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Altered Glycosidase Levels in Drug-Resistant Mouse Leukemias

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SUMMARY

Sublines of transplantable mouse leukemias made resistant to actinomycin D, vincristine, and related antitumor agents show reduced levels of 10 glycosidases when compared with parent cell lines. Since glycosidases may be involved in the metabolism of membrane glycoproteins, resistance to these drugs may be related to changes in membrane permeability.

INTRODUCTION

Evidence presented here suggests that in mouse leukemia cell lines reduced levels of 10 glycosidases are associated with the development of resistance to a group of drugs including actinomycin D, vincristine, daunomycin, and certain phthalanilides. Resistance to these drugs, which generally involves development of barriers to drug-DNA interactions (1–5), could be related to alterations in membrane glycoproteins. Such alterations could result from modifications in the levels of enzymes involved in the biosynthesis and degradation of membrane glycoproteins. In this series of experiments, only the levels of degradative enzymes were examined.

The glycosidases are a group of lysosomal enzymes responsible for cleavage of covalent bonds between adjacent sugars (or amino sugars), sugars and amino acids, or sugars and other chemical moieties (6). Such bonds are found in glycoproteins, glycolipids, glycosaminoglycans, and oligo- and polysaccharides. High levels of glycosidases occur in normal cells undergoing rapid division (7), and in the sera of patients with certain malignancies (8–11). Transformation of fibroblasts by oncogenic viruses leads to elevated glycosidase levels (12). These glycosidases are thought to be partially responsible for the properties of cell membranes and surfaces, by altering membrane glycolipids and glycoproteins (12, 13).

Data for the P388 murine leukemia and two derived sublines are reported here. P388/VCR and P388/38280 are lines made resistant to vincristine and to a phthalanilide (NSC 38280, 2-chloro-4′,4″-di(2-imidazolin-2-yl)terephthalanilide dihydrochloride). Sources of these leukemias have been described (14), as have the methods for isolation of cells from tumor-bearing animals (15). Both of the derived sublines are resistant to NSC 38280, vincristine, actinomycin D, and daunomycin,[2] all of which are drugs used clinically in the treatment of human cancer. Cell lines made resistant to any of these agents are generally found resistant to the others (3, 16, 17).

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[2] I. Wodinsky, unpublished observations.

The L1210 murine leukemia and its cytosine arabinoside-resistant line L1210/CA were also studied. Both these are lines responsive to NSC 38280 and vincristine (18).

MATERIALS AND METHODS

Cell extraction. The cells were harvested as described previously (14). The harvested cells were extracted at 4° with 3 volumes of 0.1% Triton X-100 by homogenization for 30 strokes in a TenBroeck homogenizer. The homogenate was used immediately for determination of glycosidase, acid phosphatase, and β-glucuronidase activity. Cell numbers were determined with a Coulter counter, research model B.

Protein. Protein was determined by the method of Lowry *et al.* (19). Bovine serum albumin was used as a standard. Samples were precipitated with 1% phosphotungstic acid in 0.5 N HCl and washed three times with 10% trichloracetic acid and once with ethanol–diethyl ether (2:1 by volume) before being dissolved in 1 N NaOH for the protein analysis.

Glycosidase, β-glucuronidase, and acid phosphatase assays. The assay procedures for the glycosidases, β-glucuronidase, and acid phosphatase, described previously (12, 20), are summarized below.

Portions (100 μl each) of the cell homogenate, containing about 0.9 mg of protein, were incubated for 1 hr at 37° with 6 μmoles of a *p*-nitrophenylglycoside derivative, *p*-nitrophenyl-β-glucuronide, or *p*-nitrophenyl phosphate, with 50 μmoles of sodium citrate buffer at pH 4.3, in a total volume of 1.1 ml.

The substrates used were *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, *p*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-α-D-galactopyranoside, *p*-nitrophenyl-β-D-xylopyranoside, *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-*N*-acetyl-β-D-galactosaminide, *p*-nitrophenyl-α-D-mannopyranoside, *p*-nitrophenyl-α-L-fucopyranoside, *p*-nitrophenyl-β-L-fucopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-glucuronide, and *p*-nitrophenyl phosphate. *p*-Nitrophenol was used as a standard. Each of the above compounds was purchased from Pierce Biochemicals, Rockford, Ill. The reaction was terminated by addition of 2 ml of 0.4 M glycine-NaOH buffer at pH 10.5. The mixtures were centrifuged at 5000 × *g* for 10 min, and the optical density of the released *p*-nitrophenol present in the supernatant fluid was measured at 420 mμ. From these data and a standard *p*-nitrophenol concentration curve, the rates of hydrolysis were calculated. Units used here are nanomoles of *p*-nitrophenol liberated per hour per milligram of protein. According to determinations of activity made at 10-min intervals, the reaction rates were linear for at least 4 hr. Two controls were run, in which glass-distilled water was substituted for either the *p*-nitrophenyl substrate or the enzyme. The absorbances of the two control values were added, and the total was subtracted from the experimental absorbance values. Corrections were therefore made for light scattering by the enzyme preparations and for nonenzymatic hydrolysis of the *p*-nitrophenyl derivatives. Each experiment was performed five to eight times.

