Response of methylenetetrahydrofolate levels to methotrexate in Krebs ascites cells

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Levels of methylenetetrahydrofolate in Krebs ascites cells subsequent to transplantation and the effects of methotrexate on these levels have been measured. To directly measure methylenetetrahydrofolate in tissue extracts, the cofactor was incorporated into a covalent ternary complex with thymidylate synthase and ^3H -labelled fluorodeoxyuridine monophosphate. A 3–4-day lag preceded rapid growth of the tumour cells, and this same kinetic behaviour was observed for methylenetetrahydrofolate levels in the tumour cells. Liver and kidney tissue from the same animals also showed an increase in methylenetetrahydrofolate over the same time period. The impact of methotrexate on methylenetetrahydrofolate in the tumour cells depended upon concentration and the post-transplantation time at which treatment was initiated. Levels of methylenetetrahydrofolate in the tumour cells were most sensitive to the drug at the beginning of the rapid growth phase and were more sensitive to a given level of methotrexate in the presence of phospholipids. A slight but significant increase in methylenetetrahydrofolate occurred in some cases in response to the presence of methotrexate.

The antifolate methotrexate has been effective in the treatment of a number of neoplasms (Johns & Bertino, 1973; Chabner *et al.*, 1975) and has been shown to have a high affinity for the enzyme dihydrofolate reductase (Bertino *et al.*, 1965). The antitumour action of methotrexate is presumed to result from inhibition of dihydrofolate reductase and a concomitant decrease in cellular tetrahydrofolate, and hence methylenetetrahydrofolate, causing an inadequate supply of this cofactor for the production of thymidylate via the thymidylate synthase reaction (Blakely, 1967; Hunnekens *et al.*, 1976; Goldman, 1977; Danenberg, 1977).

Ascites tumour cells grown intraperitoneally are also anticipated to respond to methotrexate through a decrease in methylenetetrahydrofolate, resulting from inhibition of dihydrofolate reductase (White & Goldman, 1981). However, the intracellular level of this folate depends upon availability of folates from the host animal as well as the metabolic disturbance caused by the antifolate. Also, in order to investigate changes caused by the presence of the drug, it is

necessary to take into account fluctuations in cofactor concentrations that occur independently of methotrexate. To address these questions, the methylenetetrahydrofolate level of both ascites cells and host tissues, and the effects of methotrexate on these levels, have been determined subsequent to transplantation.

Materials and methods

$[^3\text{H}]\text{FdUMP}$ (sp. radioactivity 18 Ci/mmol) was purchased from Moravék Biochemicals. *Lactobacillus casei* thymidylate synthase was prepared from methotrexate-resistant cells by the method of Galivan *et al.* (1975). Methotrexate was purchased from Lederle. Phosphatidylcholine was obtained from Grand Island Biochemical Co. All other reagents were purchased from Sigma.

Isolation and inoculation of Krebs ascites cells were performed under sterile conditions. Typically 5×10^6 cells were used for intraperitoneal inoculation. Upon killing and isolation, cells were found to be 99% viable by the Trypan Blue exclusion assay. Animals used were male ICR white mice that weighed 28–32 g in all cases. They were maintained

Abbreviation used: $[^3\text{H}]\text{FdUMP}$, fluoro $[^3\text{H}]$ deoxyuridine monophosphate.

in groups of six and fed standard lab chow and water *ad libitum*. Tumour weight was determined from the difference between the weight of inoculated and tumour-free control mice.

Methylenetetrahydrofolate was determined as described previously (Priest *et al.*, 1982, 1983). Tissue, other than tumour cells, was extracted by homogenization in a buffer that contained 0.05 M-Tris/HCl, pH 7.4, 1 mM-EDTA, 0.05 M-sodium ascorbate and 0.25 M-sucrose. Tumour cells were lysed by repetitive freeze/thaw in solid- CO_2 /acetone mixtures in the same buffer. After centrifugation to clear solutions, portions were introduced into reaction mixtures which contained 250 nM-[^3H]FdUMP (sp. radioactivity 18 Ci/mmol), 1.28×10^{-3} units of *L. casei* thymidylate synthase, 0.05 M-Tris/HCl, 0.05 M-N-methylmorpholine, pH 7.4, 25 mM-MgCl₂, 1.0 mM-EDTA and 6.5 mM-formaldehyde in a total volume of 100 μl . Protein concentration was determined by the method of Bradford (1976).

