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QUARTERLY

Age-related profile of β-*N*-acetylhexosaminidase glycosylation in rat liver

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β-*N*-Acetylhexosaminidase was prepared from a liver lysosomal fraction obtained from rats between 18 days of gestation (group I) and 72 weeks of age (groups II–VI). A glycan chain analysis was performed after an electrophoresis and blotting, followed by a very sensitive detection system with highly specific digoxigenin-labelled lectins. The presence of high-mannose /hybrid type glycans, as well as their fucosylated forms was shown in all the experimental groups. Complex-type glycans with terminal sialic acid or galactose were present in all the groups except for 1-week-old rats in which only a positive reaction with lectins from *Galanthus nivalis* and *Aleuria aurantia* – was observed. Thus it may be assumed that age-related changes in the glycosylation pattern occur on the first days after birth.

β-*N*-Acetylhexosaminidase (EC 3.2.1.30) (Hex) is a lysosomal hydrolase which hydrolyzes terminal β-linked *N*-acetylhexosamines from oligosaccharides, glycoproteins, glycolipids and other carbohydrate-containing macromolecules. In human tissues, there are two major forms of Hex, A and B, which are composed of two subunits ($\alpha\beta$ and $\beta\beta$), respectively. The α and β subunits are encoded by two distinct, yet closely related genes. Thus their primary structure deduced from the nucleotide sequence of cDNAs shows an overall 60% homology [1–3]. It has been shown that there are five potential glycosylation sites on the β subunit, and three sites on the α subunit [4, 5]. Being typical of glycoproteins, Hex is synthesized in endoplasmic reticulum and undergoes an extensive post-translational modification during its transit to lysosomes: N-glycosylation, intrachain disulfide bonds formation, followed by dimerization into hexosaminidase isoenzymes [1, 6]. Carbohydrate

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Abbreviations: BSA, bovine serum albumin; Dig, digoxigenin; Hex, β-*N*-acetylhexosaminidase; Man, mannose; NeuAc, sialic acid; TBS, Tris/saline buffer. Agglutinins were from: AAA, *Aleuria aurantia*; DSA, *Datura stramonium*; GNA, *Galanthus nivalis*; MAA, *Maackia amurensis*; PNA, *Arachis hypogeae*; SNA, *Sambucus nigra*.

maturation occurs in the Golgi apparatus and consists in phosphorylation of one or more oligosaccharides, of the high mannose type required for the binding of the phosphomannosyl receptor to deliver the enzyme to the lysosome. Final processing occurs in the lysosome, giving rise to mature forms of the enzyme [1, 2, 7, 8]. Glycosylation of the polypeptide usually generates a set of glycoforms. They depend on the cellular environment in which the protein is glycosylated and may therefore vary with type as well as physiological state of the organism, tissue or cell in which the glycosylation occurs [9, 10]. Several lines of evidence have demonstrated growth-dependent changes in the N-linked oligosaccharides of glycoproteins in the rat liver, as well as in the activities of sialyltransferase and galactosyltransferase in the liver Golgi membranes [11, 12].

In order to investigate the growth-dependent changes in the glycosylation profile, we studied the carbohydrate moiety of Hex isolated from the rat liver at different ages.

MATERIALS AND METHODS

Chemicals. A Glycan Differentiation Kit, containing the digoxigenin (Dig)-labelled lectins: *Galanthus nivalis* agglutinin (GNA) – a lectin specific for terminal Man (α1-2, α1-3 or α1-6) Man units; *Sambucus nigra* agglutinin (SNA) – a lectin specific for NeuAc (α2-6) Gal; *Maackia amurensis* agglutinin (MAA) – a lectin specific for NeuAc (α2-3) Gal; *Datura stramonium* agglutinin (DSA) – a lectin specific for Gal (β1-4) GlcNAc; *Aleuria aurantia* agglutinin (AAA) – a lectin specifically binding Fuc (α1-2, α1-3, α1-6) GlcNAc and *Arachis hypogeae* agglutinin (PNA) – a lectin specific for Gal (β1-3) GalNAc in O-glycans were purchased from Boehringer (Mannheim, Germany). Nitrocellulose was from Schleicher and Schuell. Other chemicals were of the highest purity and were purchased from Sigma (St. Louis, MO, U.S.A.)