The enzymes studied were *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.30), α-D-glucosidase (EC 3.2.1.20), β-D-glucosidase (EC 3.2.1.21), α-D-galactosidase (EC 3.2.1.22), β-D-galactosidase (EC 3.2.1.23), β-L-fucosidase (EC 3.2.1.--), β-L-fucosidase (EC 3.2.1.-), β-D-xylosidase (EC 3.2.1.37), α-D-mannosidase (EC 3.2.1.24), *N*-acetyl-β-D-galactosaminidase (EC 3.2.1.-), β-D-glucuronidase (EC 3.2.1.31), and acid phosphatase (EC 3.1.32). It should be pointed out that since the glycosidase activity was determined using *p*-nitrophenyl derivatives, the data may not measure activity *in vivo* for macromolecular substrates such as glycoproteins, glycolipids, and polysaccharides (21).

RESULTS AND DISCUSSION

The observations listed in Table 1 indicate that product formation was proportional to the quantity of added cell extract. Activity was negligible with heat-denatured enzyme, in unincubated reaction mixtures, or in reaction mixtures incubated at 0°.

There were no gross differences in growth rates among the various transplantable leu-

TABLE 1

Linearity and control assays for β-N-acetylglucosaminidase, β-xylosidase, and α-galactosidase in P388 and P388/VCR cells

The complete system contained 100 μl of cell extract (0.9 mg of protein) and 6.0 μmoles of *p*-nitrophenyl derivative in a final volume of 1.10 ml (0.05 M in sodium citrate buffer, pH 4.3).

Conditions	β-*N*-Acetylglucosaminidase		β-Xylosidase		α-Galactosidase	
	P388	P388/VCR	P388	P388/VCR	P388	P388/VCR
	mμmoles/hr					
Complete system	990	780	18	1.8	86	36.0
−Cell extract	0	0	0	0	0	0
+20 μl of cell extract	190	157	3	0.4	17	7.2
+40 μl of cell extract	392	306	7	0.7	37	14.7
+60 μl of cell extract	600	460	10	0.9	54	21.1
+80 μl of cell extract	789	618	15	1.5	70	29.0
+100 μl of boiled cell extract[a]	3	2	0	0	0	0
+100 μl of cell extract at zero time[b]	2	1	1	0	0	3
+100 μl of cell extract at 0°[c]	12	16	2	0	4	2

[a] Cell extracts were boiled for 2 min before assay.

[b] The assay was terminated immediately after addition and mixing of constituents.

[c] The incubation temperature was 0°.

TABLE 2

Glycosidase activity in normal and drug-resistant murine leukemias

All assays were performed on 0.1% Triton X-100 extracts of the cells, as described in the text. Results are the means of five to eight determinations on independent harvests of cells ± standard deviation.

Enzyme	P388	P388/38280	P388/VCR	L1210	L1210/Ca
	mμmoles/hr/mg protein				
α-Glucosidase	16 ± 1.7	4 ± 0.2	3 ± 0.1	14 ± 1.7	11 ± 1.0
β-Glucosidase	43 ± 4.2	22 ± 0.7	15 ± 1.7	50 ± 6.2	9 ± 0.7
α-Galactosidase	96 ± 4.3	76 ± 7.2	40 ± 2.8	168 ± 11.1	58 ± 4.1
β-Galactosidase	99 ± 8.7	34 ± 3.6	32 ± 2.7	143 ± 9.7	19 ± 1.8
α-Fucosidase	6 ± 0.2	1 ± 0.1	0	7 ± 0.2	1 ± 0.1
β-Fucosidase	5 ± 0.1	1 ± 0.1	4 ± 0.2	9 ± 0.9	0
β-*N*-Acetylgalactosaminidase	402 ± 4.7	222 ± 2.9	188 ± 16.2	651 ± 26.3	181 ± 9.9
β-*N*-Acetylglucosaminidase	1099 ± 11.9	1015 ± 7.7	872 ± 10.2	5554 ± 189.9	987 ± 49.2
α-Mannosidase	64 ± 6.2	50 ± 1.9	16 ± 1.7	183 ± 23.2	15 ± 0.8
β-Xylosidase	20 ± 1.2	7 ± 0.4	2 ± 0.1	42 ± 6.1	7 ± 0.2
Acid phosphatase	1510 ± 61.2	1515 ± 72.7	1586 ± 110.2	2694 ± 111.2	2600 ± 142.0
β-Glucuronidase	182 ± 12.1	198 ± 7.9	178 ± 18.1	432 ± 40.0	438 ± 21.5

kemias described here, as shown by the time required for a 10^6 cell inoculum to grow to 5×10^8 cells in CDF_1 mice. In general, the drug-resistant cell lines and the parent drug-susceptible lines studied had similar doubling times.

