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Advances in Brief

Acquisition of Resistance to Antifolates Caused by Enhanced γ-Glutamyl Hydrolase Activity[1]

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Abstract

A subline of H35 hepatoma cells has been developed which exhibits 80-fold resistance to 5,10-dideazatetrahydrofolate, an antifolate which inhibits glycinamideribonucleotide transformylase. The cells are cross-resistant to methotrexate, an inhibitor of dihydrofolate reductase and 10-propargyl-5,8-dideazafolate and its 2-desamino-2-methyl derivative, both inhibitors of thymidylate synthase. The resistant cells are characterized by an impaired activity of the reduced folate transport system which affects cellular import of methotrexate, 5,10-dideazatetrahydrofolate, and 2-desamino-2-methyl-10-propargyl-5,8-dideazafolate but not 10-propargyl-5,8-dideazafolate. In addition, the resistant cells exhibit a severalfold increased activity of γ-glutamyl hydrolase, the enzyme which cleaves the intracellular polyglutamate derivatives of the antifolates. Evidence for the involvement of γ-glutamyl hydrolase in resistance is derived from the observation that polyglutamate derivatives of 10-propargyl-5,8-dideazafolate in resistant cells are maintained at one-third the amount of that in parental cells in the presence of the same extracellular concentration. This is the first observation that an increase in γ-glutamyl hydrolase contributes to acquired resistance to antifolates.

Introduction

An extensive number of folate analogues have been synthesized which are antineoplastic agents. These compounds generally inhibit purine and/or thymidylate biosynthesis by acting on various folate-dependent enzymes (1–4). However, growth inhibition or cytotoxicity is often compromised by the acquisition of resistance following exposure, both *in vivo* and *in vitro*. Studies regarding acquired resistance have been directed primarily at resistance to the DHFR[3] inhibitor MTX. Several mechanisms have been identified, including gene amplification resulting in overproduction of DHFR (5), reduced binding affinity of MTX to DHFR (6), defective transport (7), and reduced FPGS activity (8). In many cases, combinations of these forms of resistance have been observed (9). Resistance against the TS inhibitor DMPDDF in human lymphoblastoid cells has been related to TS gene amplification (10). Resistance against the glycinamide-ribonucleotide-transformylase inhibitor DDATHF in CCRF-CEM cells was related to impaired transport and reduced FPGS activity (11), both of which result in a reduction in cellular DDATHF polyglutamates. Several resistant sublines of H35 rat hepatoma cells have been developed in this laboratory. One of these is characterized by a defective reduced folate transport ($H35R_1$ cells) resulting in impaired uptake of MTX (12, 13), DDATHF (14), and DMPDDF (3). H35 cell lines with DHFR overproduction ($H35R_{10}$ cells) (13) and TS overproduction (15) have also been described.

In the present study, we have developed a subline resistant to DDATHF, which exhibits impaired transport and reduced γ-glutamylation. The reduction in γ-glutamylation is related to two factors; decreased transport, presumably causing a reduction in substrate for FPGS; and an increase in γ-glutamyl hydrolase, which results in a reduction in the intracellular polyglutamates. This is the first instance of acquired resistance to antifolates which is related to an increase in γ-glutamyl hydrolase.

Materials and Methods

Materials. All media and serum were obtained from Grand Island Biological Co. (Grand Island, NY). Insulin was supplied by Eli Lilly (Indianapolis, IN). DDATHF and MTX were kindly provided by Dr. S. S. Kerwar at Lederle Laboratories (Pearl River, NY). Glycinamide-ribonucleotide and glycinamide-ribonucleotide-transformylase were a gift from Dr. J. Whiteley of the Research Institute of Scripps Clinic (LaJolla, CA). PDDF, PDDF polyglutamates, and [^{14}C]PDDF labeled with [G-^{14}C]glutamate were synthesized by the method of Nair *et al.* (16). $MTXGlu_{1-5}$ used as HPLC standards is from the National Cancer Institute. [3′,5′,7-^{3}H]MTX was purchased from Moravek Biochemicals (Brea, CA) and purified by DEAE column chromatography (12) prior to use.

Cell Culture Studies. *Mycoplasma*-free H35 rat hepatoma cells have been maintained in monolayer culture in Swim's medium containing 20% horse serum and 5% fetal bovine serum as described previously (12). The MTX-transport defective subline ($H35R_{0.3}$) that is approximately 100-fold resistant has been maintained in the presence of 0.3 μM MTX (12) and subcultured in the absence of MTX for 1 week prior to each experiment. A DDATHF-resistant subline of H35 cells (H35D) was developed by sequential stepwise increases in the concentration of DDATHF from 0.7 to 21 μM. This resistant subline was maintained in the presence of 21 μM DDATHF and subcultured in the absence of drug for 1 week prior to the experiment.