Animals. Six series of male Wistar rats (except for a prenatal group whose sex was unknown), aged 18 days of gestation, 1 week, 1, 1.5, 3 and 18 month (groups: I–VI, respectively) were used. The animals were fed *ad libidum* on a conventional diet and had free access to drinking water. The lighting conditions (12 h light :12 h dark), temperature and humidity were controlled. The animals were killed by decapitation, after having been starved for 24 h, their livers were perfused, dissected and homogenized in 5 vol. of chilled 0.25 M sucrose per 1 g of the tissue using a teflon-glass homogenizer. Preparation of the crude lysosomal extract was carried out according to the method of de Duve *et al.* [13], as described previously in detail [14].

Isolation of rat liver β-N-acetylhexosaminidase. Liver Hex from a crude lysosomal extract, obtained from rats of different age, was isolated by an immunoaffinity chromatography. IgG-type antibodies were isolated from 25 ml of rabbit antiserum against Hex by a chromatography on Protein A-Sepharose Cl 4B column (1 cm × 10 cm) in 0.1 M sodium phosphate buffer, pH 7.0 [15]. The purified antibodies were then coupled to CNBr-activated Sepharose 4B according to the recommended procedure [16]. Affinity chromatography of Hex was performed in 0.02 M Tris/HCl buffer, pH 7.4, containing 0.2 M NaCl, on anti-Hex IgG-Sepharose 4B column (1 cm × 10 cm). The column was washed with the starting buffer, and then Hex was eluted with 0.2 M glycin/HCl buffer, pH 3.0. The collected fractions were immediately neutralized by an addition of 0.7% Tris. Fractions showing the enzymatic activity were pooled, dialyzed and concentrated.

Glycan chain analysis. Glycan chain analysis of Hex was performed with the use of a Glycan Differentiation Kit according to Haselbeck *et al.* [17] on enzyme (20 μg) transferred onto nitrocellulose by Western technique [18] after polyacrylamide gel elctrophoresis in the presence of SDS under reducing condition (SDS/PAGE) [19].The process was checked

by measuring the protein remaining on the original gel as well as the proteins appearing on nitrocellulose by Ponceau S (Boehringer) staining. The protein transfer efficiency was at least 90%.

Nitrocellulose sheets were treated with a solution of 0.05 M Tris/HCl, 0.15 M NaCl, pH 7.5 (buffer A) containing blocking agent (TBS/Tween, 0.5%) for 12 h at room temperature. The blots were twice washed for 15 min in buffer A and once with the same buffer A containing 1 mM $MgCl_2$, $MnCl_2$, $CaCl_2$, respectively and were then incubated with the digoxigenin-labelled GNA, MAA, SNA, DSA, AAA or PNA for 60 min. After three times washing with buffer A, the blots were incubated with polyclonal sheep anti-digoxigenin Fab fragments conjugated with alkaline phosphatase, for 60 min. After a further washing process (3 times) the conjugated alkaline phosphatase was detected by the reduction of the 4-nitroblue tetrazolium salt in the presence of 5-bromo-4-chloro-3 indol-phosphate in buffer B (Tris/HCl 0.1 M, $MgCl_2$ 0.05 M, NaCl 0.1 M, pH 9.5) to a blue precipitate. In controls, preincubation of the lectins with the corresponding sugar (0.2 M) was performed as follows: galactose for PNA, fucose for AAA, methyl-α-D-mannopyranoside for GNA, galactose and *N*-acetylglucosamine for DSA and sialic acid for SNA and MAA.

Immunodetection of Hex. Hex (2 μg) was separated by 12.6 % SDS/PAGE [19] and was electrotransferred [18] onto nitrocellulose membrane. The blots were blocked in TBS/Tween (0.02 M Tris/HCl, pH 7.6, containing 0.15 M NaCl and 0.1% Tween 20), with 1% bovine serum albumin (BSA). Afterwards, nitrocellulose was sequentially incubated with specific primary antibodies (rabbit anti-Hex A antiserum) diluted in TBS/Tween with 1% BSA (1:4000) for 1 h, and after washing three times with TBS/Tween, was incubated with alkaline phosphatase conjugated anti-rabbit immunoglobulin G (1:4000 in TBS/Tween with 1% BSA) for 1 h. The α and β subunits on the sheet were stained with 4-nitroblue tetrazolium as a substrate.

Other methods. The hexosaminidase activity was assayed according to Kaplan & Jamieson [20]. The protein was determined by the dye-binding assay method [21] using bovine serum albumin as a standard. Gels were calibrated for molecular mass determination using the Pharmacia Standard Kit for electrophoresis in SDS.

