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REGULATION OF METHOTREXATE POLYGLUTAMINATE FORMATION IN EHRLICH ASCITES CARCINOMA CELLS BY ENDOGENOUS FOLATE POOL

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Received 17 June, 1985

The conversion of methotrexate to poly-$\gamma$-glutamyl derivatives in Ehrlich ascites carcinoma cells which are characterized by different pools of endogenous folates is described. The cells in which folate pool was high (the 5-fluorodeoxy-uridine-resistant cell line) the ability to convert methotrexate to its polyglutamate derivatives was much lower than in the cells in which folate pool was smaller (the parental cell line). When the cellular folate pool was reduced by treatment of the cells with lysol-ecithin, a similar methotrexate polyglutamate concentration in both cell lines was observed. These data suggest that cellular folate pool has a regulatory effect on methotrexate polyglutamate synthesis.

Methotrexate, a classical antifolate drug, was widely used for many years in the treatment of human malignances [1, 2, 3, 4, 5]. MTX exerts its primary action by inhibition of dihydrofolate reductase (5,6,7,8-tetrahydrofolate: NADP$^+$ oxidoreductase, EC 1.5.1.3) [6]. In recent years, the intracellular conversion of MTX to its polyglutamate derivatives has been demonstrated in a number of normal and neoplastic tissues [7, 8, 9, 10, 11].

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1 Abbreviations used:
Methotrexate, MTX; 4-amino-$N^{10}$-methylpteroylglutamic acid, 4-NH$_2$-10-CH$_3$PteGlu; methotrexate polyglutamates, 4-NH$_2$-10-CH$_3$PteGlu$_n$, where $1 < n < 6$ means number of $\gamma$-glutamyl residues; S-FdUrd, 5-fluoro-2-deoxyuridine; PBS, buffered salt solution, pH 7.4; HBSS, Hanks' balanced salt solution.
These derivatives have a longer tissue retention time than MTX alone since the polyglutamates do not cross the cell membrane [10, 11]. Their cytostatic activity is suggested by the observation that their inhibitory action on isolated dihydrofolate reductase [9, 12, 13] and deoxyuridine incorporation into DNA in intact cells [14] is the same as or higher than, that of MTX.

Variation in the rate and extent of MTX polyglutamylation among different tissues has been demonstrated and may impart some selectivity to the action of this drug [15, 16, 17]. Recent studies have thus begun to focus on those factors which regulate polyglutamylation of MTX in malignant tissues; one of those factors can be folate level in the cell. In Ehrlich ascites carcinoma cells resistant to 5-fluorodeoxyuridine developed in our laboratory [18, 19] a much higher cellular folate level was found than in Ehrlich carcinoma parental cells. Thus, a very useful model was obtained for investigation of the regulation of MTX polyglutamate formation in cells with different levels of folates. This paper presents the characterization of MTX polyglutamate synthesis by Ehrlich ascites carcinoma parental cells as compared with the 5-FdUrd-resistant subline.

MATERIALS AND METHODS

MTX (Lederle Laboratories, Pearl River N. Y. USA) and [3',5',7-3H]MTX (Amersham/Searle Arlington Highlands ILL. USA) were purified by DEAE-cellulose (Sigma Chemical Co, St. Louis MO, USA) column chromatography as described previously [12]. The concentration of MTX was determined spectrophotometrically at 302 nm. MTX polyglutamate synthetic standards were synthesized and kindly provided by Dr. John A. Montgomery from the Southern Research Institute Birmingham, AL, USA. [3',5',7,9-3H]folic acid and folate radioassay kit were obtained from Amersham International plc (Amersham, U. K.). All other chemicals were of reagent grade and purchased from Sigma Chemical Co (St. Louis MO, USA), Pharmacia P-L Biochemicals (Uppsala, Sweden) and POCh (Gliwice, Poland).

Cell culture. Ehrlich carcinoma parental and 5-FdUrd-resistant cells were transplanted into Swiss mice as described previously [18, 20].

Measurement of MTX and MTX polyglutamates from Ehrlich ascites carcinoma. Cells were harvested during the exponential growth phase (5 days after transplantation) from the peritoneal ascitic fluid into 0°C PBS and washed twice with ice-cold PBS. The cells (10^8 cells) were resuspended in 1 ml HBSS and incubated with 10 μM or 2 μM [3H]MTX (5 × 10^4 dpm/nmol) for various periods of time. The cells were cooled and washed twice with ice-cold PBS, resuspended in 1 ml ice-cold water and immediately placed for 10 min in boiling water bath, then cooled and centrifuged at 10 000 g for 10 min. For evaluation of intracellular MTX and its polyglutamates the supernatants and synthetic MTX polyglutamate used as standards were chromatographed on DEAE-cellulose as described previously [21].