Liposomes were prepared by evaporation to dryness of a 10 ml chloroform solution that contained 30 mg of egg phosphatidylcholine, 4.4 mg of cholesterol and 1.6 mg of stearylamine. The dried lipid film was removed, and liposomes were formed with 10 ml of 0.15 M-NaCl and agitation on a vortex-mixer under an N_2 atmosphere. To prepare drug-entrapped liposomes, the dried lipid film was removed with 10 ml of methotrexate (6.0 mg/ml) dissolved in 0.15 M-NaCl.

Results

Fig. 1 shows the change in methylenetetrahydrofolate levels in liver, kidney and tumour cells of mice after intraperitoneal inoculation with Krebs ascites cells. Growth of tumour cells over the same period is also shown. It can be seen that a lag in the growth rate of the ascites cells occurs initially, with maximal growth occurring after day 4, as has been observed previously with Ehrlich ascites cells (Eckhardt *et al.*, 1982). This rapid phase of growth occurs concomitantly with an increase in the methylenetetrahydrofolate level of the tumour cells and two major organs of the mouse as well. As the growth rate slows and host death approaches, methylenetetrahydrofolate in liver, kidney and the tumour cells returns to near baseline levels.

Fig. 2 shows that the concentration of methylenetetrahydrofolate responds to the presence of methotrexate 24 h after administration. The co-factor level is diminished in a strict dose-dependent manner if the drug is administered on day 4 after transplantation but not on day 8. Also, the cells are more drug-sensitive on day 4 compared with day 8. In fact a slight increase in cofactor level results from low drug levels at the latter post-transplantation

Fig. 1. Changes in methylenetetrahydrofolate levels in liver (●), kidney (△) and tumour cells (○) and in tumour weight (□) after intraperitoneal transplantation of Krebs ascites cells

Approx. 5×10^6 cells were inoculated into each mouse. After removal, cells were washed in 0.15 M-NaCl and counted in a haemocytometer; 7×10^7 cells were suspended in 1.0 ml of buffer, which contained 0.05 M-Tris/HCl (pH 7.4), 1 mM-EDTA, 0.05 M-sodium ascorbate and 8.5% sucrose. Cells were lysed by the freeze/thaw method in a solid- CO_2 /acetone mixture. Tissue (2 mg of liver, 3.5 mg of kidney) was homogenized in the same buffer. Cell-free extracts in each case were centrifuged at 20000 g for 20 min. Reaction mixtures (100 μl) contained 250 nM-[^3H]FdUMP, 1.28×10^{-3} units of *L. casei* thymidylate synthase in 0.05 M-Tris/HCl, 0.05 M-N-methylmorpholine, pH 7.4, 10 mM-2-mercaptoethanol, 25 mM-MgCl₂, 8.5% sucrose, 1 mM-EDTA, 6.5 mM-formaldehyde, and the cell-free extract. Reaction mixtures were incubated for 30 min at 25°C, and complexed [^3H]FdUMP was separated from free [^3H]FdUMP by Sephadex G-25 chromatography. Points represent the average of duplicate assays from a single animal at each time shown. All experiments (except kidney determinations) were repeated entirely at least twice. Some quantitative variation occurred, but general trends were reproducible in every case.

time. Finally, administration of relatively high drug levels early or late could not diminish methylenetetrahydrofolate levels to less than 50% of the pretreatment level.

The slight increase in methylenetetrahydrofolate level at low methotrexate concentrations was investigated further by monitoring the change in methylenetetrahydrofolate concentration at earlier times after drug administration. Relatively high and low concentrations of methotrexate were used on days 4 and 8 after transplantation. Fig. 3 shows that, during

Fig. 2. Changes in methylenetetrahydrofolate levels in Krebs ascites cells as a function of methotrexate on the 4th (O) and 8th (●) day after transplantation

Assays were carried out, and the concentrations of methylenetetrahydrofolate calculated and presented, as in Fig. 1.

the first few hours, low drug levels resulted in an increase in methylenetetrahydrofolate for cells treated on both day 4 and day 8. High methotrexate levels, on the other hand, caused the expected decrease in cofactor level for cells treated 8 days after transplantation, but the more sensitive day-4-treated cells responded by a marked increase over the first few hours after drug treatment.