RESULTS

Under reducing conditions, Hex migrated in PAGE/SDS as two bands as shown by probing with specific antibodies against Hex A. The protein bands of higher (52100 Da) and lower molecular mass (26800 Da) corresponded to the α and β subunits, respectively (Fig. 1). De-

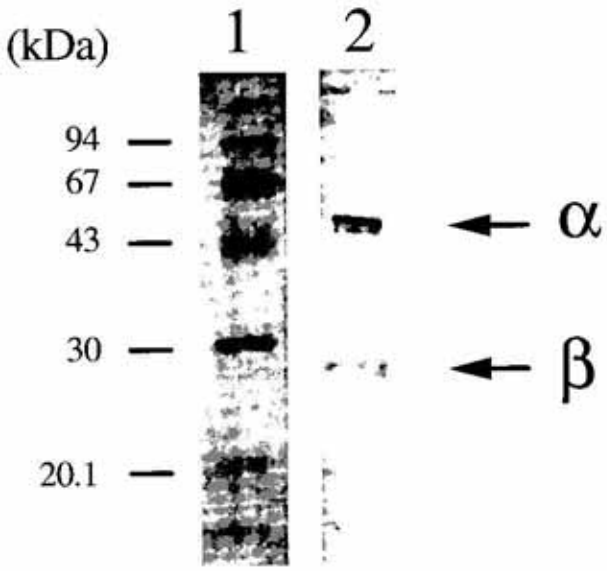

Figure 1. β-*N*-Acetylhexosaminidase (2 μg) run on a 12.6% PAGE/SDS.

The subunits identified by immunoblotting are indicated. Lane 1 – molecular mass markers (kDa): phosphorylase *b* – 94, albumin – 67, ovalbumin – 43, carbonic anhydrase – 30, trypsin inhibitor – 20.1, α-lactalbumin – 14.4. Lane 2 – α- and β-Hex subunits.

spite a strong reaction of both subunits with antibodies, only the band corresponding to the α subunit showed reaction with the lectins used.

A positive reaction with GNA, a mannose-specific lectin, was observed in all the experimental groups. This suggested the presence of glycans of the high mannose/hybrid type as a predominant form. The described reaction was more pronounced in older animals (groups III–VI) than in young ones (groups I and II) (Fig. 2).

Apart from oligosaccharides of the high mannose-type, the positive reaction with MAA and SNA – lectins specific for sialic acid – suggested the presence of complex-type glycans in all the groups tested except group II (1-week-old rats) (Fig. 2). Their presence was further confirmed by their reaction with DSA, a lectin which recognized the Gal β(1-4) GlcNAc linkage. All the groups, except for 1-week-old rats were DSA-positive. A reaction with AAA, a lectin specific for fucose-linked α (1-2,1-3,1-6) to the innermost *N*-acetylglucosamine unit, was observed in all the groups, but it was the most pronounced in 1-week-old rats (Fig. 2). The lack of reaction with PNA, a lectin which recognizes Gal β(1-3) GalNAc in O-linked gly-

	GNA	SNA/MAA	DSA	PNA	AAA	immuno
prenatal	+	+	+	–	+	
1 week	+	–	–	–	+	
1 month	+	+	+	–	+	
1.5 month	+	+	+	–	+	
3 month	+	+	+	–	+	
18 month	+	+	+	–	+	

Figure 2. Twenty microgram of Hex was run on a 12.6% PAGE/SDS, blotted onto nitrocellulose and probed with Dig-labelled lectins: GNA; SNA and MAA; DSA; PNA; AAA.

Immuno, Hex probed with antibodies. (+) Positive reaction with lectin, (-) negative reaction. Typical results are shown which were repeated at least 3 times.

cans, indicated the absence of glycans of that kind in rat liver Hex.

DISCUSSION

Glycoproteins may contain Asn-linked (N-linked) or Ser/Thr-linked (O-linked) oligosaccharides or a mixture of both. N-glycans are generally classified as high-mannose, hybrid or complex types.

In the present study a combination of highly specific lectins and a sensitive detection system provided valuable information on the age-dependent changes in the glycosylation profile.

Up till now, studies on glycan structure of the liver Hex have not been performed. Only human Hex from the placenta [3, 22, 23] and fibroblasts [24, 25] has been investigated, so far.

Our results indicate that rat liver Hex contains predominantly oligosaccharides of the high-mannose type. Some of the oligosaccharides were of the complex-type, terminated with sialic acid or galactose. Fucosylated forms were also observed.

This is in agreement with the finding of O'Downen *et al.* [23] on oligosaccharide structure of placental Hex.

Complex-type oligosaccharides, have also been found in human fibroblasts by Hasilik & von Figura [25]. The latter authors examinated the distribution of oligosaccharides in Hex and cathepsin D, synthesized in cultured human fibroblasts using endo-β-*N*-acetylglucosaminidase H. In both those enzymes, endo-H-resistant (complex) and cleavable oligosaccharides were found.