For growth inhibition studies, cells were seeded as 1×10^4/200 μl/well in a 96-well plate (Corning Glass Works, Corning, NY) and incubated in the presence of each antifolate for 72 h. The outgrowth was measured by the modified method (17) of Finlay *et al.* (18) and expressed as a percentage of control which is the growth in the absence of drug.

For transport studies, cells were grown in Swim's medium containing 20% horse serum and 5% fetal bovine serum for 96 h (stationary phase), and the medium was changed to folate-free Swim's without serum for the next 24 h. One h prior to the experiment, the medium was replaced with Hanks' balanced salts solution and then 10 μM [^{3}H]MTX (specific activity, $2–5 \times 10^5$ dpm/nmol) was added. The uptake was measured at 1, 2, and 4 min, during which linear unidirectional influx occurs. The rate of transport was expressed as pmol/mg cell protein/min.

Polyglutamate formation was measured in cells which were grown as above for 54 h, and the medium was changed with folate-free Swims medium supplemented with insulin (10 mU/ml). At 68 h, 1 μM [^{3}H]MTX (specific activity, 1×10^6 dpm/nmol) was added for 4 h. The metabolites of MTX in cell extracts were analyzed by HPLC as previously described (19). Glutamylation was calculated as the total number of glutamate residues added per mg cell protein. The same system was used for the analysis of [^{14}C]PDDF (specific activity, 5.1 $\times 10^4$ dpm/nmol) polyglutamates with a linear gradient of 4% to 24% acetonitrile in 0.1 M sodium acetate over 40 min (17). Complete recovery of poly-

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[3] The abbreviations used are: DHFR, dihydrofolate reductase; DDATHF, 5,10-dideazatetrahydrofolate; DMPDDF, C^2-desamino-C^2-methyl-N^{10}-propargyl-5,8-dideazafolic acid; FPGS, folylpolyglutamate synthetase; MTX, methotrexate; 4-NH_2-10-CH_3PteGlu$_n$, methotrexate polyglutamates, where *n* indicates the total number of glutamate residues; PDDF, 10-propargyl-5,8-dideazafolate; TS, thymidylate synthase; HPLC, high-performance liquid chromatography.

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Table 1 *Growth inhibition in H35 and resistant cells*

Cells were cultured and incubated in the presence of drug for 72 h, and the outgrowth was measured as described in "Materials and Methods."

	IC_{50} (μM)[a]			
	DDATHF	MTX	PDDF	DMPDDF
H35	0.68 ± 0.04[b]	0.01 ± 0.001	3.9 ± 0.4	0.08 ± 0.01
H35D	55 ± 3.5	0.55 ± 0.04	46.3 ± 5.3	4.9 ± 0.6

[a] IC_{50}, concentration of antifolate needed to cause 50% inhibition of untreated growth.
[b] Mean ± SD (n = 3 or 5).

glutamate standards of MTX or PDDF added during cell extraction was observed, indicating that hydrolysis of these species did not occur during this process.

Enzyme Assays. GARTF in crude cell extracts was assayed according to the method of Young *et al.* (20) using 10-formyldideazafolate as a substrate. FPGS was assayed by the method of McGuire *et al.* (21) using MTX as substrate, and γ-glutamyl hydrolase was assayed by the method of O'Connor *et al.* (22) using 4-NH_2-10-CH_3 $PteGlu_2$ as a substrate. TS activity of the cell extract was assayed according to the method of Roberts by tritium release from [5-^{3}H]deoxyuridylate (23). DHFR was assayed by the method of Mathews and Huennekens (24). The protein of cell extracts was measured by the method of Lowry *et al.* (25).

Results and Discussion

H35 cells resistant to DDATHF have been developed by sequential increases in drug concentration from 0.7 to 21 μM. These cells have an IC_{50} that is 80-fold greater than that of the parental cells (Table 1). They have a doubling time of approximately 20 h, which is equivalent to that of parental cells. As shown in Table 1, DDATHF-resistant cells (H35D) exhibit cross-resistance to MTX (54-fold), DMPDDF (60-fold), and PDDF (12-fold). The resistance to antifolates having three different target enzymes suggests that increases in respective enzymes might not play an important role in H35D cells. The activity of GARTF and TS in H35D cell extracts was essentially the same when compared to H35 parental cells, although a modest increase in DHFR (3-fold) was noted.[4]

