Effect of Methotrexate (MTX) on NAD(P)+ Dehydrogenases of HeLa Cells: Malic Enzyme, 2-Oxoglutarate and Isocitrate Dehydrogenases

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The effects of methotrexate (MTX) on oxygen uptake by permeabilized HeLa cells were evaluated. MTX did not inhibit state III respiration when the oxidizable substrate was succinate, but when the substrates were 2-oxoglutarate or isocitrate the respiration decreased about 50 per cent at 1.0 mM concentration of the drug. This effect was explained by inhibition of 2-oxoglutarate and isocitrate dehydrogenases by MTX. No effect was observed on succinate dehydrogenase. An evaluation of the effects of MTX on malic enzyme activity as measured by pyruvate plus lactate production in intact cells supplied with malate showed a decrease of about 40 per cent in metabolite production using 0.4 mM MTX. HeLa cell malic enzyme, as observed for other tumour cells, is compartmentalized in mitochondria and cytosol, and is another example of a dehydrogenase inhibited by MTX. © 1997 John Wiley & Sons, Ltd.

INTRODUCTION

The biochemical mechanism by which methotrexate exerts its antineoplastic activity has been attributed to its stoichiometric linkage to high affinity dihydrofolate reductase binding sites in mammalian cells. The saturation of these sites with MTX results in the blockage of tetrahydrofolate synthesis from dihydrofolate with consequent decrease in the formation of single carbon units for the synthesis of purine nucleotides and thymidilic acid, thus inhibiting DNA synthesis and cell replication. The binding of MTX to dihydrofolate reductase is favoured by increased levels of NADPH + H+. According to present day clinical trends, MTX is usually administered in high doses, which allows its cytoplasmic accumulation in concentrations higher than those required to saturate dihydrofolate reductase and so also affect other metabolic pathways. In fact, an action of MTX on folate-independent pathways has been suggested by several studies. It has been shown, for example, that MTX inhibits oxygen uptake in some tumour cells as well as in isolated mitochondria of normal cells. In HeLa cells, this chemotherapeutic drug decreases the transplasmamembrane redox activity, and the malate–aspartate shuttle. An action of MTX on calcium fluxes in normal liver mitochondria and HeLa cell mitochondria has been described. It has also been demonstrated that some of these effects in liver mitochondria are related to MTX inhibition of 2-oxoglutarate, isocitrate, malate and pyruvate dehydrogenases.

Recently it was shown that administration of glutamine to animals receiving high-dose MTX
therapy, favours host tolerance to MTX and increases its tumoricidal effectiveness. The authors suggested that the glutamine effect was related to a decrease of GSH levels in tumour tissue and to an increase of the GSH level in normal tissue. Glutamine is the major energy source for tumour cells and is metabolized by tumour and normal cells by different pathways. The difference is related to the utilization of malate derived from glutamine or glutamate by the malic enzyme in a pathway that represents an important source of NADPH for tumour cells, which can be used by glutathione reductase to maintain the reduced state of cell glutathione.

Considering the role of the malic enzyme in tumour cell metabolism and the effects of MTX on the above-mentioned dehydrogenases, the principal aim of our study was to determine if the malic enzyme of HeLa cells could be a target for this chemotherapeutic agent. It was also investigated whether the effect of MTX on HeLa cell citric acid cycle dehydrogenases is similar to that described for liver mitochondria.

MATERIALS AND METHODS

HeLa cells (ATCC) were grown as monolayers in flasks containing Eagle’s medium supplemented with 10 per cent calf serum, with sufficient 7.5 per cent sodium bicarbonate to adjust the pH to 7.4 and then collected as described by Bastos et al. Determinations of cell viability were carried out with Trypan blue according to Phillips. Only preparations with cell viability of >95 per cent were used.

Methotrexate (4-amine-N pteroylglutamic acid) was supplied as sodium salt by Cyanamid Co. (Pearl River, U.S.A.).

Polarographic Determinations

Reactions were carried out in a 1.2-ml water-jacketed closed chamber with magnetic stirring at 30°C. Oxygen consumption was measured polarographically using a Clark-type electrode with a Gilson oxygraph. The respiratory rate, using succinate, 2-oxoglutarate or isocitrate as oxidizable substrate is expressed as nmoles of oxygen consumed per min per 10^6 cells. The reaction medium was phosphate buffered saline (PBS) containing 136-mM NaCl, 81-mM Na₂HPO₄ and 1.46-mM KH₂PO₄, pH 7.4. When it was necessary to permeabilize the cells, digitonin was added directly to the chamber, according to Granger and Lehninger. The effect of MTX on oxygen consumption was evaluated after 10 min pre-incubation (30°C) with the cells, prior to addition of substrates and digitonin. Controls were performed in absence of MTX. The reaction systems are described in the legends of the figures.

Enzymatic Activities

All enzymatic activities were monitored with an Aminco-Chance dual wavelength(split-beam recording spectrophotometer.

