Presence of an atypical thermolabile species of β -hexosaminidase B in metastatic-tumour tissue of human liver

Jack A. ALHADEFF and Robert T. HOLZINGER

Department of Neurosciences, School of Medicine, University of California San Diego, La Jolla, CA 92093, U.S.A.

(Received 8 June 1981/Accepted 9 September 1981)

An atypical thermolabile species of Hex B (hexosaminidase B) has been found in metastatic-tumour sites of human liver which has a thermostability curve similar to that of Hex A (hexosaminidase A), which is present in decreased amounts relative to the Hex A isoenzyme, and which exhibits decreased relative activity at acidic pH values (2.6–3.6) when compared with control-liver Hex B. This atypical Hex B isoenzyme has a normal apparent Michaelis constant (0.6 mM) for 4-methylumbelliferyl 2-deoxy-2-acetamido- β -D-glucopyranoside. The presence of this atypical Hex B suggests that variant β -chains are being produced in metastatic-tumour tissue.

β -Hexosaminidase (β -2-acetamido-2-deoxy-D-glucoside: *N*-acetamidodeoxy-D-glucosylhydrolase, EC 3.2.1.30) exists in many human tissues and has both *N*-acetylglucosaminidase and *N*-acetylgalactosaminidase activity. The enzyme has two major isoenzymes, a thermolabile form (Hex A) with an isoelectric point (pI) near 5.4 and a thermostable form (Hex B) with a pI near 7.9 (Robinson & Stirling, 1968; Beutler, 1979). Hex A appears to be composed of two different polypeptides ($\alpha\beta$)_n, whereas Hex B appears to contain only one type of polypeptide ($\beta\beta$)_n (Beutler, 1979). According to this model, mutations in the α -chain lead to deficiency of Hex A (and Tay–Sachs disease), whereas mutations in the β -chain lead to deficiency of both Hex A and Hex B (and Sandhoff's disease).

In the present study we have documented the presence of, and characterized, an atypical thermolabile species of Hex B in metastatic-tumour tissue. This atypical Hex B has a thermostability curve similar to that of Hex A, is present in decreased amounts relative to Hex A, exhibits decreased relative activity at acidic pH values (2.6–3.6) when compared with control-liver Hex B, but has a normal apparent Michaelis constant for 4-methylumbelliferyl 2-deoxy-2-acetamido- β -D-glucopyranoside.

Materials and methods

General

All procedures were carried out at 2–4°C unless

Abbreviations used: Hex A and Hex B, hexosaminidases A and B.

otherwise stated. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. β -Hexosaminidase activity was assayed as previously described (Alhadeff, 1978) with 6 mM-4-methylumbelliferyl 2-deoxy-2-acetamido- β -D-glucopyranoside (Koch–Light Ltd., Colnbrook, Bucks., U.K.). A unit of activity is defined as the amount of enzyme that hydrolyses 1 nmol of substrate/min at 37°C.

Tissue preparation

Human livers which appeared normal on gross pathological examination and livers containing metastatic-tumour sites were obtained from autopsied individuals and stored at –20°C until used. The primary tumour site varied in the patients as did the amount of metastatic tumour present in each liver (Ingraham & Alhadeff, 1978). Control tissue was obtained either from histologically uninvolved adjacent areas of livers which contained metastatic sites (at least 3 cm from metastatic sites) or from non-pathological control livers (Ingraham & Alhadeff, 1978). Supernatant fluids were prepared from metastatic tumour and liver tissue as follows: 50–200 mg of tissue was homogenized (1:5, w/v) in 10–150 mM-citric acid/sodium citrate buffer, pH 4.4, by using seven to nine strokes of a motor-driven, glass/Teflon Potter–Elvehjem homogenizer. The homogenates were centrifuged at 16200 g for 30 min to give the supernatant fluids. The procedures which involved human tissues were approved by the Committee on Investigations/Activities Involving Human Subjects of the School of Medicine, University of California San Diego.

