THE EFFECT OF METHOTREXATE ON THE FOLATE COENZYME POOLS IN HUMAN
HEPATOMA CELLS IN CULTURE

John Galivan¹, Myung Rhee¹, David G. Priest¹, Marlene Bunni², James H. Freisheim³, and John M. Whiteley⁴

¹Wadsworth Center for Laboratories and Research
New York State Department of Health
Albany, NY 12201

²Department of Biochemistry and Molecular Biology
Medical University of South Carolina
Charleston, South Carolina 29405

³Department of Biochemistry and Molecular Biology
Medical College of Ohio
Toledo, Ohio 43699

⁴Department of Molecular and Experimental Medicine
Research Institute of Scripps Clinic
La Jolla, California 92037

INTRODUCTION

Recent studies on the activity of antifolates have been directed at the perturbations in folate pools caused by inhibitors of dihydrofolate reductase. For the most part these studies have been restricted to the effects of methotrexate, but the effects of trimetrexate have also been examined. A constant feature of these results is the elevation of dihydrofolate which accompanies inhibition by both these agents (1-7). A variable result is the extent to which the reduced folate coenzymes are depleted following exposure to the antifolates. In several cell lines the reduction in tetrahydrofolate is quite modest (less than 40%) with the 10-formyltetrahydrofolate being maintained (1-5). In other cases such as L1210 cells in vivo there is a marked depletion of the reduced folate coenzyme pools (6). Preliminary studies with a rodent hepatoma cell line indicated that the reduced folate coenzymes are depleted by 80% or greater and this is compensated for by an increase in H₂PteGlu and 10-HCO-H₂PteGlu (7). The current study extends these investigations to the effect of methotrexate on the folate coenzymes in human HEPG2 hepatoma cells in vitro.

METHODS

Cell culture. Human HepG2 cells were grown in monolayer culture in Dulbecco's MEM containing 10% FBS for 72 hr for all experiments. The cells were pulsed with MTX for 2 hr, washed twice and placed in fresh medium for 2 hr or 16 hr prior to analysis.

Thymidylate and purine biosynthesis. [6-³H]Deoxyuridine incorporation into DNA was used as a measure of de novo thymidylate biosynthesis (8). De novo purine biosynthesis was measured by the incorporation of [¹⁴C] glycine into DNA according to the method of Cadman et al. (9). The results were expressed as dpm/mg cell protein.

Cellular reduced folates. 5,10-CH₂H₂PteGlu, H₂PteGlu, and H₂PteGlu in the cell extracts were measured by the ternary complex formation of [³H]FdUMP-TS-5,10-CH₂H₂PteGlu, as previously described (10, 11). 10-HCO-
H₄PteGlu and 10-HCOH₂PteGlu were measured following conversion to H₅PteGlu in the presence of glycineamideribonucleotide (GAR) and GAR transformylase (GARTF), or in the presence of DHFR and GARTF, respectively (12). 5-CH₃H₄PteGlu was reacted with methylenetetrahydrofolate reductase to form 5,10-CH₂H₂PteGlu (13). 5-HCOH₄PteGlu was reacted with 5,10-methenyltetrahydrofolate synthetase in the presence of ATP (0.5 mM) (14). The product, 10-HCOH₄PteGlu (formed at neutral pH from 5,10-CH₂H₂PteGlu) is converted to H₅PteGlu with GAR and GARTF as above. Individual reduced folate species were calculated by the appropriate subtractions based on each enzymatic conversion.

RESULTS AND DISCUSSION

The effects of methotrexate on human HEPG2 cells were examined over a 72 hr culture period. Cultures exposed to methotrexate continuously over this period result in 50% growth inhibition at a concentration of 8.1 nM. In order to determine the effects of methotrexate on the cellular folate coenzymes, shorter incubation periods are necessary. The concentration chosen for these studies is based upon the fact that 25 μM methotrexate causes not less than 90% inhibition of glycine and deoxyuridine incorporation after a 4 and 18 hr incubation (Table 1). In these studies methotrexate is present for only the first two hours of the incubation. It is then removed and the incubation continued for 2 or 16 hr. The intracellular concentration of total methotrexate is 9.4 and 7.0 μM respectively at the 4 and 18 hr time points. The composition of methotrexate is greater than 80% polyglutamates and is predominately tri- and tetraglutamates.

The folate pools in control cultures and those exposed to methotrexate as described in Table 1 were evaluated. Control cultures contained primarily 10-HCOH₄PteGlu with lesser amounts of 5-CH₃-, 5,10-CH₂-, and H₅PteGlu (Table 2). Following methotrexate exposure for 4 hr there was a near complete depletion of all reduced folates with an increase in H₂PteGlu and 10-HCOH₂PteGlu to approximately 20 pmol/mg. The folate profile after a 16 hr exposure to methotrexate is essentially the same as that at the 4 hr point. Complete recovery of the folate pool was not observed in these experiments. However, the amount of folate recovered after methotrexate exposure has to be corrected upward by 25% to account for the loss of total cellular folate caused by methotrexate. This results in the recovery of total folates of approximately 60% the methotrexate treated cells are compared with untreated cultures.

The results of these studies demonstrate that treatment of HEPG2 cells with methotrexate causes an extensive and nearly complete loss of reduced folate coenzymes which is accompanied by an increase in the dihydro- species. The latter does not completely account for the loss in the tetrahydro-derivatives, and the reason for this is under investigation. Analysis of similar studies with other cell lines reveals

Table 1. Effect of methotrexate on glycine and deoxyuridine incorporation by HepG2 cells.

<table>
<thead>
<tr>
<th>MTX (μM)</th>
<th>Time of exposure (hr)</th>
<th>[6H] UdR x10³ dpm/mg (%</th>
<th>[14C] glycine x10³ dpm/mg (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-b</td>
<td>-</td>
<td>121.6 ± 33.5 (100)</td>
<td>23.6 ± 2.8 (100)</td>
</tr>
<tr>
<td>25</td>
<td>4</td>
<td>1.15 (0.95)</td>
<td>2.3 (9.9)</td>
</tr>
<tr>
<td>25b</td>
<td>18</td>
<td>0.83 ± 0.19 (0.71)</td>
<td>0.89 ± 0.40 (4.76)</td>
</tr>
</tbody>
</table>

a. HepG2 cells are cultured in Dulbecco's MEM containing 10% FBS for 72 hr. The cells were exposed to MTX for 2 hr and removed for 2 (4 hr exposure) or 16 hr (18 hr exposure), respectively. Glycine and deoxyuridine incorporation was measured as described in "Methods".

b. n = 3 ± SD.
c. average of 2 experiments.