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Alterations of asparagine-linked sugar chains of *N*-acetyl β-D-hexosaminidase during human renal oncogenesis: a preliminary study using serial lectin affinity chromatography

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Abstract

Enzymatic properties and asparagine (Asn)-linked sugar-chain structures of *N*-acetyl β-D-hexosaminidase A (Hex A) were compared in human tissues between normal renal cortex and renal cell carcinoma (RCC). No significant differences between the two Hex A preparations were observed with respect to enzymatic properties such as molecular mass, Michaelis–Menten value or optimal pH. With RCC preparations, relatively more Hex A passed through the concanavalin A (Con A) column, bound weakly to Con A, or bound strongly to Con A and also to the wheat germ agglutinin (WGA) column, than with preparations from normal renal cortex. In contrast, relatively less Hex A bound strongly to the Con A column, but passed through the WGA column with RCC preparations than with those from normal renal cortex. Asn-linked sugar-chain structures might apparently be altered during human renal oncogenesis.

Keywords: *N*-Acetyl β-D-hexosaminidase; Hexosaminidase A; Sugar chains

1. Introduction

Representative enzymes and/or proteins have been specifically associated with various human organs, such as alkaline phosphatase (ALP) in bone and hepatobiliary tracts [1–4], acid phosphatase in prostate (PAP) [5], gamma glutamyl-transferase (GGT) in liver [6], α-amylase in parotid gland [7], α-fetoprotein in embryonic liver and yolk sac [8], human chorionic gonadotropin in placenta [9], and *N*-acetyl β-D-hexosaminidase (Hex, also known as NAG) in kidney [10]. Disease-associated alterations in activities or concentrations of these proteins in body fluids are used widely as diagnostic tools and indices of therapeutic responses. Recently, cancer-associated changes in the sugar chains of various enzymes or other proteins have been reported [11,12], suggesting that studies of alterations in sugar chains of these proteins may become possible diagnostic tools for human cancers. Although human renal cell carcinoma (RCC) is believed to originate from the proximal tubules of the kidney [13], in which high activities of *N*-acetyl β-D-hexosaminidase A (Hex A) are present [14], no definitive tumor markers have been identified for RCC.

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With this goal in mind, we compared the enzymatic properties of Hex A purified from RCC with those purified from normal renal cortex, and also compared sugar-chain structures of these two Hex A preparations by lectin affinity chromatography using concanavalin A (*Canavalia ensiformis*, Con A)–sepharose and wheat germ agglutinin (*Triticum vulgaris*, WGA)–agarose columns.

2. Experimental

2.1. Reagents

Sephacryl S-400, Sephacryl S-200, DEAE–cellulose, Con A–sepharose 4B, and polyacrylamide gradient electrophoretic gels (8–25%) were obtained from Pharmacia Chemicals (Uppsala, Sweden). WGA–agarose was obtained from E Y Labs. (San Mateo, CA, USA) and α-methyl-D-mannoside (α-MM) was purchased from Sigma Chemical (St. Louis, MO, USA). High-performance liquid chromatography (HPLC) materials (TSK DEAE-5PW and TSK-Gel 3000) were obtained from Toso (Tokyo, Japan). A kit for measuring hexosaminidase activity was purchased from Shionogi (Osaka, Japan). All other reagents were of analytical grade and were purchased from Wako (Osaka, Japan).

2.2. Samples

Six samples of normal renal cortex and 12 of RCC tissues were obtained from total nephrectomy specimens with RCC. Patient ages ranged from 45 to 73 years. All patients consented to the use of their resected renal tissue for this study. RCC tissues were dissected carefully to exclude necrotic areas and surrounding normal renal tissue. Samples were dissected in ice-cold Tris–HCl buffer (100 mmol/l, pH 7.4) containing 1 mmol/l benzamidine chloride and 0.3 mmol/l phenylmethylsulfonyl fluoride. After dissection, all samples were frozen immediately in the operating room and kept at −80°C until used.