Isoelectric focusing

Isoelectric focusing was performed essentially as previously described (Alhadeff *et al.*, 1975) on 90–360 μ l of liver supernatant fluid (20–300 units of β -hexosaminidase) on a 40 ml column with 1% pH 3.5–10.0 ampholytes (LKB Produkter, Stockholm, Sweden) and a gradient of 0–67% (w/v) sucrose. The specific activity of β -hexosaminidase in the samples before isoelectric focusing ranged from 66 to 250 units/mg of protein. Electrofocusing was conducted for 16–19 h at a starting current of 3.0–4.5 mA and at 600 V. Fractions (approx. 0.4 ml) were collected, the pH values determined with a Beckman digital pH-meter, and portions (10–50 μ l) of the fractions were assayed for β -hexosaminidase activity for 10 min at 37°C. The recovery of enzymic activity was similar (60–70%) for metastatic-tumour sites and control-liver tissues. The relative amounts of Hex A and Hex B were determined by cutting out the corresponding peaks from the isoelectric profiles and weighing them on a Mettler analytical balance. In certain experiments the peaks corresponding to Hex A and Hex B were separately combined, dialysed against 10 mM-citric acid/sodium citrate buffer, pH 4.4, and concentrated by vacuum dialysis in a Collodion-bag apparatus with 25 000 mol.-wt.-retention Collodion bags (Schleicher and Schuell, Keene, NH, U.S.A.). These separated Hex A and Hex B isoenzyme peaks were characterized kinetically.

Kinetics studies on Hex A and Hex B

The kinetic studies were performed on separated Hex A and Hex B from metastatic tumours and control-liver tissue. Apparent Michaelis constants (K_m values) were determined graphically by the Lineweaver–Burk (1934) method, with 4-methylumbelliferyl 2-deoxy-2-acetamido- β -D-glucopyranoside as substrate. For these studies, the enzyme preparations were incubated in duplicate for 5 min at 37°C in 150 mM-citric acid/sodium citrate buffer, pH 4.4, containing various concentrations (0.1–6.0 mM) of substrate. The β -hexosaminidase isoenzyme pH-optima profiles were determined by using 150 mM-citrate/phosphate buffers with various pH values (2.6–7.0). Incubations were carried out in duplicate for 5 min at 37°C. pH-stability studies were also done on the separated isoenzymes preincubated at pH values of 2.6, 3.0 and 4.4 (in 150 mM-citrate/phosphate buffers) at 37°C for 5 min, followed by assay for enzymic activity at pH 4.4.

Thermal-stability studies were performed on Hex A and Hex B from metastatic-tumour and control-liver tissue after preincubation for up to 3 h at 43°C. Substrate was added to the preincubated samples and they were assayed in duplicate after incubation at 37°C for 5 min. Thermal-stability studies were

also performed as above on: (1) Hex B from control liver mixed with metastatic-tumour supernatant fluids and (2) Hex B from metastatic-tumour tissue mixed with control-liver supernatant fluids. In some experiments, human serum albumin (2.5 mg/ml final concn.) was added to the β -hexosaminidase preparations before preincubation.

Results

Fig. 1 depicts typical isoelectric-focusing profiles of β -hexosaminidase in metastatic-tumour sites (a) and histologically uninvolved host liver tissue (b). Isoelectric focusing of β -hexosaminidase in non-pathological control livers gave profiles similar to that of uninvolved host liver (Fig. 1b). The relative amounts of Hex A and Hex B were determined by cutting out the corresponding peaks from the profiles and weighing them on an analytical balance. Since the average Hex B/Hex A ratio (mean $\pm$ M.D.) was 0.60 ± 0.38 for 12 metastatic-tumour-tissue profiles and 1.27 ± 0.37 for 15 control-liver profiles, it appears that decreased relative amounts of Hex B are present in metastatic-tumour tissue from human liver.

Isoelectric focusing was used to separate Hex A

Fig. 1. Isoelectric-focusing profiles of β -hexosaminidase in metastatic-tumour-site (a) and histologically-uninvolved-host-liver (b) supernatant fluids. See the Materials and methods section for details.

Fig. 2. pH-optimum curves of Hex A in metastatic-tumour tissue (●) and control liver (■), and Hex B in metastatic-tumour-tissue (○) and control-liver (□) supernatant fluids

See the Materials and methods section for details.

