distinctly different dose-response relation-
ships for at least some of these effects, it seems most likely
that the overall set of BeWo cytodifferentiative changes in-
duced by methotrexate results from the concatenation of a
number of separate cellular actions.

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Fig. 1. Control BeWo culture. An enlarged STL cell is surrounded by a number of small CTL cells. Fibrillar bundles are visible in the nucleus of the STL cell. x 400.

Fig. 2. A MTX-STL cell. Giant, multinucleated STL cells were induced by treatment of BeWo cultures with 1 µM methotrexate for 4 days. Nuclear enlargement, nuclear filament bundles, and multiple nuclei can be seen. x 315.

Fig. 3. MTX-STL cells. A field of STL cells in various stages of enlargement and multinucleation is shown following 4 days of incubation with 1 µM methotrexate. One of the cells in the center of the field shows extensive micronucleation. x 315.

Fig. 4. Scanning electron micrograph of a control BeWo culture. A single enlarged STL cell is surrounded by a group of smaller CTL cells. The STL cell is flattened and covered with microvilli while the CTL cells are rounded and possess ruffles as their primary surface projection. x 700.

Fig. 5. Scanning electron micrograph of CTL cells in a 4-day-old control BeWo culture showing surface ruffling and filopodial contacts between cells. x 1,318.

Fig. 6. Scanning electron micrograph of a BeWo culture treated for 3 days with 1 µM methotrexate. The cells are greatly enlarged and flattened and have already transformed into the STL phenotype. Cell surfaces are covered with microvilli, while ruffling is restricted to the lamellar edge. x 1,275.
Fig. 7. Scanning electron micrograph of a control CTL cell surface showing surface ruffles and long microvilli. × 6,640, tilt 75 degrees.

Fig. 8. Scanning electron micrograph of MTX-STL cell surface showing short microvilli and lack of ruffles. × 6,813, tilt 75 degrees.

Fig. 9. Control CTL cell showing perinuclear filament array, oval mitochondria, and a dilated endoplasmic reticulum cisterna. × 13,330.

Fig. 10. CTL intercellular junction. Desmosomal and fascia adherens junctions couple adjacent CTL cells in control BeWo cultures. Filamentous bundles, free ribosomes, mitochondria, Golgi apparatus, and an occasional dilated RER cisternae are also shown. × 20,281.

Fig. 11. Intercellular junction between MTX-STL cells. Adjacent cells are adhered by regions of plasma membrane interdigitation. Desmosomes and fascia adherens junctions are almost entirely absent. × 5,900.
Fig. 12. Enlarged and multilobed nucleus of a MTX-STL cell. x 3,886.

Fig. 13. MTX-STL cell showing dilated endoplasmic reticulum cisternae, free ribosomes, smooth vesicles, isolated filament bundles, and an elongated mitochondrion. x 11,910.

Fig. 14. MTX-STL cells showing microvilli, Golgi apparatus, ribosomes, and microtubules and filament bundles beneath the plasma membrane. Coated vesicles are seen at the base of the microvilli. The electron-clear space is presumably a site of glycogen deposition which is not preserved by the fixative procedure. x 21,015.

Fig. 15. Phase-contrast micrograph of an STL cell treated for 4 days with methotrexate (1 μM) and then replated at low density in the continued presence of the drug. Under these conditions, extensive micronucleation occurs and multiple nucleoli develop. x 1,300.

Fig. 16. Phase-contrast micrograph of a cell recovering from methotrexate. The culture was incubated for 4 days with 1 μM methotrexate and then subcultured at low density in the absence of further drug treatment. During recovery, STL cells largely disappear and are replaced by CTL cells. Cell configurations like the one illustrated are common during the recovery period, and they suggest that CTL cells may be formed by the production of multiple, mononucleated daughters by a single multinucleated STL cell. x 1,136.
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