2.3. Enzyme assay

The activity of Hex was measured using a kit (see *Reagents*) that utilizes a colorimetric assay employing an artificial substrate (sodio-*m*-cresol sulphonfutaleinyl *N*-acetyl β-D-glucosaminide, or MCP–NAG), as previously reported [15]. In brief, 50 μl of specimen was added to 1 ml of 2.75 mmol/l MCP–NAG dissolved in citrate–phosphate buffer (50 mmol/l, pH 4.9). After incubation at 37°C for 15 min, the reaction was terminated by adding 2.0 ml of a 0.3 mol/l Na_2CO_3 solution. The absorbance of liberated *m*-cresol purple by Hex activity was measured at 580 nm using an Hitachi 0-3210 spectrophotometer. The liberated *m*-cresol purple was stable for at least 1 h. One unit was defined as the amount of enzyme which liberated 1 μmol of *m*-cresol purple per minute at 37°C.

2.4. Purification of the enzyme

Tissues from each sample were prepared as follows. First, the tissues were homogenized with an ultradisperser (Yamato, Tokyo, Japan) in four volumes of ice-cold Tris–HCl buffer (100 mmol/l, pH 7.4) containing 150 mmol/l NaCl. The homogenate was centrifuged at 20 000 *g* for 30 min at 4°C to obtain a crude extract. The resulting supernatant was centrifuged at 105 000 *g* for 60 min at 4°C to obtain a cytosol fraction. The cytosol fraction was fractionated further by 60% saturation with ammonium sulfate, and the precipitate was suspended in ice-cold Tris–HCl buffer (100 mmol/l, pH 7.4). The resulting preparation was applied to a Sephacryl S-400 column (2.7×100 cm) and eluted with Tris–HCl buffer (100 mmol/l, pH 7.4). Fractions containing Hex activity were pooled and concentrated using a minicon device (Amicon, Beverly, MA, USA). Then aliquots were applied to a DEAE–cellulose column (2.7×45 cm) equilibrated with 10 mmol/l sodium phosphate buffer (pH 7.4). Hex A activity retained on the column was eluted using a linear gradient of NaCl (0 to 500 mmol/l) at a flow-rate of 1 ml/min. Further purification of Hex A was performed by HPLC with TSK-Gel G-3000 equilibrated with 10 mmol/l sodium phosphate buffer (pH 7.4) and eluted at a flow-rate of 1 ml/min. Enzyme activity with a molecular weight of 140 kDa was further chromatographed using TSK DEAE 5-PW with a linear gradient of NaCl (0 to 500 mmol/l) at a flow-rate of 1 ml/min, yielding a single protein band demonstrable by polyacrylamide gel electrophoresis.

2.5. Lectin affinity chromatography

A column (0.5×10 cm) of Con A–sepharose or WGA–agarose (2 ml) was equilibrated with 10 mmol/l Tris–HCl-buffered saline (TBS, pH 8.0). An enzyme preparation with a specific activity of approximately 100 U was applied to the Con A column and allowed to stand at room temperature for 3 h. The column was then washed with TBS until the activity of Hex in the fraction became undetectable. The initial elution was carried out with 10 mmol/l α-MM in TBS, and subsequent elution was carried out with 500 mmol/l in TBS. Three fractions were obtained by use of the two different concentrations of α-MM: an unbound fraction (fraction I), a weakly bound fraction (fraction II), and a strongly bound fraction (fraction III). Because less significant Hex activities were detectable in fractions I and II, only fraction III was further applied to the WGA column. The unbound and bound fractions, designated A and B, were separated by use of 500 mmol/l *N*-acetylglucosamine (GlcNAc) as the elution buffer (Fig.1). The relative amount of Hex was calculated by dividing the Hex activity in each fraction by the sum of the Hex activities in all fractions.

2.6. Histopathologic examination of RCC

Microscopic sections were evaluated by a pathologist who classified the tumor according to the scheme of the Union Internationale Contre le Cancer (UICC) and the American Joint Committee on Cancer (AJCC) [16]. Nuclear grade was determined according to Fuhrman et al. [17] using the area showing the highest grade.