Fig. 3. Thermostability curves (after preincubation at 43°C) of Hex A (●) and Hex B (▲) from: (a) metastatic-tumour tissue; (b) metastatic-tumour tissue [with preincubation in the presence of human serum albumin (2.5 mg/ml)]; (c) uninvolved host tissue; (d) non-pathological control-liver tissue

See the Materials and methods section for details.

and Hex B, and the separated isoenzymes were independently combined, dialysed, concentrated and subjected to kinetic analysis. Fig. 2 depicts pH-optimum curves for Hex A and Hex B from metastatic tumour and control-liver tissue typical of three livers studied. The curves for Hex A and Hex B from metastatic-tumour and control-liver tissue are similar from pH 4.0 to 7.0. However, for both Hex A and Hex B the metastatic-tumour-tissue isoenzymes consistently retain less relative activity at acidic pH values (2.6–3.6) when compared with control-liver tissue. This differential activity at low pH values is more pronounced for Hex B than for Hex A. pH-stability studies of Hex A and Hex B from metastatic-tumour and control-liver tissue indicated that both Hex B isoenzymes were completely stable after preincubations at low pH values (2.6, 3.0), suggesting that the differences in the Hex B pH-optimum curves are due to catalytic-activity differences. On the other hand, under comparable preincubation conditions, both Hex A isoenzymes lost considerable activity. Furthermore, metastatic-tumour Hex A lost sufficiently more activity than control-liver Hex A to account for their different pH-optimum curves, suggesting that the curve differences are due to differential stability at low pH values.

Thermostability curves for Hex A and Hex B from various liver tissues are depicted in Fig. 3. The Hex B isoenzyme curves for non-pathological-control and uninvolved-host liver tissues are very similar (Figs. 3c and 3d): Hex B is thermostable and retains 80–90% of its initial activity after 3 h preincubation at 43°C. Under comparable conditions, Hex A retains 22–28% of its initial activity for these tissues. However, the metastatic-tumour-tissue Hex B was thermolabile and gave curves comparable with those for Hex A. The thermolabile Hex B, but not Hex A, became thermostable when preincubated in the presence of human serum albumin (2.5 mg/ml) (Fig. 3b). The presence of

human serum albumin did not affect the thermostability curves of Hex A and Hex B from normal control-liver tissue. Hex A from metastatic-tumour tissue lost 50% of its activity more quickly (15 min preincubation) than Hex A from control-liver tissues (90 min preincubation) (Figs. 3a, 3c and 3d), but this effect was also reversed by the addition of human serum albumin (2.5 mg/ml). Mixing Hex B from metastatic-tumour tissue with three different control-liver supernatant fluids gave thermostability curves

very similar to that for metastatic-tumour Hex B alone (Fig. 3a). However, mixing Hex B from control liver with five different metastatic-tumour supernatant fluids gave inconsistent results: thermostability curves included those that were similar to: (1) control Hex B alone (Fig. 3c); (2) crude metastatic-tumour supernatant fluid alone; and (3) curves intermediate between (1) and (2). Although the overall results of the thermostability studies are very difficult to interpret unambiguously, they suggest that the thermostability of metastatic-tumour Hex B is due to an alteration of the enzyme itself and not to factors in the crude metastatic-tumour-tissue extracts.

Apparent K_m values of Hex A and Hex B were determined with 4-methylumbelliferyl 2-deoxy-2-acetamido- β -D-glucopyranoside as substrate for three metastatic-tumour tissues and three non-pathological control livers. The Lineweaver-Burk double-reciprocal plots yielded straight lines with similar average K_m values (mean $\pm$ M.D.): 0.6 ± 0.1 and 0.7 ± 0.1 mM for Hex A and 0.6 ± 0.1 and 0.5 ± 0.1 mM for Hex B from the metastatic-tumours and normal-liver tissues respectively.

