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Lysosomal Isozyme Patterns in Ethylnitrosourea-induced Brain Tumors*

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Abstract—*Isozyme patterns for five acid hydrolases, acid phosphatase (AP), arylsulfatase (AS), β-glucuronidase (β-Glu), N-acetylglucosaminidase (NAG) and β-galactosidase (β-Gal), were studied in isolated lysosomes from ethylnitrosourea (ENU)-induced gliomas and compared with normal and newborn rat brains. With polyacrylamide gel electrophoresis (PAGE), AP was separated into three bands, acidic (A), intermediate (B) and basic (C). In tumors and newborn brains there was a decrease in A and C but a significant increase in B. For NAG the acidic form was elevated by 9–19% in tumors, while newborn brains showed a 19% decrease. Even though the band intensities of β-Glu in tumors and newborn brains were increased, the relative distribution remained similar to normal brain. With isoelectric focusing, five hydrolases were separated into four to five distinct forms. In ENU-induced gliomas the intensities of all peaks were considerably increased, but in most cases the number of isozymes remained the same. In tumors the isolectric points were shifted towards the acidic side and smaller peaks in the basic regions merged into more acidic peaks. This effect was especially evident for AP and Gal. In the cases of AS, β-Glu and NAG, consistently more activity was associated with acidic peaks than with the basic ones. Our data indicates that there is a significant increase in acidic forms of some of the lysosomal hydrolases studied in ENU-induced brain tumors.*

INTRODUCTION

A NUMBER of acid hydrolases of normal rat kidney and brain lysosomes are shown to migrate as two to five distinct forms on polyacrylamide gel electrophoresis [1] and the acidic forms of these hydrolases, which are biologically more active, can be released from the lysosome matrix, whereas the basic forms are more firmly bound to the insoluble lysosomal residues [2, 3].

Through the use of ion exchange chromatography, basic forms of acid phosphatase have been found to be increased in rat hepatoma and squamous cell carcinoma [4]. On the contrary, in prostatic tissues an acidic form seems to be related to a cancerous state [5].

Kakizoe *et al.* [6] have reported that normal rat liver contained six types of β-glucuronidase (numbered I to VI in order of decreasing mobility towards the cathode), while Yoshida ascites heptomas and fetal liver contained only type II β-glucuronidase. Abnormalities in hexosaminidase isozymes have also been demonstrated in tumor tissues. Weber *et al.* [7] reported that hexosaminidase isozyme patterns of fast-growing hepatomas in the rat were similar to those of brain or fetal liver (HexA). More recently, several investigators reported that hexosaminidase isozymes in normal kidney and in normal human and rat colon mucosa showed a preponderance of HexA over HexB, whereas colon and renal carcinoma tissues contained a higher proportion of HexB [8–10].

All of these workers used acid hydrolase preparations obtained by total homogenization and extraction of the tissues, which mixes lysosomal with extra-lysosomal enzymes. It is possible, however, that hydrolases from multiple sources may vary independently, particularly in tumors. Also, to the best of our knowledge no-one has studied isoenzyme patterns of lysosomal hydrolases in ENU-induced brain tumors. Therefore, in the present investigation isozyme patterns of 5 acid hydrolases, acid phosphatase, arylsulfatase, β-glucuronidase, *N*-acetylglucosaminidase and β-galactosidase, have been studied in lysosomes isolated from ENU-induced nervous system tumors and compared with similar preparations from normal and newborn brains.