Succinate Dehydrogenase (E.C.1.3.99.1). HeLa cells were disrupted by a freeze–thawing treatment, homogenized in a Van Potter apparatus and then centrifuged at 310 g for 10 min (4°C). The homogenate was centrifuged at 8000 g for 15 min. The resulting pellet was suspended in a phosphate buffer (50-0 mM, pH 7.5) and used as the enzyme source. The reaction system was: phosphate buffer (50-0 mM, pH 7.5), EDTA (2.0 mM), sodium succinate (20-0 mM), sodium cyanide (1.0 mM), rotenone (20-0 µM) and 24 µg ml⁻¹ mitochondrial protein fraction. After 10 min incubation the reaction was initiated by addition of 60-0 µM dichlorophenol-indo-phenol (DPIP) and 1-0 mM phenazine methosulfate (PMS). The reduction of DPIP was monitored spectrophotometrically at 660 nm (28°C). The results are expressed as nmoles of DPIP reduced min⁻¹ mg⁻¹ protein.

Isocitrate Dehydrogenase (E.C.1.2.4.2). Cells recently collected and suspended in PBS containing 1-0 mM dithiotreitol (DTT), were disrupted by a freeze–thawing treatment, homogenized and centrifuged at 310 g for 10 min (4°C). The supernatant was used directly as the enzyme source. The activity of isocitrate dehydrogenase was measured spectrophotometrically at 340 nm (28°C) as described by Denton et al. using the following reaction medium: 3-(n-morpholine) propanesulphonic acid buffer (50-0 mM, pH 7-0), MgCl₂ (1-0 mM), rotenone (20-0 µM), ADP (1-0 mM), NADP⁺ (0-4 mM) and 0-68 mg ml⁻¹ cell free protein extract. The reaction was initiated by addition of 0-4 mM sodium isocitrate. The specific activity is expressed in nmoles of NADP⁺ reduced min⁻¹ mg⁻¹ protein.

2-Oxoglutarate Dehydrogenase (E.C.1.2.4.2). Cells recently collected and suspended in phosphate buffer solution (PBS) containing 1-0 mM
DTT were disrupted by a freeze–thawing treatment, homogenized and centrifuged at 310 g for 10 min (4°C). The supernatant was used as the enzyme source. The activity of 2-oxoglutarate dehydrogenase was measured spectrophotometrically according to McCormack and Denton,\textsuperscript{28} using the following reaction medium: phosphate buffer (35.0 mM, pH 7.4), rotenone (21.0 μM), sodium cyanide (2.0 mM), sodium malonate (13.0 mM), DTT (0.8 mM), CoASH (67.0 μM), sodium 2-oxoglutarate (8.6 mM), sodium malate (0.47 mM) and HEPES (0.08 mM) at 0°C. The reaction was initiated by addition of 3.0 mM sodium malate. As controls, and for each individual situation, incubation systems were set up in the absence of MTX or malate. At the maximum dose, the inhibition was about 50 per cent. The reaction was initiated by addition of 3.0 mM sodium malate. The results are expressed as nmoles of NAD$^+$ reduced min$^{-1}$ mg$^{-1}$ protein.

Malic Enzyme (E.C.1.1.1.40). Recently collected cells were suspended in PBS containing 0-25 mM sucrose, homogenized and centrifuged at 310 g for 15 min (4°C). The supernatant was used as the enzyme source. When necessary the crude extract was centrifuged at 36,000 g for 15 min and the malic enzyme activity was also measured in the mitochondrial pellet and supernatant fractions. The marker enzyme for the mitochondrial fraction was succinate dehydrogenase and for the cytosolic fraction was lactate dehydrogenase. The activity of the malic enzyme was measured at 340 nm (28°C) according to Hsu and Lardy.\textsuperscript{29} The reaction system was: triethanolamine buffer (0.07 MM pH 7.0), MgCl$_2$ (4.0 mM), NADP$^+$ (0.3 mM) and cellular protein in an adequate concentration. The reaction was initiated by addition of 3-0 mM sodium malate. The results are expressed as nmoles of NADP$^+$ reduced min$^{-1}$ mg$^{-1}$ protein.

**Determination of Pyruvate and Lactate Production**

HeLa cell suspension (3.6 × 10$^6$ cells ml$^{-1}$) was prepared in PBS, containing 20-0 mM sodium fluoride plus 5-0 mM sodium arsenite. MTX (0-4 mM) was added to the cell suspension and aerated for 10 min (25°C). The reaction was initiated ($T_0$) by addition of 1-0 mM sodium malate. As controls, and for each individual situation, incubation systems were set up in the absence of MTX or malate. At $T_0$ and $T_{30}$ aliquots were transferred to tubes with perchloric acid (0-47 M) and HEPES (0.08 M) at 0°C, vortexed for 30 s, and placed in ice until the end of experiment. Perchloric acid extracts were neutralized (at 0°C) by addition of 5-0 mM KOH until a pH of 6.5–7.5 was achieved and used in determinations of pyruvate and lactate. All metabolite assays were carried out on the same day as the experiment.