2.7. Protein determination

Protein was measured according to Bradford [18], using bovine serum albumin as a standard.

2.8. Statistical analysis

Values are expressed as the mean±standard deviation. Statistical analyses were performed using the Mann–Whitney U test and the Spearmann rank correlation test. A $P \leq 0.05$ was considered statistically significant.

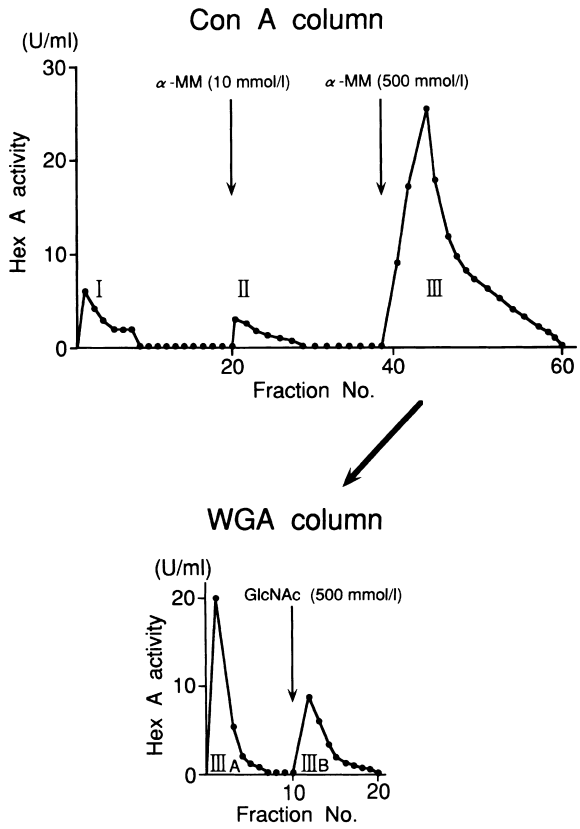

Fig. 1. Separation of enzyme preparations into four fractions by serial lectin affinity chromatography. Fractions I, II, and III obtained from the concanavalin A (Con A) column are shown at the top. Fraction III was subsequently applied to a wheat germ agglutinin (WGA) column (bottom) as described in the Experimental section. Hex A activity, hexosaminidase A activity; α-MM, α-methyl-D-mannoside; GlcNAc, *N*-acetylglucosamine. Arrows indicate the starting points of elution.

3. Results

3.1. Histopathologic examination of RCC

Six out of the 12 tumors were classified as conventional RCC, three represented the papillary type, and three were of chromophobe type. Seven

were assigned to nuclear grade I, three were grade II, and two were grade III.

3.2. Hex activity in the crude extracts of the normal renal cortex and RCC tissues

The Hex activities for MCP–NAG in the extracts of normal renal cortex and RCC tissues were 3.14±0.63 and 2.99±1.64 U/mg protein, respectively (no significant difference). However, Hex activity in histologically conventional RCC (4.22±1.22 U/mg protein) was significantly higher than in normal renal cortex (P=0.0009) or chromophobe type (1.03±0.21 U/mg protein; P=0.0033). No significant differences were observed between papillary types (2.42±1.73 U/mg protein) and conventional or chromophobe types. Tumors did not differ in activity by nuclear grades (grade I, 3.34±1.70 U/mg protein; grade II, 2.94±2.02 U/mg protein; grade III, 1.95±1.20 U/mg protein).

3.3. Studies of optimal pH and Michaelis–Menten constant in Hex A purified from normal renal cortex and RCC

Purity of the final enzyme preparations was confirmed by polyacrylamide gel electrophoresis, revealing a single band. Purified Hex A from normal renal cortex and RCC tissues appeared at the same position with polyacrylamide gel electrophoresis (Fig. 2). Using purified enzyme preparations, the following enzymatic properties were compared between normal renal cortex and RCC samples.

