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INHIBITION OF GAMMA-GLUTAMYL HYDROLASES IN HUMAN CELLS BY 2-MERCAPTOMETHYLGLUTARIC ACID

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Summary: Cultured human lymphocytes and fibroblasts accumulate methotrexate during 24 hours and synthesize methotrexate polyglutamates up to the hexaglutamate, with the triglutamate predominating. In the interval after incubation with methotrexate, drug is lost, metabolites are converted to longer chain-lengths, and methotrexate pentaglutamate predominates. 2-Mercaptomethylglutaric acid, an inhibitor of neutral pH gamma-glutamyl hydrolases in vitro, had little effect on polyglutamate synthesis during incubation of the cells with methotrexate, but maintained for 48 hours almost all the methotrexate as the pentaglutamate when added after the removal of the drug. These findings demonstrate that inhibition of gamma-glutamyl hydrolases is an effective approach to alter the distribution of polyglutamate forms of methotrexate in vivo and indicate that enzymatic hydrolysis may contribute to regulation of polyglutamate chain lengths in human cells.

Introduction

Methotrexate (MTX), an analog of folate, and an important anticancer agent, shares with folates the property of being a substrate for the enzyme folylpolyglutamate synthetase (1). This enzyme converts it to MTXPGs which appear to be critical to the cytotoxic action of MTX (2). Degradation of the gamma-polyglutamate tail of MTXPGs is effected by gamma-glutamyl hydrolases (3). These exist in two main classes: acid hydrolases contained in lysosomes and enzymes with neutral pH optima present in the cyto-

Abbreviations: MTX = methotrexate, 4-NH_2-10-CH_3PteGlu; MTXPG = MTX polyglutamate; $MTXGlu_{2-6}$ = 4-NH_2-10-$CH_3PteGlu_{2-6}$; 2-MMGA = 2-mercaptomethylglutaric acid.

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plasm or in the jejunal brush-border. We describe the action of 2-mercaptomethylglutaric acid (4), a new inhibitor of neutral pH gamma-glutamyl hydrolases, on MTXPG formation in transformed human lymphocytes and in human diploid fibroblasts.

Materials and Methods

Chemicals were obtained as follows: 3',5'-7-[^{3}H]MTX from Moravek Biochemicals (Brea, CA); Hanks balanced salt solution (Hanks) and Eagles minimal essential medium (MEM) from Flow Laboratories (Mississauga, Ontario), fetal calf serum (FCS) from Gibco Co. (Burlington, Ontario); tetrabutylammonium phosphate from Eastman Kodak Co. (Rochester, NY); and Ready-Solv HP liquid scintillant from Beckman Instruments Co. (Montreal, Quebec). All other chemicals were of reagent or HPLC grade. Standards of $MTXGlu_{2-7}$ were obtained from Dr. C.M. Baugh. Department of Biochemistry, University of South Alabama, Mobile, AL.

2-MMGA was synthesized as previously described (4) and will be published in detail elsewhere. Transformed human lymphoctes were incubated at a density of 5 million cells in 2 ml culture medium in P-35 culture dishes. Human diploid fibroblasts in confluence were incubated in 3 ml culture medium in P-60 culture dishes. Culture medium consisted of MEM containing 10% FCS and added glycine, adenosine and thymidine. Incubations were at 37° in 95 % O_2 + 5 % CO_2. Cells were incubated in culture medium for an additional 24 or 48 h. 2-MMGA was added to test cultures in concentrations of 0.1 and 0.4 mM. Cells were harvested, counted, lysed by sonication (Fisher sonic dismembrator, Model 150); protein was precipitated with 10% trichloracetic acid and the supernatant frozen at -20°. Addition of 2-MMGA during cell harvesting and extraction had no effect on the distribution of MTX and MTXPGs.