Impaired transport has been one of the recognized mechanisms for the resistance to antifolates (7, 12, 13). MTX, DMPDDF, and DDATHF share entry in H35 cells by the reduced-folate/MTX carrier system (3, 12–14). This transport system was examined by measuring the unidirectional influx of [^{3}H]MTX (10 μM). As shown in Table 2, the rate of uptake in H35D cells was reduced by 80% compared to parental cells. The loss of transport was also verified by the uptake of [^{3}H]folinic acid, which was 90% less in H35D cells than in parental cells.[4]

In addition to transport, the growth-inhibitory activity of these three antifolates depends upon their capacity to be γ-glutamylated in cells. [^{3}H]MTX was utilized as a probe to determine the γ-glutamylation capacity of H35D cells. When glutamylation rates (the total number of glutamate residues added per mg protein) are compared between H35 and H35D cells, the rate was 87% less in H35D cells (Table 2). In addition to the reduction in total glutamylation, the distribution of polyglutamate derivatives was altered in H35D cells. The triglutamate was predominant, whereas the tetraglutamate was the major species in wild-type cells. The impairment in γ-glutamylation observed with MTX can be related in part to defective transport since an earlier transport-resistant subline ($H35R_{0.3}$) is defective in glutamylation (12, 26). That may be related to a reduction in the cellular concentration of MTX available for glutamylation. This rationale could also be applied to the glutamylation of DDATHF and DMPDDF, but not to PDDF. It has previously been shown that PDDF enters the cell by a mechanism independent of MTX since it is equally toxic to parental cells and a MTX transport-resistant cell (3, 27). Supportive evidence for that result is the observation that addition of a 10-fold excess of PDDF has no effect on the initial uptake of [^{3}H]MTX.[4]

The formation of polyglutamates depends upon the cellular level of substrates and the function of two enzymes, FPGS and γ-glutamyl hydrolase. Resistance to DDATHF in CCRF-CEM cells has been related to reduced FPGS activity (11). The fact that H35D cells also were resistant to PDDF suggested that its glutamylation might be altered by a mechanism that is independent of the transport process. For these reasons, the activity of FPGS and γ-glutamyl hydrolase in extracts of H35 and H35D cells was measured (Table 3). The activity of FPGS was identical in the two cell lines, but surprisingly the activity of γ-glutamyl hydrolase was increased 7-fold in H35D cells. The routine γ-glutamyl hydrolase assay in this study used 4-NH_2-10-$CH_3PteGlu_2$ as a substrate. An identical increment in γ-glutamyl hydrolase activity in H35D cells relative to wild-type cells was also observed when 4-NH_2-10-$CH_3PteGlu_5$ was used as the substrate.[4] The incubation of γ-glutamyl hydrolase from H35D cells with 4-NH_2-10-$CH_3PteGlu_5$ results in the exclusive appearance of 4-NH_2-10-$CH_3PteGlu_1$,[4] which is similar to the pattern of hydrolysis in wild-type cells (22). γ-Glutamyl hydrolase is extensively secreted by H35 cells (22), and H35D cells show a 3-fold increase in the amount of enzyme in conditioned medium compared to the parental cells.[4] These results suggest that the enhanced cellular activity of γ-glutamyl hydrolase may be responsible for the resistance to PDDF (Table 1) by hydrolyzing PDDF polyglutamates and causing lower intracellular concentrations of these cytotoxic species. This may also be the case with MTX and DDATHF, but this is difficult to test with intact cells because H35D cells are also transport defective, a condition which itself can result in impaired glutamylation (12, 26).

In order to clarify these possibilities, the glutamylation of PDDF was compared in H35, H35D, and transport-resistant $H35R_{0.3}$ cells. PDDF has already shown to be converted primarily to Glu_4 and longer-chain derivatives in H35 cells (28). Measurement of FPGS and γ-glutamyl hydrolase in $H35R_{0.3}$ cells revealed that the activity of both enzymes in crude extracts was similar to that in H35 cells.[4] As expected from these results the PDDF polyglutamate profile in $H35R_{0.3}$ cells is similar to that in H35 cells (Table 4). However, the

Table 2 *Transport and glutamylation of MTX by H35 and resistant cells*

Transport and glutamylation were measured as described in "Materials and Methods." The polyglutamate distribution was derived from the same experiment for glutamylation.

	H35	H35D
Transport (pmol/mg/min)	0.81 ± 0.07[a]	0.17 ± 0.02
Glutamylation (pmol/mg)	107.5 ± 7.6	14.8 ± 0.9
Polyglutamates (pmol/mg)		
Glu_2	2.1 ± 0.2	1.6 ± 0.2
Glu_3	15.6 ± 0.8	3.5 ± 0.4
Glu_4	20.2 ± 1.5	1.7 ± 0.2
Glu_5	3.4 ± 0.6	0.3 ± 0.04

[a] Mean ± SD (n = 3).