Permeable and sealed cells. The cells were made permeable with lysolecithin by the modification of the previously described procedures [22, 23]. Cells were
harvested and washed twice with ice-cold PBS, then 10^8 cells per ml were incubated, with 0.1% lysolecithin, 0.25 M sucrose, 10 mM Tris/HCl, pH 7.4, 10 mM EDTA and 10 mM MgCl_2 at 0°C for 5 min (parental cells) or 10 min (5-FdUrd-resistant cells) and after that at 37°C for 5 or 10 min (parental or 5-FdUrd-resistant cells, respectively). This treatment resulted in 100% cell permeation. To obtain sealed cells, the permeable cells were incubated at 37°C for 16 h in 1 ml HBSS containing 1 - 2 mU/ml of insulin. Under these conditions all cells were trypan blue-negative as were the untreated control cells.

Intracellular folate level. For evaluation of intracellular level of folate, a commercial kit for estimation of folates was used. Total folate level was measured in boiled extracts after treatment with conjugase (folate γ-glutamyl hydrolase, EC 3.4.22.12).

Folate polyglutamate synthesis in Ehrlich ascites carcinoma cells. The synthesis of folate polyglutamate was studied by the same procedure as the synthesis of MTX polyglutamate, except that [3',5',7-3H]folic acid was used instead of radioactive MTX. For separation of folates and folate polyglutamates Sephadex G-25 (medium) column was used as described previously [24]. The results of these studies are expressed in μM based upon measurements of intracellular water by the procedure of Kletzian [25].

Cell viability. Cell viability was measured with trypan blue by a modification of the method described by Williams (26).

RESULTS

Measurement of folate level and folylpolyglutamate synthesis in Ehrlich ascites carcinoma and 5-FdUrd-resistant cells. The level of total folates found in boiled extracts of Ehrlich parental cells was three times lower than in 5-FdUrd-resistant cells (Table 1). In contrast the level of folates in sealed cells from both lines was

Table 1

Cell folate level and % of folylpolyglutamate formation in control and sealed Ehrlich carcinoma parental and 5-FdUrd-resistant cells.

Cells were incubated for 24 h with 10 μM [3H]folic acid; folate polyglutamates were separated by Sephadex G-25 gel filtration (see Material and Methods) (Mean of two estimations are given).

<table>
<thead>
<tr>
<th>Cells</th>
<th>Level of total folates (μM)</th>
<th>Polylpolyglutamates formed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>parental</td>
<td>70</td>
<td>20</td>
</tr>
<tr>
<td>5-FdUrd-resistant</td>
<td>210</td>
<td>15</td>
</tr>
<tr>
<td>Sealed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>parental</td>
<td>50</td>
<td>75</td>
</tr>
<tr>
<td>5-FdUrd-resistant</td>
<td>50</td>
<td>68</td>
</tr>
</tbody>
</table>
similar and amounted to 50 μM. In separate experiments total folyglutamate formation in both lines in the control and sealed cells was followed. The results demonstrate small differences in folypolyglutamates formation in the control cells of both lines and 4 times higher formation of folypolyglutamates in the sealed cells of both lines.

**Cellular MTX polyglutamate composition in Ehrlich ascites carcinoma parental and 5-FdUrd-resistant cells.** Incubation of Ehrlich carcinoma parental and 5-FdUrd-resistant cells with 10 μM MTX resulted in a rapid uptake of MTX. After 2 h of drug uptake 15% of total cellular MTX was converted to polyglutamate derivatives in both lines. When the incubation was extended to 24 h, MTX polyglutamates made up to 60% and 30% of the total cellular MTX pool in the parental and 5-FdUrd-resistant cells, respectively (Table 2).

**Table 2**

**Cellular concentration of MTX and its polyglutamates in Ehrlich carcinoma parental and 5-FdUrd-resistant cells after 24 h incubation with MTX.**

Cellular composition of MTX and MTX polyglutamates was based upon the radioactivity in each peak from the DEAE-cellulose chromatography and the specific radioactivity of [3H]MTX (see Materials and Methods). The number in parentheses indicate the percentage of the particular MTX glutamate derivatives, — — non detectable

<table>
<thead>
<tr>
<th>Compound</th>
<th>Parental cells</th>
<th>5-FdUrd-resistant cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>External concentration of MTX (μM)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>4-NH₂-10-CH₃PteGlu</td>
<td>4 (20)</td>
<td>8 (42)</td>
</tr>
<tr>
<td>4-NH₂-10-CH₃PteGlu₂</td>
<td>— —</td>
<td>1 (5)</td>
</tr>
<tr>
<td>4-NH₂-10-CH₃PteGlu₃</td>
<td>2 (10)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>4-NH₂-10-CH₃PteGlu₄</td>
<td>7 (35)</td>
<td>5 (26)</td>
</tr>
<tr>
<td>4-NH₂-10-CH₃PteGlu₅</td>
<td>7 (35)</td>
<td>4 (22)</td>
</tr>
</tbody>
</table>

The 24 h incubation of parental cells with 10 μM MTX resulted in formation of the MTX derivative with three additional γ-glutamyl residues (4-NH₂-10-CH₃PteGlu₄) as the major form (25%) of total polyglutamates with slightly smaller formation of 4-NH₂-10-CH₃PteGlu₃ (20%). At the lower concentration of MTX in the medium (2 μM) both 4-NH₂-10-CH₃PteGlu₃ and 4-NH₂-10-CH₃PteGlu₄ each amounted to 35% of total polyglutamate pool with a smaller amount of 4-NH₂-10-CH₃PteGlu₃.