MTX and $MTXGlu_{2-6}$ were separated by HPLC using the method of Jolivet et al. (5). Thawed extracts were concentrated and clarified by passage through a SepPak C18 cartridge (Waters Associates, Milford, Mass.). The cartridge was prepared by washing with 2.0 ml methanol, 5.0 ml water, 5.0 ml solvent A (5.0 mM tetrabutylammonium phosphate in 10 mM KH_2PO_4 buffer, pH 5.5) and 5.0 ml water. After the 2.0 ml cell extract was applied, the cartridge was rinsed with 5.0 ml water and then MTX and MTXPGs were eluted in 2.0 ml methanol. After drying under N_2, the precipitate was resuspended in 200 ul of solvent A. There was no preferential loss of MTX or MTXPGs during passage through the SepPak.

Aliquots of 50-100 µl were mixed with 2 µl of a standard mixture of MTX and $MTXGlu_{2-7}$ and injected on a Radial-Pak C8 column contained in a Z-module (Waters Associates, Milford, Mass.). Product was eluted with a gradient starting with a mixture of 80% solvent A and 20% acetonitrile and ending with 64% solvent A and 36% acetonitrile. The gradient was formed in 4 linear steps on an Automated Gradient Controller. Elution was at 2.0 ml/min, collecting 1.0 ml fractions. Slight changes were made to the gradient to maintain separations as columns aged. Standards were monitored spectrophotometrically at a fixed wavelength of 313 mu. Fractions were mixed with 10 ml Ready-Solv and counted in a RackBeta liquid scintillation counter (LKB Wallac Co.).

Table 1. Levels of MTX and MTXPGs in transformed human lymphocytes in the presence and absence of 2-MMGA. Cells were incubated with 1.0 μM [^{3}H]MTX for 24 h then in MTX-free medium for 24 and 48 h with and without addition of 0.1 mM 2-MMGA. Cells were then lysed and levels of MTX and MTXPGs determined by HPLC (see Methods). Results are means of triplicate determinations

Addition of 2-MMGA during MTX medium			Total MTX nmol/	No. of Glu residues (%)						ratio $\frac{Glu_5 + Glu_6}{Glu_3 + Glu_4}$
24h	24h	48h	10^9cells	1	2	3	4	5	6	
-			1.22	9.7	22.2	39.5	21.2	7.5	ND	0.12
+			1.38	11.8	14.8	33.4	24.9	15.1	ND	0.26
-	-		0.82	2.4	3.1	17.3	32.4	42.7	2.1	0.90
-	+		0.77	2.2	2.3	8.5	17.6	67.0	2.3	2.66
-		-	0.50	11.0	8.4	24.8	29.6	24.4	1.8	0.48
-		+	0.62	7.0	3.3	8.9	15.9	62.1	2.8	2.61

ND = not detected

Results

2-MMGA at concentrations of 0.1 - 0.4 mM had no deleterious effect on cell growth or on MTXPG formation. After 24 h incubation in 1.0 μM [^{3}H]MTX, lymphocytes accumulated MTXPGs with as many as 6 glutamate residues (Table 1). The predominant species formed was $MTXGlu_3$. With subsequent incubation for 24 and 48 hours in the absence of MTX there was a fall in the level of total drug and $MTXGlu_4$ and $MTXGlu_5$ became the predominant species. Addition of 0.1 mM 2-MMGA to MTX during the initial 24 h incubation produced a 10-15% shift of MTXPGs toward longer chain-lengths in half of 10 experiments. In the remainder no change in the distribution of MTXPGs was seen. When cells were incubated in 0.1 mM 2-MMGA during the 24 or 48 hours after exposure to MTX the fall in total MTX was similar to that seen

Table 2. Levels of MTX and MTXPGs in cultured human diploid fibroblasts in the presence and absence of 2-MMGA. Cells were incubated with 1.0 μM [^{3}H]MTX for 24 h then in MTX-free medium for 24 and 48 h with and without addition of 0.4 mM 2-MMGA. Cells were then lysed and levels of MTX and MTXPGs determined by HPLC (see Methods). Results are means of triplicate determinations