An attempt was made to use liposomal encapsulation of methotrexate to gain a more rapid uptake and therefore obtain a more rapid response to the drug (Gregoriadis, 1978). Fig. 4 shows that liposomal preparations enhanced the response observed with methotrexate. However, approximately the same decrease in methylenetetrahydrofolate was observed regardless of whether methotrexate had been encapsulated into liposomes or simply administered in the presence of phospholipid. Thus, methotrexate in the presence of lipid had a greater impact on lowering methylenetetrahydrofolate levels than in the absence of lipid, but uptake enhancement was probably not due to liposomal encapsulation of the drug.

Discussion

In order to replicate, ascites cells grown intraperitoneally must acquire folate from the host. This folate may already be in a usable reduced form or could be metabolized to such a form after uptake. This acquisition, at least with regard to methylenetetrahydrofolate, does not occur immediately

Fig. 3. Kinetics of the change in methylenetetrahydrofolate of tumour cells after the introduction of methotrexate

Two doses of methotrexate, 1.8 mg/kg body wt. (●) and 36 mg/kg body wt. (○), were introduced 4 days after transplantation, and the same two doses, 1.8 mg/kg body wt. (■) and 36 mg/kg body wt. (□), were introduced 8 days after transplantation. Assays were carried out, and concentrations of methylenetetrahydrofolate calculated and presented, as in Fig. 1.

Fig. 4. Kinetics of the change in methylenetetrahydrofolate of tumour cells treated with free methotrexate (O) (18 mg/kg body wt.), liposome-encapsulated methotrexate (Δ) (18 mg/kg body wt.) and liposomes plus methotrexate (●) (18 mg/kg body wt.) on the 8th day after transplantation

Assays were carried out, and methylenetetrahydrofolate concentrations calculated and presented, as in Fig. 1.

upon transplantation (see Fig. 1). A lag of several days precedes an increase in the intracellular level of this cofactor form. A similar lag precedes rapid growth of the tumour mass. Since many other events are also required for tumour growth, it cannot be concluded that the lag is due to an inadequate supply of methylenetetrahydrofolate alone, but it is possible that this is at least one of the contributing factors to the delay in attainment of a rapid growth rate.

Since the only source of folate to support tumour cell growth is the host animal, levels of cofactor in selected mouse tissues were examined in the present study. Rather than depletion of host methylenetetrahydrofolate pools in the liver and kidney, as had been expected, the presence of the tumour caused an increase in this cofactor form in both tissues. Since there was no change in dietary availability of folate to these animals, it is probable that the presence of the tumour cells resulted in a diversion from the total folate pool into the methylenetetrahydrofolate form to support the rapid growth of the tumour cells. This could suggest that reduced folates are available to tumour cells for uptake and utilization without extensive metabolism. However, it is also possible that the same circulating factor(s) is capable of diverting tumour-cell, as well as liver and kidney, folates into the methylenetetrahydrofolate form.

The methylenetetrahydrofolate level in tumour cells responded to the antifolate, methotrexate. The greater sensitivity during the early stages of growth could possibly be related to lower intracellular levels of methylenetetrahydrofolate at this time. During later stages there is a higher concentration of methylenetetrahydrofolate and cells have passed the point of maximum growth. At this later stage other factors may become limiting, and thus reliance on methylenetetrahydrofolate is diminished.

The initial effect of methotrexate in these cells is an increase in the methylenetetrahydrofolate level. This effect diminishes with post-inoculation age of the tumour cells. Such a methotrexate-caused increase is in direct contradiction to the presumed primary effect of this antifolate (i.e. inhibition of dihydrofolate reductase resulting in decreased methylenetetrahydrofolate). The mechanism by which this apparent initial enhancement occurs is unclear and requires further investigation in the future.

An effort was made to enhance the uptake rate of methotrexate, to see if differences in the methylenetetrahydrofolate response could be related to drug uptake kinetics. Indeed an earlier impact of methotrexate on methylenetetrahydrofolate was observed in the presence of the liposomal preparations. However, the effect was apparently not related to encapsulated drug uptake, but rather was due to a general effect of lipid on cell membranes, as observed by Balinska *et al.* (1982) with rat hepatoma cells. Thus, pursuit of this approach to separate uptake effects from anti-metabolite effects was terminated, since the apparently general effect of lipid on transmembrane absorption was deemed too broad to be useful for this purpose.

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