A similar incubation of 5-FdUrd-resistant cells with 2 μM or 10 μM MTX resulted in smaller intracellular pool of MTX polyglutamyl derivatives and in the shorter γ-glutamyl chain length of predominant derivative than observed in the parental cells (Table 2). At 2 μM MTX 4-NH₂-10-CH₃PteGlu₃ and 4-NH₂-10-CH₃PteGlu₄ amounted to 25% and 15% of total intracellular pool, respectively
(Table 2). After 24 h incubation with 10 μM MTX, its predominant polyglutamate species was 4-NH$_2$-10-CH$_3$PteGlu$_3$ with a small amount of 4-NH$_2$-10-CH$_3$PteGlu$_2$ (Table 2).

**Table 3**

*Cellular concentration of MTX and its polyglutamates in sealed Ehrlich carcinoma parental and 5-FdUrd-resistant cells after 24 h incubation with 10 μM MTX.*

Experimental conditions and calculation are identical as those described in Table 2 except that sealed cells are being examined. The numbers in parentheses indicate the percentage of the particular MTX derivative, — — non detectable.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cellular concentration (μM)</th>
<th>5-FdUrd-resistant cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Parental cells</td>
<td></td>
</tr>
<tr>
<td>4-NH$_2$-10-CH$_3$PteGlu</td>
<td>4.5 (15)</td>
<td>4.5 (15)</td>
</tr>
<tr>
<td>4-NH$_2$-10-CH$_3$PteGlu$_2$</td>
<td>— —</td>
<td>— —</td>
</tr>
<tr>
<td>4-NH$_2$-10-CH$_3$PteGlu$_3$</td>
<td>6 (22)</td>
<td>6 (22)</td>
</tr>
<tr>
<td>4-NH$_2$-10-CH$_3$PteGlu$_4$</td>
<td>10.5 (35)</td>
<td>10.5 (35)</td>
</tr>
<tr>
<td>4-NH$_2$-10-CH$_3$PteGlu$_5$</td>
<td>7.5 (28)</td>
<td>7.5 (28)</td>
</tr>
</tbody>
</table>

*Cellular MTX polyglutamates composition in sealed Ehrlich carcinoma parental and 5-FdUrd-resistant cells.* Under the same experimental conditions as described above using permeated and sealed cells after 24 h incubation with 10 μM MTX, the MTX polyglutamate pool was higher in 5-FdUrd-resistant cells and similar to that found in the parental line, with 4-NH$_2$-10-CH$_3$PteGlu$_4$ as a predominant species, accompanied by slightly smaller amounts of 4-NH$_2$-10-CH$_3$PteGlu$_3$ and 4-NH$_2$-10-CH$_3$PteGlu$_2$ (25% and 20%, respectively) (Table 3). A similar incubation of cells of both lines with 2 μM MTX resulted in conversion of 90% of intracellular MTX to γ-glutamyl derivatives while 4-NH$_2$-10-CH$_3$PteGlu$_4$ and 4-NH$_2$-10-CH$_3$PteGlu$_5$ were each 35% of the total pool (data not shown).

**DISCUSSION**

The efficiency of cytotoxic action of MTX depends on the ability of cells to synthesize polyglutamate derivatives of this drug, which are preferentially retained by the cells [10, 21, 27-29]. Most cultured cancer cells, including Ehrlich ascites carcinoma cells [30, 31] can form MTX polyglutamates. Previous studies have shown that methotrexate and folates compete for the same enzyme for glutamylation [28, 32] and folate cofactors are much better substrates than MTX for polyglutamate synthetase [32]. Thus, intracellular folate pool might play an important role in regulation of MTX polyglutamate synthesis.

In attempting to understand the effect of cellular folates pool on MTX polyglutamate synthesis two Ehrlich ascites carcinoma cell lines with different folate pool were examined. The 5-FdUrd-resistant cell line was characterized by a high
folate pool while in the parental line the endogenous folate pool was three times lower. Both lines exhibited the same linear (for 5 min) initial rate of MTX flux i.e. 0.7 mmol/g of cellular protein per minute (results not shown), and similar percentage (15\%) of conversion of MTX polyglutamate in early stage (up to 2 h) of MTX polyglutamate synthesis. The only difference in MTX polyglutamate pool was found when the accumulation of MTX polyglutamate was saturated [31]. The 5-FdUrd-resistant cell line accumulated 60\% less of MTX polyglutamates than the parental one. Since that the endogenous folate pool was the only difference between these two lines, it seems that high cellular folate pool caused diminishing accumulation of MTX polyglutamate by 5-FdUrd-resistant cells. To check this possibility the reversibly permeable cells partly depleted of the cellular folate pool were used and MTX polyglutamates synthesis was studied. The results demonstrate that MTX polyglutamates accumulation by the folates-depleted cells of both lines was similar. These data show that the ability to convert MTX to its polyglutamate derivatives depends on the physiological folate pool in the cell.

The authors gratefully acknowledges valuable discussions and suggestions of Dr John Galivan and Dr Wojciech Rode.

REFERENCES