Addition of 2-MMGA during MTX medium			Total MTX pmol/g	No. of Glu residues (%)						ratio $\frac{Glu_5 + Glu_6}{Glu_3 + Glu_4}$
24h	24h	48h	protein	1	2	3	4	5	6	
-			6.81	6.2	10.9	29.8	24.2	25.6	3.2	0.53
+			9.04	7.1	14.0	33.4	21.8	22.2	1.4	0.43
-	-		4.26	2.8	1.9	8.5	25.8	55.6	5.4	1.78
-	+		5.04	1.8	1.4	3.2	8.5	81.0	4.2	7.27
-		-	3.79	4.7	4.0	13.5	26.6	48.5	2.6	1.28
-		+	3.33	3.0	1.5	3.3	5.1	78.4	8.7	10.36

without 2-MMGA. However, most of the remaining MTX was in the form of $MTXGlu_5$. Compared to cells incubated without 2-MMGA, there was a marked reduction in $MTXGlu_3$ and $MTXGlu_4$. This is illustrated by the increase in ratio of $MTXGlu_{5+6}$ to $MTXGlu_{3+4}$ (Table 1).

Similar results were obtained when confluent human diploid fibroblasts were incubated with and without 0.4 mM 2-MMGA (Table 2). These cells tended to form more long-chain polyglutamates than lymphocytes and the proportion of $MTXGlu_5$ present 24 and 48 hours after MTX incubation was also greater. 2-MMGA had no significant effect when added during incubation with MTX. When added after MTX however, 2-MMGA caused almost all of the drug which remained to be in the form of $MTXGlu_5$. In fibroblasts, the ratio of $MTXGlu_{5+6}$ to $MTXGlu_{3+4}$ was even greater than in lymphocytes (Table 2).

Discussion

Gamma-glutamyl hydrolases are present throughout nature in microorganisms, plants and animals (3). In most animal tissues their pH optimum has been in the acidic range and these enzymes are believed to be lysosomal in origin. In addition, neutral pH gamma-glutamyl hydrolases have been found in human and animal plasma (6). They are believed to be cytoplasmic in origin. Evidence has been found for two hydrolases in human (7) and bovine (8) liver. Human jejunal mucosa also contains two hydrolases, one soluble and intracellular with an acid pH optimum, the other bound to membrane in the brush border (9) with a neutral pH optimum. Fractionated lymphocytes were reported not to contain gamma-glutamyl hydrolases (10), while phytohemagglutinin-stimulated peripheral blood lymphocytes had high levels (11). Most gamma-glutamyl hydrolases appear to function as exopeptidases, removing successive single glutamyl residues, and longer polyglutamates are the preferred substrates (3).

Recently, 2-MMGA was synthesized as a potential inhibitor of carboxypeptidases with glutamic acid side-chain specificity and found to be a potent inhibitor of partially purified gamma-glutamyl hydrolase from chicken pancreas, assayed at pH 7.2 (4). In contrast, there was no inhibition of a partially purified lysosomal hydrolase from hog kidney, assayed at pH 4.7 (4).

If neutral pH gamma-glutamyl hydrolases are present and active in cells, their inhibition by 2-MMGA would be expected to result in accumulation of longer-chain MTXPGs. Evidence of this was obtained during incubation with MTX, but changes were small and inconstant, suggesting either weak enzyme activity or weak inhibition. In the 24 h interval following MTX exposure, further metabolism of MTXPGs to longer chain-lengths (12,13) was evident from the absolute increase in $MTXGlu_5$ in both cells. Addition of 2-MMGA exagerated this process so that almost all the MTX became $MTXGlu_5$. These results indicate that gamma-glutamyl hydrolases are present in these cells and appear to function as exopeptidases, removing successive glutamyl residues from $MTXGlu_5$ and converting it to shorter chain-length derivatives.

There is indirect evidence that single carbon metabolism may be regulated by changes in the chain-lengths of folate polyglutamates. Such changes have been seen related to fetal development, to folate depletion, and in response to alcohol, infection and following partial hepatectomy (14). The present results provide direct evidence that such regulation may occur through the competing actions of folylpolyglutamate synthetase and gamma-glutamyl hydrolase.

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