Pyruvate Determination

Pyruvate was determined as described by Czok and Lamprecht.\textsuperscript{30} The test media contained: Tris–HCl buffer (0-1 M, pH 7.4), NADH (85-0 μM), 5 U of lactate dehydrogenase and 0-3 ml of neutralized extract. After 15 min incubation, NADH oxidation was measured spectrophotometrically at 340 nm. The results are expressed as nmoles of pyruvate per 10$^6$ cells.

Lactate Determination

Lactate was determined as described by Hohorst.\textsuperscript{31} The assay media contained: 0-5 mM glycine–0.2 M hydrazine buffer (pH 9.0), NAD$^+$ (1-5 mM), 10 U of lactate dehydrogenase and 0.05 ml of acid extract. After 90 min incubation (28°C), reduction of NAD$^+$ was measured spectrophotometrically at 340 nm. The results are expressed as nmoles of lactate per 10$^6$ cells.

**Protein Determination**

The protein of HeLa cell extracts was assayed by the method of Lowry et al.\textsuperscript{32} using bovine serum albumin as standard.

**Statistical Analysis**

Results are reported as means ± SD. Differences of $p < 0.05$ by the Student’s $t$-test were considered to be significant.

**RESULTS AND DISCUSSION**

Figure 1 shows the effect of MTX on oxygen consumption in digitonin-permeabilized HeLa cells. Since these experiments were carried out in the presence of ADP, the respiratory activity measured is equivalent to state III respiration. In this situation the succinate oxidation was not affected. On the other hand, MTX in a dose-dependent fashion, was able to inhibit oxygen consumption when the substrate was 2-oxoglutarate or isocitrate. With a maximum dose, the inhibition was about 50 per cent for both substrates. Taking into account that MTX in liver mitochondria inhibits several dehydrogenases,\textsuperscript{8,14–16} the results shown in Figure 1 may also be a consequence of the action of MTX on substrate oxidation. To clarify this proposition, the effect of the drug on the activities of 2-oxoglutarate, isocitrate and succinate...
dehydrogenases of HeLa cells was evaluated. The results presented in Figure 2 (A and B) show an inhibition of 100 per cent for 2-oxoglutarate and isocitrate dehydrogenases at 0.1 mM and 0.5 mM MTX respectively. No inhibition of succinate dehydrogenase was observed. In spite of the 100 per cent inhibition of the NAD\(^+\)-enzymes (Figure 2), the maximal decrease in oxygen consumption was 50 per cent (Figure 1). A possible explanation could be that the drug dilution among several affinity sites present in permeabilized cells used in the polarographic experiments was high and insufficient to inhibit the enzymes completely or this effect may reflect a permeability barrier across the mitochondrial membranes.\(^8\) These results are quite similar to those reported by Yamamoto \textit{et al.}\(^7,8\) for normal rat liver mitochondria, but with higher drug concentrations.

Figure 3 shows the effect of MTX on the pyruvate plus lactate production by intact HeLa cells supplied with malate. In this experiment, sodium fluoride was included to block pyruvate production by endogenous metabolism through the glycolytic pathway and sodium arsenite to block pyruvate utilization by pyruvate dehydrogenase. It was also necessary to evaluate lactate production by the cells because lactate dehydrogenase was unblocked. Thus, the amount of pyruvate plus lactate measured represents an evaluation of the malic enzyme in intact cells. It can be observed that in HeLa cells, substantial amounts of malate are metabolized by the malic enzyme and in the presence of MTX, the metabolite production was about 70 per cent in relation to the control, suggesting an action of MTX on the malic enzyme.

Table 1 shows that HeLa cells had a mitochondrial malic enzyme corresponding to about 20 per cent of the total enzyme activity of the cell-free extracts. In mitochondria where the matrix volume is very small and the protein concentration very high\(^33\) this value represents significant enzyme
activity in HeLa cell mitochondria, allowing pyruvate formation in the mitochondrial matrix. This observation is similar to that for Ehrlich tumour cells and other tumours. The inhibitory effect of MTX on the NADP⁺-malic enzyme of HeLa cells can also be seen in Figure 4. The results shown represent a measure of the total enzyme. Considering the 100 per cent inhibition of the enzyme of cell-free extracts (total cell enzyme) observed at 0.4 mM MTX, it is possible to suggest that malic enzyme of the mitochondria and cytosolic compartments are similarly affected by MTX. According to Sauer et al., the malic enzyme is a tumour progression-linked enzyme. Its inhibition and that of the citric acid cycle dehydrogenases by MTX would affect the general energy metabolism of HeLa cells and also the availability of NADPH in the cell. In this case, it may be possible that the decreased levels of GSH in the tumoural tissues of MTX-treated animals, as described by Rose et al., could be related to wider effects of this chemotherapeutic drug, that contribute to cell death.

It has been reported in several studies that other NAD(P)⁺-dependent dehydrogenases are inhibited by MTX. The general inhibition of these enzymes by MTX, which is observed in the reduction and oxidation of adenine nucleotides, suggests the existence of a domain capable of binding MTX, that is very similar in all these proteins.

The inhibition of malic enzyme described in this article is important because it occurred at therapeutic levels of the MTX. Moreover, additional experimental work, dealing with the enzymes involved in the homeostasis of cellular glutathione is highly desirable and is in progress.

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