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Influence of intracellular folates on methotrexate metabolism and cytotoxicity

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The antifolate methotrexate (MTX) is given clinically in high doses to overcome tumor resistance. This therapy is administered in association with 5-formyltetrahydrofolate (folinic acid; leucovorin; ^5N-CHO-H_4PteGlu) to prevent antifolate toxicity [1]. Increased intracellular folate levels are thus potentially achieved not only in normal but also in tumor cells. This could possibly affect the sensitivity of the latter to subsequent MTX therapy since folate repletion of tumor cells inhibits MTX polyglutamate (MTXPG) formation [2, 3]. In the present report, we have examined the effects of varying the tumor cell intracellular folate pool size on MTX cytotoxicity and have confirmed that tumor cell sensitivity to MTX decreases as folate pools rise because of impaired MTXPG formation.

Methods and results

Chemicals. [3′,5′,7-^{3}H]MTX (sp. act. 20 Ci/mmol) and [3′,5′,7,9-^{3}H]folic acid (sp. act. 18 Ci/mmol) were purchased from Moravek Biochemicals, Inc. (Brea, CA). (6*S*)-[3′,5′,7,9-^{3}H]Folinic acid and unlabeled (6*S*)-folinic acid were synthesized by the method of Moran and Colman [4]. Unlabeled MTX was obtained from the National Cancer Institute (Bethesda, MD). All other chemicals were of reagent grade and purchased from the Fisher Scientific Co. (Pittsburgh, PA) or the Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) was obtained from Gibco Laboratories (Chagrin Falls, OH) and treated with dextran-coated charcoal (cFBS) at room temperature until less than 1% of the [^{3}H]thymidine added before the procedure remained.

Propagation of cells in culture. MCF-7 cells, a line of human breast cancer cells in continuous monolayer culture [5], were obtained from the American Type Culture Collection (Rockville, MD). The cells were grown in Dulbecco's modification of Eagle's medium (DMEM) (Flow Laboratories) supplemented with 10% FBS, penicillin (200 μg/ml) and streptomycin (200 μg/ml) under 5% CO_2 at 37°. Prior to all experiments, MCF-7 cells were folate depleted by transferring them to folate-free DMEM (Flow Laboratories) containing 10% charcoal-treated FBS (cFBS) for 10 days until cell growth stopped. The cells were then refed with the same medium containing either added 10 μM folic acid or 10 μM (6*S*)-folinic acid, and experiments were performed approximately 5 days later when both refed cell types were at the same logarithmic cell growth rate. In all experiments, the folate-containing medium was removed, and the cells were washed and transferred to folate-free DMEM to remove exchangeable folate 1 hr prior to MTX exposure.

MTX cytotoxicity. We first examined the consequences of two different folate exposures on MTX cytotoxicity. Results are illustrated in Fig. 1. Folic acid refed cells were significantly more sensitive to MTX than folinic acid refed cells with an IC_{50} of 0.04 μM in the folic acid cells compared to 0.25 μM for the folinic acid refed cells.

Intracellular folates. We next examined the consequences of the two different folate exposures on total intracellular folate pools in MCF-7 cells. Total intracellular folates and intracellular monoglutamated folates were assayed after logarithmic growth had resumed following folate depletion and refeeding with either 10 μM [^{3}H]folic acid (final sp. act. 2.25 Ci/mmol) or 10 μM [^{3}H]-(6*S*)-folinic acid (final sp. act. 0.11 Ci/mmol). The methods used were described recently by Allegra *et al.* [6]. The following results represent the mean ± SD of three experiments: total folate pools after 10 μM (6*S*)-folic acid exposure were over 10-fold lower (7.22 ± 0.74 nmol/g) than after 10 μM folinic acid exposure (89.2 ± 20.1 nmol/g). The distribution of intracellular folates was then determined by HPLC (N = three experiments): 5-CH_3H_4PteGlu pools represented a slightly higher percentage of intracellular folates in folic acid compared to folinic acid refed cells (53.7 ± 2.88% vs 45.4 ± 0.74%), whereas the combined H_4PteGlu-10-CHO-H_4PteGlu pools made up a greater portion of intracellular folates in folinic acid refed cells (46.0 ± 0.57 vs 33.5 ± 1.90%). Intracellular folinic acid represented 8.59 ± 1.13% of intracellular folates in folinate-refed cells and 2.03 ± 0.65% in folate-refed cells. Intracellular folic acid was only identified in cells exposed to folic acid (10.8 ± 1.43% of intracellular folates).