Discussion

Considerable research interest has focused on the biochemical genetics of human β -hexosaminidases primarily because of Tay-Sachs disease and other related ganglioside-storage diseases which result from absence of activity of various isoenzymes of the enzyme (Beutler, 1979). Two major β -hexosaminidase isoenzymes are found in most human tissues and the major distinguishing physico-chemical property between these two isoenzymes is with regard to thermostability: Hex A is considerably more thermostable than Hex B. However, atypical thermostable Hex B isoenzymes have recently been found in tissues from patients with ganglioside-storage diseases. A Japanese Tay-Sachs-disease patient has been identified whose serum contains thermostable Hex B and Hex I (the intermediate isoenzyme) components (Momoi *et al.*, 1978). The variant Hex B exhibited a normal apparent K_m value (0.65 mM) for 4-methylumbelliferyl 2-deoxy-2-acetamido- β -D-glucopyranoside. Another Tay-Sachs-disease patient of Yugoslavian parents has been reported whose fibroblast Hex B exhibited atypical electrophoresis on cellulose acetate and significantly decreased thermostability at 50°C (Burck *et al.*, 1980). In addition, a carrier of Sandhoff's disease in a South-African family has been identified with an apparent variant allele for the β -chain, leading to a thermostable serum Hex B isoenzyme (Lane & Jenkins, 1978). The presence of these thermostable Hex B isoenzymes has led to the suggestion (Momoi *et al.*, 1978) that methods other

than heat-inactivation (O'Brien *et al.*, 1970) should also be employed for prenatal diagnosis of ganglioside-storage diseases.

In studying the relative amounts and kinetic properties of Hex A and Hex B in metastatic-tumour tissue, we have found a thermostable species of Hex B which has a thermostability curve very similar to that of Hex A. This labile Hex B became thermostable when preincubated in the presence of human serum albumin suggesting that its decreased apparent thermostability could be due to an increased proteinase activity of metastatic-tumour liver tissue. This possibility is unlikely, since we have previously shown that metastatic-tumour tissue and uninvolved control tissue from some of these same livers have comparable proteinase activities (Ingraham & Alhadeff, 1978). Furthermore, mixing studies suggest that the thermostability of metastatic-tumour tissue Hex B results from an alteration of the isoenzyme itself rather than from factors in the crude extracts. An interesting possible explanation for the decreased thermostability is that altered β -chains (and consequently altered Hex B) are being synthesized in metastatic-tumour tissue. These altered β -chains could also be incorporated into Hex A in metastatic-tumour tissue and account for its somewhat abnormal thermostability curve (Fig. 3a). Whatever the reason for the alteration of Hex B, its greatly increased thermostability may partially account for the present findings of decreased relative amounts of it in metastatic-tumour tissue.

Previous investigators have reported altered β -hexosaminidase isoenzyme compositions and altered isoenzyme properties in cancerous tissue. Tucker *et al.* (1980) found increased relative amounts of the intermediate isoenzymes (Hex I₁ and Hex I₂) in the sera of patients with various types of primary carcinoma, particularly in those patients with bone metastases. Ellis *et al.* (1978) have reported that the leucocytes of acute-lymphoblastic-leukaemia patients have increased amounts of Hex I and increased ratios of Hex I to Hex A. Brattain *et al.* (1979) have reported that Hex B from cancerous rat colon is more thermostable than normal Hex B and gives thermostability curves similar to those for normal Hex A. They found that this atypical Hex B has less activity at low pH (3.0) than Hex B from normal colon. Their findings are very similar to ours for Hex B from metastatic-tumour tissue of human liver. The fact that no significant alterations were found in the apparent K_m values for Hex A and Hex B from metastatic-tumour tissue in our studies argues against the occurrence of major structural alterations of these isoenzymes. Nevertheless, our results suggest that variant Hex B (and possibly variant Hex A) isoenzymes with some altered properties exist in patients other than those with ganglioside-storage diseases. The significance of

altered β -hexosaminidase isoenzymes in metastatic-tumour tissue is not presently known and awaits further experimentation. Characterization of these isoenzymes in a purified state should lead to an understanding of the reason(s) for their unusual properties.

This study was supported in part by grants from the U.S. National Institutes of Health (N.I.H.) (AM 20409 and AM 21245). J. A. A. is recipient of Research Career Development Award (no. KO4 AM 00257) from the N.I.H.

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