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MATERIALS AND METHODS

Preparation of lysosomal fractions

Experimental nervous system tumors in rats were induced by the method of Koestner *et al.* [11]. Pregnant Sprague–Dawley rats were injected with ethylnitrosourea, 30–50 mg/kg, via the lateral caudal vein on the 20th day of gestation. All offspring developed nervous system tumors after an average of 211 days. Tumors were placed on ice-chilled Petri dishes immediately after their removal, and were dissected free of necrotic and normal tissues. Blocks of each tumor sample were fixed in 10% formalin and stained routinely by hematoxylin and eosin, Nissl and Luxol fast blue–periodic acid Schiff methods. When indicated, the following stains were also used: phosphotungstic acid hematoxylin, Gridley's reticulum and Anderson's glial fiber stain. Many of the cerebal gliomas showed the usual mixed appearance of tumors of subependymal origin. Rarely, cells with ependymal characteristics were seen. For the present purpose (Table 1), tumors are identified by the prevailing cellular types corresponding to the samples used for biochemical studies. The classification system of Koestner *et al.* [11] was generally followed. The remaining tumor samples, usually weighing 100–400 mg, were used for the lysosomal preparations. Normal adult brain tissue from Sprague–Dawley rats was used as control. For newborn rat brains, normal Sprague–Dawley rats were bred in the laboratory and sacrificed within 24 hr after birth. Only the cerebral cortices from normal and neonatal brains were utilized for lysosomal preparations.

Tissues were chopped into small pieces, suspended in 9 vol of 0.32 M sucrose and homogenized in a Potter–Elvehjem type glass homogenizer by 3–5 rapid passes with a motor-driven Teflon pestle. Slightly modified procedures of Koenig [12] and Allen [13] were utilized for the isolation of the different fractions. The homogenate was centrifuged at 900 ***g*** for 10 min to give a crude nuclear fraction. The resulting supernatant was centrifuged at 25,000 ***g*** for 10 min to obtain a crude mitochondrial–lysosomal fraction. This crude mitochondrial–lysosomal fraction was further fractionated by discontinuous sucrose density gradient centrifugation. Pellets were resuspended in 1–3 ml of 0.32 M sucrose, carefully layered on top of the gradient system consisting of 6 ml each of 0.8 M and 1.4 M sucrose, and centrifuged in the Spinco swinging bucket type 40 rotor at 63,000 ***g*** for 2 hr. Lysosomal pellets so obtained were frozen at −70° C until further use. Such lysosomal preparations contained less than 6% of total cytochrome oxidase and less than 2% choline phosphotransferase activities, which are markers for mitochondria and microsomes respectively [14].

Extraction of acid hydrolases

Lysosomal pellets were resuspended in 0.15 M glycine–NaOH buffer, pH 9.0, containing 0.5% Triton X-100, and subjected to 3–4 cycles of mild freezing and thawing. The suspension was sonicated for 2 min in a Branson sonifier (Branson Instruments, Inc.), with the tube constantly being immersed in superchilled water and centrifuged in a Spinco 40 rotor for 30 min at 100,000 ***g***. The supernatant was used for polyacrylamide gel electrophoresis and isolectric focusing after protein determination by the method of Lowry *et al.* [15].

Polyacrylamide gel electrophoresis

Disc electrophoresis in 5% polyacrylamide gel was carried out in 0.2 M glycine–Tris buffer, pH 8.9, by the method of Ornstein and Davis [16] for β-glucuronidase (β-Glu). For acid phosphatase (AP), arylsulfatase (AS), *N*-acetylglucosaminidase (NAG), and β-galactosidase (β-Gal), the acidic gel system of Lim and Tadayyan [17] was employed. Enzyme extracts containing 0.05–0.15 mg protein were layered directly on top of the polymerized gels in 0.2 ml of 20% sucrose. Electrophoresis was conducted for 90 min at 2.5 mA/gel at 4°C. In a typical run, all the gels were loaded with equal amounts of protein and the tracking dye (bromophenol blue for basic and methyl green for acid system) was allowed to travel exactly the same distance (approximately 55 mm from the origin). Gels were then transferred to beakers containing ice cold distilled water and stored overnight in the refrigerator.