MTX uptake. To determine the mechanisms underlying the decrease in MTX cytotoxicity observed in the folinic acid refed cells, we examined if higher intracellular folate

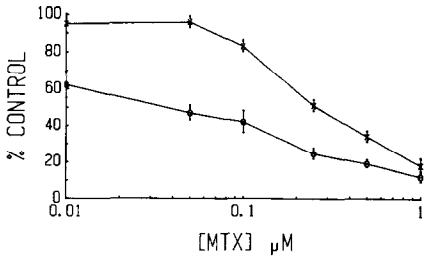

Fig. 1. MTX cytotoxicity. MCF-7 cells were folate depleted and refed with either 10 μM folinic or (6*S*)-folinic acid. The cells were then exposed for 24 hr to 0.01, 0.05, 0.1, 0.25, 0.5 or 1 μM MTX after which they were washed three times with phosphate-buffered saline (PBS) and transferred to DMEM containing 10% cFBS. Cell growth was determined after 7 days by visual counting of trypsinized cells with a hemacytometer. The mean results and standard deviation bars of three experiments done in triplicate are illustrated. Results are expressed as percentage of control growth. Total number of cells for the control was 4.5×10^6. Key: (○) folic acid pretreated cells, and (×) folinic acid exposed cells.

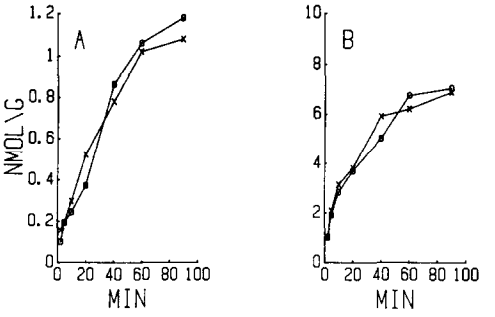

Fig. 2. MTX uptake. MCF-7 cells were folate depleted, refed with either 10 μM folic or (6*S*)-folinic acid and exposed to folate-free DMEM containing 10% cFBS and either 0.05 (sp. act. 20 Ci/mmol) or 1 μM [^{3}H]MTX (final sp. act. 4.5 Ci/mmol). At various time intervals, MTX uptake was measured as described by Schilsky *et al.* [7]. Results are expressed in nmol/g of protein. MTX uptake after exposure to 0.05 and 1 μM MTX are illustrated in panels A and B respectively. Mean results of four experiments are shown. Key: (○) folic acid refed cells, and (×) folinic acid refed cells.

pools led to decreased MTX uptake. As shown in Fig. 2, MTX uptake was identical at both MTX concentrations tested and under both folate repletion states.

MTX metabolism. To determine if the differences in cytotoxicity observed were secondary to decreased MTXPG formation, we next measured MTX metabolism by HPLC as previously described [8]. As shown in Fig. 3, MTXPG formation was depressed after exposure to either 0.05 or 1 μM [^{3}H]MTX of cells refed with folinic acid. There was no cytotoxicity seen after 0.05 μM MTX in folinate-refed cells, and no retainable MTXPGs (MTXPGs containing a total of from 3 to 5 glutamyl residues; MTXGlu$_{3-5}$) were formed. There was a greater than 50% decrease in cell growth under the same conditions in the folic acid refed cells, and 21.4% of intracellular MTX was in the form of MTX-Glu$_{3-5}$. Furthermore, after 24 hr in drug-free medium, these metabolites were retained and still made up 28.7% of the intracellular drug. After 1 μM [^{3}H]MTX incubations, MTX-Glu$_{3-5}$ represented 50.8 and 29.8% of intracellular MTX after folic and folinic acid refeedings, while cell growth was decreased by 85 and 75%, respectively, compared to control cells. There was significant retention of MTX-Glu$_{3-5}$ under both circumstances, and the metabolites represented 87.4 and 64.2% of intracellular drug after folic and folinic acid refeedings, 24 hr after transfer in drug-free medium.