The optimal pH was determined under standard assay conditions in 0.2 mol/l citrate–phosphate buffer for the pH range from 3.0 to 7.5 and in 0.2 mol/l Tris–HCl buffer for the pH range from 7.2 to 8.2. The activity of Hex A from normal renal cortex gave a pH profile with maximal activity at pH 4.8. A similar optimal pH value was also obtained from RCC tissues, regardless of tumor classification or nuclear grade.

The Michaelis–Menten constants (K_m) were determined from reciprocal plots with substrate (MCP–NAG) concentrations ranging from 2.75 μmol/l to 2.75 mmol/l at optimal pH values. The K_m of Hex A from normal renal cortex ranged from 0.172 to 0.183 mmol/l (mean 0.177±0.004 mmol/l); similarly, the

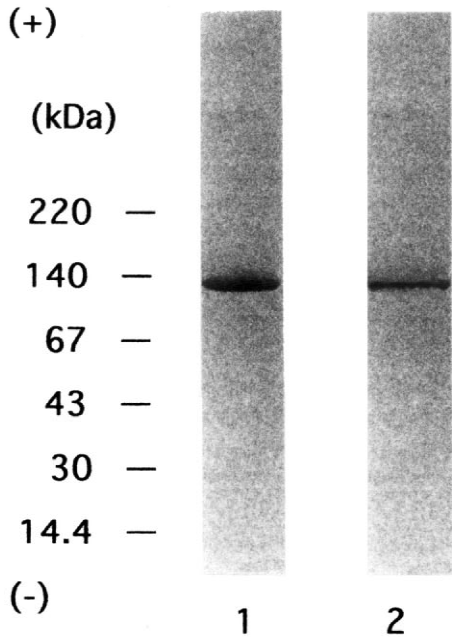

Fig. 2. Representative polyacrylamide gel electrophoresis of final preparations of *N*-acetyl-β-D-hexosaminidase A (Hex A). Lanes 1 and 2 indicate the representative electrophoresis of final enzyme preparations obtained from normal renal cortex and renal cell carcinoma (RCC), respectively. A single band stained with Coomasie Brilliant Blue R-250 was seen in each lane, demonstrating the purity of the enzyme. Purified Hex A from the normal renal cortex and RCC tissues appeared at the same position, representing approximately 140 kDa.

K_m shown by the RCC enzyme ranged from 0.168 to 0.187 mmol/l (mean 0.177±0.006 mmol/l). No significant difference was observed in K_m between tumor histologic subgroups or between nuclear grades.

3.4. Lectin affinity chromatography

The four fractions separated by the Con A and the WGA affinity technique were as follows: I, the fraction that passed through the Con A column; II, the fraction that bound weakly to the Con A column; IIIA, the fraction that was bound strongly to the Con A column but passed through the WGA column; and IIIB, the fraction that was bound strongly to the Con A column and was also bound to the WGA column. The total activity recovered after separation by the

lectin binding studies was more than 97% of the initial activity.

The K_m study of Hex A after lectin affinity chromatography ranged from 0.170 to 0.188 mmol/l for the substrate, which was identical to that obtained before lectin affinity studies. The relative amounts of Hex A activity of the four fractions in normal renal cortex and RCC are shown in Table 1.