Demonstration of enzyme activities

The following enzyme activities were demonstrated by incubating the gels in appropriate substrates at 37° C for 30–60 min, as described by Patel and Koenig [1]: acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2), β-glucuronidase (β-D-glucuronide glucuronohydrolase, EC 3.2.2.31) and arylasylfatase (arylsulfate-sulfohydrolase 3.1.6.1). *N*-acetylglucosaminidase (β-2-acetamido-2-deoxy-D-glycoside acetamido-deoxy-glucohydrolase, EC 3.2.1.30) was detected by incubating gels in 0.75 mM naphthol AS-BI-*N*-acetyl-β-D-glucosaminide and 10 mg of fast red violet LB salt in 0.1 M citrate buffer, pH 4.2, at 37°C for 30 min [18]. β-Galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23)

Table 1. Distribution of acid phosphatase and N-acetylglucosaminidase isozymes in cerebral cortex, newborn brains and nervous system tumors

Group	Description	Relative percentages: Acid phosphatase A	Acid phosphatase B	Acid phosphatase C	*N*-Acetylglucosaminidase A	*N*-Acetylglucosaminidase B
Adult cerebrum	Mean ± S.E.M. (4)*	61.1 ± 1.3	35.9 ± 1.3	3.1 ± 0.09	27.1 ± 1.5	72.9 ± 1.5
Newborn brain	Mean ± S.E.M. (4)	49.8 ± 2.5	49.0 ± 2.0	1.3 ± 0.45	8.4 ± 1.1	91.6 ± 1.1
Astrocytomas	RT-361: anaplastic	—†	—	—	35.8	64.2
	RT-359B: well differentiated protoplasmic		—	—	41.4	58.6
	RT-414A: well differentiated fibrillary	—	—	—	36.4	63.6
	Mean ± S.E.M. (3)	—	—	—	37.9 ± 1.5	62.1 ± 1.5
Oligodendrogliomas	RT-352: anaplastic	30.0	69.2	0.8	—	—
	RT-410A: anaplastic	51.9	48.1	—	40.9	59.1
	RT-412: anaplastic	52.5	47.5	—	41.7	58.3
	RT-358A: differentiated	50.1	49.9	—	41.1	58.9
	RT-366: well-differentiated	—	—	—	34.9	65.1
	Mean ± S.E.M. (4)	46.1 ± 4.6	53.7 ± 4.5	0.2 ± 0.15	39.7 ± 1.4	60.3 ± 1.4
Mixed gliomas (oligodendroastrocytomas)	RT-353: anaplastic	—	—	—	40.5	59.5
	RT-357B: anaplastic	50.8	48.7	0.5	45.9	54.1
	RT-362: anaplastic	50.0	49.1	0.9	—	—
	RT-378B: anaplastic	48.4	49.6	2.0	43.8	56.2
	RT-380B: anaplastic	24.0	75.0	1.0	—	—
	RT-359A: anaplastic	49.1	50.9	—	42.0	58.0
	RT-407: anaplastic	—	—	—	29.4	70.6
	RT-370A: differentiated	50.5	49.5	—	40.0	60.0
	RT-415: well-differentiated	88.7	11.3	—	—	—
	RT-492B: well-differentiated	—	—	—	41.5	58.5
	Mean ± S.E.M. (7)	51.6 ± 6.6	47.7 ± 6.5	0.63 ± 0.21	40.4 ± 1.8	59.6 ± 1.8
Sarcoma	RT-375: perivascular	42.2	57.0	0.8	—	—

*Number of samples analyzed.
†Signifies not done.

was demonstrated by incubating the gels in 0.056 mM 4-methyl umbelliferyl-β-D-galactopyranoside in 0.1 M citrate–phosphate buffer, pH 3.6, for 15 min at 37°C. Fluorescence was developed by adding 25% NH_4OH after washing the gels with water.

Enzyme activities of separated bands were scanned with quick scan densitometer (Helena Laboratories) at 540 nm (AP) and 500 nm (GAD and β-Glu). The areas under the peaks were carefully measured with a planimeter. Relative percentages were calculated by dividing the area under each peak by the total area (sum of all peaks) and multiplying by 100.