Table 2 shows the activities of the glycosidases, acid phosphatase, and β-glucuronidase in the normal and drug-resistant murine leukemias. The changes are similar whether expressed per milligram of protein or on a per-cell basis. In the P388 cells, hydrolase

activity fell into three categories: enzymes with high activity (400 mμmoles/hr/mg of protein or higher), i.e., β-*N*-acetylgalactosaminidase, acid phosphatase, and β-*N*-acetylglucosaminidase; enzymes with intermediate activity (between 40 and 400 mμmoles/hr/mg of protein), i.e., β-glucosidase, β-galactosidase, β-galactosidase, α-mannosidase, and β-glucuronidase; and enzymes yielding less than 40 mμmoles/hr/mg of protein or no detectable activity, i.e., α-glucosidase, α- and β-fucosidase, and β-xylosidase. P388 cells had low but measurable α- and β-fucosidase activity. In the L1210 cell line the activity was generally higher than in the P388 cell line (Table 2). L1210 enzymes with high activity (400 mμmoles/hr/mg of protein) were β-*N*-acetylglucosaminidase, acid phosphatase, β-*N*-acetylgalactosaminidase, and β-glucuronidase; enzymes with intermediate activity (less than 400 but greater than 40 mμmoles/hr/mg of protein) were α-mannosidase, α-galactosidase, β-galactosidase, β-glucosidase, and β-xylosidase; and enzymes with low activity (less than 40 mμmoles/hr/mg of protein) or no activity were α-glucosidase and α- and β-fucosidase. The finding of α- and β-fucosidase activity and β-xylosidase in mammalian tissues is rare. Many mammalian tissues do not possess α- or β-fucosidase or β-xylosidase but contain most other glycosidases (20). HeLa S-1, 3T3, and 3T6 (normal mouse fibroblasts) and SV-3T3, PY-3T3, and SV-PY-3T3 (oncogenic virus-transformed fibroblasts) do not possess α- or β-fucosidase or β-xylosidase (12). The finding that the *N*-acetylhexosaminidases are highest in activity in these cells is consistent with data on hydrolase distribution for most mammalian tissues and cell lines (12, 20).

The data indicate that for each glycosidase the activity in the drug-resistant cell lines, P388/38280 and P388/VCR, is lower than in the parent drug-susceptible P388 cell line, from which these cells were derived. Similarly, glycosidase levels in the L1210/CA cell line were lower than those in the L1210 cell line. Acid phosphatase and β-glucuronidase activities were virtually constant in each of the three P388 cell lines and the two L1210 cell lines studied, indicating that the lowered glycosidase levels are not accompanied by a decrease in all lysosomal enzymes. Thus the presence of lower glycosidase levels in the drug-resistant cell lines is a phenomenon peculiar to the glycosidases.

The exact nature of drug resistance in P388/VCR and P388/38280 is unknown. Both cell lines exhibit an impaired capacity to accumulate and retain actinomycin D *in vivo* (2). In P388/38280, the accelerated efflux of daunomycin was related to barriers to drug-DNA interaction (3). Such barriers might be membrane-mediated, depending on physical and chemical properties of the cell and nuclear membrane material. Whether the drug resistance is really due to decreased drug uptake is in some instances unclear.

Since little is actually known above the function of the glycosidases *in vivo* it is difficult to determine whether the decreased activity in drug-resistant cells reported here is a cause of the drug resistance, a consequence of the drug resistance, a determining factor in the function of drug-resistant cells, or an unrelated phenomenon. Decreased levels of the glycosidases might cause changes in membrane structure. Such membrane structural changes might be involved in development of permeability barriers to these drugs in the drug-resistant sublines. Furthermore, if the glycosidases do function to cleave plasma membrane glycoproteins and glycolipids (12), alteration of glycosidase levels could affect cell invasiveness, communication, adhesiveness, contact inhibition, and charge properties.

The conclusion of the study is that activity of the glycosidase enzymes is lowered in the drug-resistant cell lines as opposed to the drug-sensitive cell lines. Whether a causal relationship between drug resistance and the decrease in glycosidase levels exists or whether the decrease is simply a result of the genetic selection process for drug resistance is not known.

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