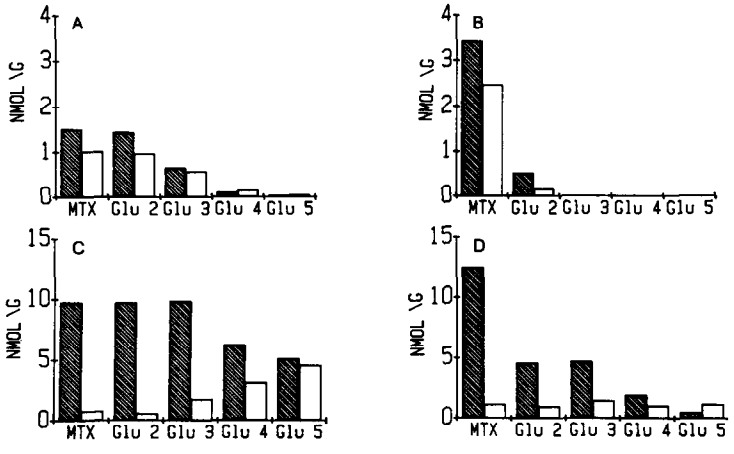

Fig. 3. MTXPG formation and retention. MCF-7 cells were folate depleted and refed with folate and exposed to folate-free DMEM (containing 10% cFBS and either 0.05 (sp. act. 20 Ci/mmol) or 1 μM [^{3}H]MTX (final sp. act. 4.5 Ci/mmol) for 24 hr. MTXPG formation was determined by HPLC at the end of incubation (hatched bars) and 24 hr after transfer to drug-free DMEM containing 10% cFBS (open bars). Results are expressed in nmol/g of protein precipitate. MTXPGs formed and retained after exposure of 10 μM folic acid refed cells to 0.05 and 1 μM MTX are illustrated in panels A and C respectively. MTXPGs in cells refed with 10 μM (6*S*)-folinic acid after exposure to 0.05 and 1 μM MTX are illustrated in panels B and D respectively. Glu$_2$ to Glu$_5$ represent MTXPGs containing a total of 2 to 5 glutamyl residues. The mean results of three experiments are shown.

Discussion

Expansion of intracellular folate pools by folinic acid exposure can decrease tumor cell sensitivity to MTX probably because high cellular folate contents decrease the capacity of the cells to metabolize MTX to polyglutamate derivatives. Indeed, MTXPGs are thought to be very important to the antitumor activity of the parent drug by adding important new characteristics to MTX [9]. Associations between impaired capacity to produce MTXPGs, decreased intracellular retention of the metabolites and decreased MTX sensitivity have been made previously in a number of experimental models [9]. Our findings extend earlier reports of the inhibition of MTXPG formation in cells by increased folate pools [2, 3] by showing that decreased metabolism leads to impaired cytotoxicity. However, the mechanism underlying the inhibition of MTXPG formation by intracellular folates remains uncertain. Possible sites of interaction between folates and antifolates leading to decreased MTX metabolism include membrane transport and folylpolyglutamate synthetase. Since MTX uptake was identical under both folate repletion states examined, folates and MTX probably interacted at the enzymatic level. The folinic acid refed cells had 10-fold higher folate levels than the folic acid refed cells with a similar distribution of reduced folates under both conditions. Reduced folates are better substrates for folylpolyglutamate synthetase than MTX and can decrease MTXPG synthesis *in vitro* when both substrates are coincubated [10] and possibly *in vivo* as suggested by our experiments. Since high-dose MTX therapy is followed clinically by rescue with folinic acid, it would be important to know what effect this folate exposure has on intracellular tumor cell folate pools. If the latter remained elevated until subsequent MTX administration, they might decrease MTX metabolism and cytotoxicity during subsequent MTX therapy.

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Hydroxyurea, methotrexate and adriblastine can mediate non-enzymatic reduction of nitroblue tetrazolium with NADH which is inhibited by superoxide dismutase

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Free radical mediated reactions are known to induce a number of types of cell damage [1]. Among them are highly degradative processes of lipid peroxidation exerted primarily within plasma membranes. Increasing number of drugs are suggested to cause their toxic effects through the interference with free radical reactions. Among anticancer agents quinone containing anthracycline antibiotics are known to exert their cytotoxicity through generation of free radicals and their side toxic actions *in vivo* have been attributed to their ability to induce peroxidative injury [2]. However, several non-specific, possibly peroxidative, toxic effects induced by treatment with anticancer agents unrelated structurally to anthracycline antibiotics, have been reported recently.

Although it is generally believed that the primary mechanism of HU cytotoxicity is the inhibition of ribonucleotide reductase, an additional mechanism of its toxicity is considered [3, 4]. This is based on the hypothesis of DeSesso [5] formulated to explain the teratogenic action of this drug and rapid cell killing and suggesting that it may exert side toxic action through free radical reactions. The hypothesis has been supported by several observations indicating that HU* treatment can induce changes in osmotic fragility, methemoglobim formation and sensitivity to oxidant stress in erythrocytes [6], suppress phagocytic activity [7] and migration rate (I. Szczepańska, J. Malec; unpublished) of leucocytes and impair lysosomal stability [8, 9]. Moreover, the exposure of L5178Y lymphoblasts to higher concentrations of HU induced X-ray-like post-treatment giant cell formation and blockage of DNA synthesis [10]. All these toxic effects could be substantially prevented by radical scavengers, among them tocopherol—the most potent physiological membrane antioxidant. Despite these effects suggesting peroxidative mechanism of HU-induced cell damage, our attempts to detect malondialdehyde formation in HU-treated cells were unsuccessful.

MTX is a recognized antifolate antagonist. However, there were observed several MTX-induced toxic effects which are difficult to explain by the antifolate mechanism. They were: occurrence of methemoglobim formation in intact erythrocytes [11] and diene conjugation in erythrocyte ghosts (W. M. Przybyszewski and J. Malec; unpublished), suppression of phagocytic activity [12] and of migration rate of leucocytes which could be prevented by

* Abbreviations used: HU, hydroxyurea; MTX, methotrexate; 5-FU, 5-fluorouracil; ADB, adriblastine; SOD, superoxide dismutase; NBT, nitroblue tetrazolium.