Isoelectric focusing and enzyme assays

Lysosomal extracts containing 3–10 mg protein were immediately fractionated by isoelectric focusing on 2.5% ampholine gradient in the pH range 10–3 (cathode at the bottom) using an LKB Model 7100 column of 110 ml capacity with cooling mantle and platinum electrodes (LKB Products Co., Piscataway, NJ), essentially according to the method of Vesterberg and Svensson [19]. The enzymes, acid phosphatase, arylsulfatase, β-glucuronidase, *N*-acetylglucosaminidase, and β-galactosidase, were assayed as previously described by Needleman and Koenig [20].

Terminology

In keeping with prevailing usage, multiple molecular forms of acid hydrolases are referred to as A, B, C, etc., with the first letter denoting closest proximity to the anode. These descriptions compared to 1, 2, 3, etc. of the system recommended by the Commission on Biochemical Nomenclature.

RESULTS

The initial phase of this investigation utilized polyacrylamide gel electrophoresis (PAGE) to study the isozyme patterns of lysosomal hydrolases in ethylnitrosourea (ENU)-induced nervous system tumors. In a typical run, lysosomal extracts from normal and newborn rat brains were always included as controls for the tumor samples being analyzed for each enzyme. In any one run, protein concentrations were kept constant for all samples and tracking dye was allowed to travel about the same distance in all gels.

Lysosomal acid phosphatase from rat brain separated into three distinct bands, A, B and C, in order of increasing mobility towards the cathode. Average relative percentages for A, B and C from normal adult brains were 61.1 ± 1.3, 35.9 ± 1.3 and 3.1 ± 0.09 respectively (Table 1). In lysosomal preparations from ENU-induced gliomas, the A band contained 24–52% of the total acid phosphatase activity, while 47–75% was associated with B band. In most cases band C either had completely disappeared or contained less than 1% of the total activity (Table 1). The relative percentages of lysosomal acid phosphatase bands in tumors generally resembled those of newborn rat brain and corresponded to decreases of 10–14% and 1.8–2.9% in the A and C bands respectively, with an average 13–18% increase in the intermediate (B) form (Table 1). The exception was a well-differentiated mixed glioma (RT-415, Table 1) which deviated not only from normal rat brain but also from other brain tumors and newborn rat brain. Finally, when the results were examined with regard to tumor classification and histological composition, no major trend was noted. Comparison of anaplastic vs well-differentiated tumors gave no clear indication of any deviation (Table 1). The differences between normal brain vs tumor and newborn brain were highly significant ($P > 0.001$).

N-Acetylglucosaminidase activity in lysosomal preparations from rat brain separated into two forms. In normal adult brain the distribution was 27 and 73% in acidic and basic forms respectively (Table 1). All tumors showed an 8–19% increase in the acidic form, with the exception of one anaplastic mixed glioma (RT-407) which was not significantly different from the normal rat brain. The basic form of this enzyme was dominant in newborn rat brain lysosomes. The differences between normal brain vs tumor and normal brain vs newborn brain were again highly significant ($P > 0.001$).

β-Glucuronidase appeared as a single band in our rat brain lysosomal preparations. In tumors and newborn rat brain the intensity of the peak was increased, but the position remained the same when compared to gels containing the same amount of protein from the normal rat brain. β-Galactosidase and arylsulfatase from normal rat brain lysosomes were resolved into two peaks, A and B. In brain tumors and newborn rat brain the acidic form of β-galactosidase was increased, whereas there was a decrease in the acidic form of arylsulfatase (data not shown).