Table 3 *FPGS and γ-glutamyl hydrolase activity of cell extracts of H35 and resistant cells*

FPGS and γ-glutamyl hydrolase in cell extracts were assayed as described in "Materials and Methods."

	FPGS	γ-glutamyl hydrolase
	(nmol/mg/h)	
H35	0.61 ± 0.03[a]	1.0 ± 0.2
H35D	0.69 ± 0.03	7.1 ± 0.8

[a] Mean ± SD (n = 3).

[4] M. Rhee and J. Galivan, unpublished data.

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Table 4 *PDDF metabolites in H35 and resistant cells*

Stationary-phase cultures were incubated with 10 μM [^{14}C]PDDF for 6 or 24 h, and then the cellular PDDF metabolites were analyzed by HPLC as described in "Materials and Methods."

	PDDF polyglutamates (pmol/mg)		
	Glu_2	Glu_3	Glu_4[a]
6-h incubation			
H35		0.3[b]	6.4
$H35R_{0.3}$	0.5	0.1	7.9
H35D	0.3		2.0
24-h incubation			
H35	0.2	8.1	10.7
$H35R_{0.3}$	0.8	7.2	14.1
H35D	0.8	1.4	4.5

[a] All species of Glu_4 and longer forms.
[b] The average of two experiments.

amount of PDDF polyglutamates in H35D cells, which differs in having a markedly increased activity of γ-glutamyl hydrolase, is impaired by 70%, which occurs in spite of sufficient amounts of PDDF for glutamylation. At 6 h and 24 h the H35D cells have 18 and 25 pmol/mg, respectively, which exceeds that found in H35 cells. The decreased cellular level of polyglutamates of PDDF is constant over a 24-h period, which is consistent with continual cleavage of PDDF polyglutamates by the increased activity of γ-glutamyl hydrolase in H35D cells.

These findings are the first in which cell line with acquired antifolate resistance has exhibited an increase in γ-glutamyl hydrolase. The specific effects of the increase in γ-glutamyl hydrolase on growth inhibition and glutamylation of an antifolate are best evaluated with PDDF, since this compound enters the cells independently of MTX, DMPDDF, and DDATHF (3, 14, 27). There is a 70% reduction in the glutamylation of PDDF which is accompanied by a 12-fold decrease in the growth inhibition of H35D cells by PDDF. Thus, an inverse correlation is shown between an acquired increase in γ-glutamyl hydrolase and a decrease in cellular antifolyl polyglutamates and cytotoxicity. It is interesting to note that resistance to DDATHF in all cases studied to date involves an impairment in the glutamylation of the drug and not changes in the target enzyme. In two cell lines, CCRF (11) and H35, transport is defective, which can result in defective glutamylation. In each of these lines a second alteration, either FPGS deficiency or a γ-glutamyl hydrolase increase, appeared, which further reduced cellular DDATHF polyglutamates. These results underscore the importance of glutamylation in the pharmacological activity of DDATHF.

These results relate to the question of the role of γ-glutamyl hydrolase in folate and antifolate homeostasis. Attempts to answer this question have been impeded by the absence of (*a*) mutant cell lines lacking the enzyme and (*b*) potent and specific inhibitors of the enzyme. An attempt at the latter approach was made by Whitehead *et al.* (29), who found that 2-mercaptomethylglutaric acid inhibited a cytosolic form of the enzyme, resulting in an increase of cellular MTX-polyglutamates. However, that compound did not appear to inhibit lysosomal hydrolase. Barrueco *et al.* (30) have recently described the properties of the lysosomal hydrolase system in terms of interactions with antifolyl polyglutamates. Their study showed that the hydrolysis of $PteGlu_n$ was related directly to the accumulation of intralysosomal polyglutamates. The present study has shown that a 7-fold increase in the cellular γ-glutamyl hydrolase causes a reduction in cellular PDDF polyglutamates, demonstrating an inverse relationship between these two variables. Although this result is consistent with the general understanding of the activity of γ-glutamyl hydrolase, this is the first instance in which it has been established in a cell system and, more specifically, in relation to acquired drug resistance.

A number of questions are unanswered. Is the increase in activity related to enzyme amplification or an enzyme that is catalytically more active? Is the increased activity the same as the acidic endopeptidase, which is the predominant activity in H35 cells (21)? Is the enhanced activity in the cells primarily lysosomal? What effect does the increase in γ-glutamyl hydrolase have on the level and turnover of cellular folates? These experimental questions are currently being actively scrutinized.

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