In preparations from normal renal cortex, the relative amounts of Hex A in the four fractions were as follows: fraction IIIA showed the greatest value, followed in decreasing order by fractions IIIB, I, and II. The mean relative amount of Hex A activity in fraction I was 6.3±1.8% (range 6.0–8.0%); the activity in fraction II was 1.3±0.2% (range 1.0–1.6%); the activity in fraction IIIA was 78.7±1.3% (range 76.5–80.4%); and the activity in fraction IIIB was 13.6±1.6% (range 10.5–15.0%). Significant differences were seen in the relative amounts of Hex A activity between the four fractions. Similar observations were made in RCC preparations: relative amounts of Hex A activity were greatest in fraction IIIA, followed in decreasing order by fractions IIIB, I, and II. Relative amounts of Hex A activity in fraction I ranged from 6.1 to 30.7% (mean 16.6±8.7%); the activity in fraction II ranged from 3.8 to 18.2% (mean 10.3±5.2%); the activity in fraction IIIA ranged from 31.0 to 63.9% (mean 50.4±12.0%); and the activity in fraction IIIB ranged from 20.2 to 39.3% (mean 28.2±1.8%). As with the normal renal cortex, significant differences were observed between the four tumor-derived fractions. When the normal renal cortex and RCC were compared with respect to relative amounts of Hex A activity in each fraction, the activity in fractions I, II, and IIIB of the normal renal cortex were significantly smaller than in RCC. In contrast, the activity in fraction IIIA of normal renal cortex was significantly greater than in the corresponding fraction from RCC. No significant correlations occurred between the relative amount of Hex A in the fractions and tumor classification or nuclear grade.

4. Discussion

Hex is widely distributed in mammalian tissues [14,16–19]. Two major Hex isoenzymes, designated A and B, have been reported. Hex A is the main enzyme in the proximal tubules of the kidney [14], while Hex B is the main form in the reproductive organs [20]. In the present study we observed a similar molecular weight, K_m, and optimal pH for Hex A purified from normal renal cortex and RCC tissues. These results suggest that the molecular mass and catalytic sites of the enzyme were unaffected by the process of oncogenesis in the proximal tubules resulting in RCC. In contrast, lectin affinity chromatography showed elution patterns that differed between the two Hex A sources.

Based on prior reports of lectin affinity chromatography [21–24], the sugar-chain structures of the present four fractions represent: I, a multiantennary complex type and a complex type with bisecting GlcNAc; II, a biantennary complex type; IIIA, a high-mannose type or hybrid type with fucose linkages to the innermost GlcNAc; IIIB, a hybrid type without fucose linkages to the innermost GlcNAc. In both normal tissue- and tumor-derived Hex A, fractions IIIA and B showed the greatest relative activity, while activities in fractions I and II were small. This is not surprising since most sugar chains associated with lysosomal enzymes consist of Asn-linked chains of high-mannose type which bind strongly to Con A. Differences were observed between the two Hex A sources among the four

Table 1
Relative amounts of the four fractions of *N*-acetyl β-D-hexosaminidase A activity in human normal renal cortex and renal cell carcinoma

	Normal renal cortex (M±SD%)	Renal cell carcinoma (M±SD%)	Significance[a]
Fraction I	6.3±1.8	16.6±8.7	$P=0.0123$
Fraction II	1.3±0.2	10.3±5.2	$P=0.0008$
Fraction IIIA	78.7±1.3	50.4±12.0	$P\leq0.0001$
Fraction IIIB	13.6±1.6	28.2±1.8	$P\leq0.0001$

[a] Comparison between normal renal cortex and renal cell carcinoma.

fractions. In RCC-derived Hex A, the high-mannose type or hybrid type with fucose linkages to the innermost GlcNAc was significantly decreased, while complex types and the hybrid type without fucose linkage to the innermost GlcNAc were increased relative to Hex A derived from normal renal tissues. These observations suggest that sugar-chain structures of Hex A might be altered during oncogenesis.

In recent years alterations in the organ- and species-specific sugar-chain structure of enzymes have been found in carcinomas. For instance, in GGT, a bisecting GlcNAc structure never seen in normal human or rat liver has been detected in rat hepatoma as well as in the sera of hepatoma patients [25,26]. In ALP, a hybrid type without fucose linkages to the innermost GlcNAc is increased in human hepatoma [27]. A fucose residue is completely missing from the sugar chains in preparations of α-amylase from lung and ovarian carcinomas [28]. Similarly, our present data suggest that posttranslational glycosylation of Hex A might be altered during malignant transformation in the human kidney. We believe that further studies of these alterations in sugar-chain structures of Hex A between normal renal cortex and RCC might represent a new clinical diagnostic marker for RCC.

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