We have also utilized isoelectric focusing for separation of isozymes. This technique produces a distribution of isozymes according to their isoelectric points in a pH gradient formed by the carrier ampholytes. The resolving power for this separation method is better

than 0.2 pH units. The results obtained for the five acid hydrolases from lysosomal preparations derived from normal rat brain and ENU-induced gliomas are presented in Fig. 1. Even though the protein concentrations for normal brain and tumors were kept the same, the tumor enzyme activity was always much higher than the level seen in normal brain. These increases in peak intensity were most pronounced for arylsulfatase, β-glucuronidase and *N*-acetylglucosaminidase (Fig. 1). In addition, the acidic peaks of these enzymes consistently had more activity than the basic ones (Fig. 1). Acid phosphatase from normal rat brain resolved into two major peaks with pIs of 6.6 and 5.0, along with three minor peaks (pIs 4.4, 7.7, 8.8). Arylsulfatase separated into two major peaks and one minor peak, with pIs of 7.9, 5.9 and 4.8 respectively. *N*-Acetylglucosaminidase and β-galactosidase both resolved into four peaks each, two major and two minor ones (NAG 4.9, 6.8, 8.1, 8.9; β-Gal 5.2, 6.3, 7.5, 8.1). β-Glucuronidase was separated into two major and one minor peaks, with pIs of 7.9, 5.9 and 4.8 respectively (Fig. 1). In general, the number of isozymes remained the same in lysosomal preparations from ENU-induced gliomas. In tumors, pIs were shifted towards the acidic side and smaller peaks in the basic regions merged into more acidic peaks. This effect was most noticeable for acid phosphatase and β-galactosidase.

DISCUSSION

Elevated activities of β-glucuronidase, *N*-acetylglucosaminidase, acid phosphatase and arylsulfatase have been found in human tumors as well as in ethylnitrosourea (ENU)-induced rat nervous system tumors [21, 22]. Soluble or non-sedimentable activities were not increased, indicating that almost all of the hydrolases remained particle bound [21, 22]. In the present investigation we have studied the isoenzyme patterns of these four enzymes along with β-galactosidase in isolated lysosomes from ENU-induced gliomas and compared them with those from the normal rat brain.

Our PAGE analysis has shown a substantial increase in the intermediate form of acid phosphatase in lysosomes isolated from ENU-

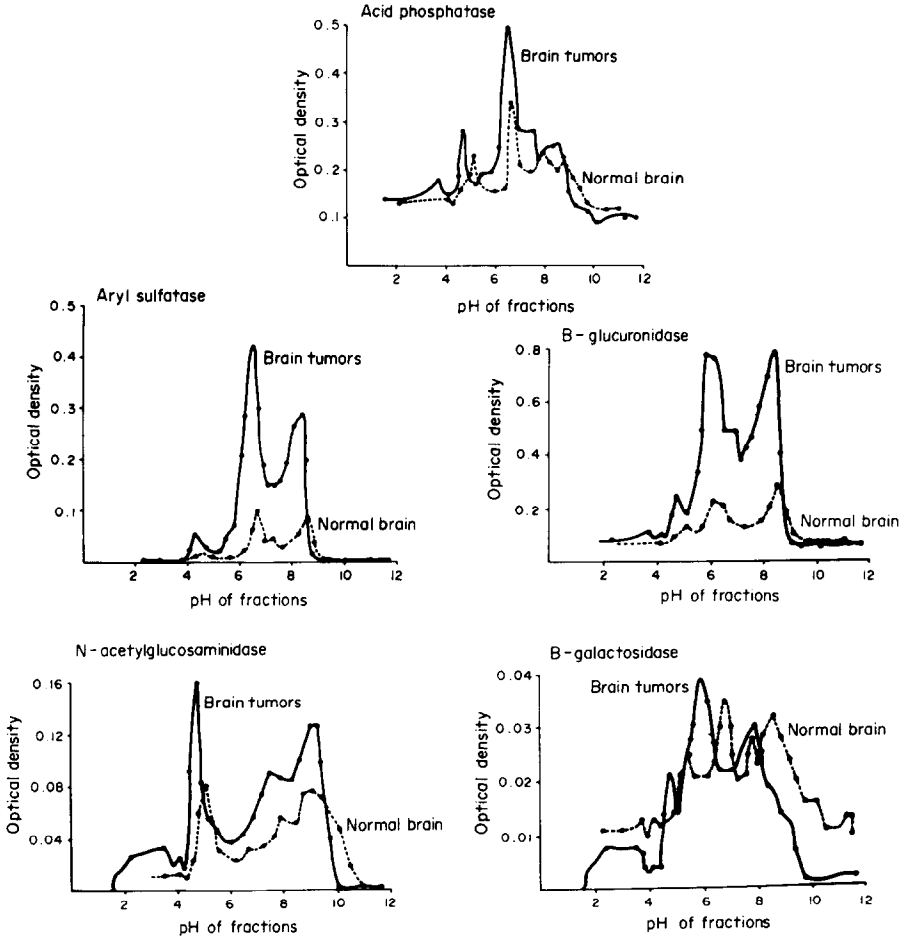

Fig. 1. Acid hydrolase isozymes separated by isoelectric focusing (number of samples analyzed—3 for normal rat brains and 7 for brain tumors).

induced nervous system tumors and newborn rat brain. Results from other tumor systems differ considerably from our findings. Moore and Angeletti [4], using rat hepatoma, and Nicholson and Davies [23], studying squamous cell carcinoma, have demonstrated a predominance of basic forms of acid phosphatase in these tumors. Similar results were obtained for ascites hepatoma and fetal liver by Kaneko *et al.* [24]. Recently, Fischer and Gevers [5] have reported a predominance of the acidic form of acid phosphatase in prostate cancer. However, our isoelectric patterns for the most part agree with several other investigators [20, 25, 26]. In addition to a generalized increase in enzyme activity in tumors, there was a slight but definite shift of basic isozymes to the acidic side.

The present investigation, using PAGE analyses, have revealed an increase in the acidic form of *N*-acetylglucosaminidase in ENU-induced tumors. Similar abundance of the acidic form of this enzyme in hepatoma and fetal liver has been reported by Weber *et al.* [7]. In contrast, the basic form was predominant in renal and colonic carcinomas [8, 9, 10]. In our studies the basic form was prevalent in newborn rat brain. This change is in general agreement with the data presented by Harzer and Sandhoff [27]. Their studies also indicated higher basic/acidic ratios in newborn human brain than in fetal brain. Upon isoelectric focusing, *N*-acetylglucosaminidase was resolved into four peaks. Activity in the most acidic peak was always found to be increased in tumors. However, pIs for NAG major peaks resembled those reported in other tumor systems [8, 9].

When PAGE was performed, β-glucuronidase appeared as a single band in our rat brain lysosomal preparations, as was previously reported for kidney [2] and liver [28], and for brain lysosomes [1]. In tumors and newborn rat brain the intensity of the peak was increased but the position remained unchanged. A single, sharp and intense peak has also been reported in squamous cell and Ehrlich ascites carcinomas [29, 30]. Using cellulose acetate electrophoresis, the basic forms of β-glucuronidase have been shown to increase in fast-growing and highly deviated Yoshida ascites hepatomas, as well as in fetal liver [6]. However, our isoelectric focusing studies indicated an increase in the acidic form of this enzyme (Fig. 1). In brain tumors the most acidic peak was consistently broader and had higher activity associated with it compared to the normal rat brain.

Isoelectric focusing of arylsulfatase separated it into two major peaks, with slightly higher activity associated with the acidic peak in gliomas.

The most remarkable finding of this study has been the unique distribution of isozymes of five lysosomal hydrolases in ENU-induced rat brain tumors. These isozyme patterns differ considerably from the other tumor systems, as has been mentioned above. These differences are either due to different (characteristic) lipid contents of each tumor, as described by Okochi *et al.* [10], or due to the mixing up of lysosomal and non-lysosomal hydrolases during the extraction of enzymes, because a majority of the investigators whose work has been quoted here used acid hydrolase preparations obtained by total homogenization and extraction of the tissue, which mixes lysosomal with extra-lysosomal enzymes. It is possible, however, that hydrolases from multiple sources may vary independently, particularly in tumors.

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