The mature chain of β subunit of lysosomal Hex was composed of three peptides designated as "a", "b" and "c", linked through disulfide bonds that have apparent molecular mass of 29, 23 and 12 kDa, respectively [1, 4, 6, 7].

In our study we have observed a reaction of Hex with antibodies in the region of 26.8 kDa, but no reaction with lectins has been detected. The absence of such a reaction, may be explained by a partial or complete degradation of oligosaccharides in lysosomes, as was already shown [22, 23], or by a decreased amount of oligosaccharide – below the detection level. Mahuran [3] suggested that in lysosomes the glycans of β-a chain were rapidly degraded to a single GlcNAc residue, not recognized by the lectin used.

At present studies on the developmental patterns of lysosomal enzymes have been described in a few reports only. Olea & Nagata [12] correlated the significant increase in the number and size of lysosomes in the mouse kidney from day 1 after birth and till day 7 with the rapid growth and development of an animal at that stage of development. The activity of Hex increased during development, having reached the peak between days 4 and 7 after birth; and later on, it dropped to an almost fetal level in adults [26].

The results of Kato *et al.* [11], who studied the postnatal changes in the N-linked oligosaccharides of glycoproteins in the rat liver, indicate that tissue glycoproteins from the 2-week-old rat liver contain a higher proportion of complex-type species as compared with those from other groups (1-, 3-, 5-week-old), moreover they suggest that oligosaccharide processing of glycoproteins in the liver may be altered at 2 weeks *post partum*.

The sialic acid/galactose ratio in plasma membrane N-glycans was approximately 1 throughout the postnatal period, which suggests that most of the galactose residues were sialylated. When the activity of sialyltransferase and galactosyltransferase in liver Golgi membranes was determined, age-dependent changes were found. The activity of galactosyltransferase increased immediately after birth, whereas that of sialyltransferase remained at a low level for 2 weeks and then increased to reach a constant level after 4 weeks [27].

Hex is transported to lysosomes in a phospho-mannosyl-dependent manner. Sosa *et al.* [28] found important differences in the bind-

ing of the fetal Hex compared to that of 90-day-old rats which suggests that lysosomal enzymes and their receptors undergo significant molecular changes in the rat liver during the perinatal period [28].

Our results confirm some earlier findings that the most pronounced differences in the glycosylation profile occur around the first week of age. The liver Hex from one week old rats reacts with GNA and AAA only, the reaction with AAA being the most pronounced.

REFERENCES

1. Sagherian, C., Poroszlay, P., Vavougios, G. & Mahuran, D. (1993) Proteolytic processing of the proβ chain of β-hexosaminidase occurs at basic residues contain within an exposed disulfide loop structure. *Biochem. Cell. Biol.* **71**, 340-347.
2. Church, W.B., Swensen, L., James, M.N.G. & Mahuran, D. (1992) Crystallization of human β-hexosaminidase B. *J. Mol. Biol.* **227**, 577-580.
3. Mahuran, D.J. (1990) Characterization of human placental β-hexosaminidase I_2. *J. Biol. Chem.* **265**, 6794-6799.
4. Sonderfeld-Fresko, S. & Proia, R.L. (1989) Analysis of the glycosylation and phosphorylation of the lysosomal enzyme β-hexosaminidase B, by site-directed mutagenesis. *J. Biol. Chem.* **264**, 7692-7697.
5. Wietz, G. & Proia, R.L. (1992) Analysis of the glycosylation and phosphorylation of the α-subunit of the lysosomal enzyme, β-hexosaminidase A, by site-directed mutagenesis. *J. Biol. Chem.* **267**, 10039-10044.
6. Quon, D.V.K., Proia, R.L., Fowler, A.V., Bleibaum, J. & Neufeld, E.F. (1989) Proteolytic processing of the β-subunit of the lysosomal enzyme, β-hexosaminidase, in normal human fibroblasts. *J. Biol. Chem.* **264**, 3380-3384.
7. Mahuran, D.J., Noete, K., Klavins, M.H., Leung, A. & Grave, R.A. (1988) Proteolytic processing of pro-α and pro-β precursors from human β-hexosaminidase. *J. Biol. Chem.* **263**, 4612-4618.
8. Mahuran, D. & Lowden, J.A. (1980) The subunit and polypeptide structure of hexosaminidase from human placenta. *Can. J. Biochem.* **58**, 287-294.
9. Kornfeld, S. (1990) Lysosomal enzyme targeting. *Biochem. Soc. Trans.* **18**, 357-374.
10. Kobata, A. (1992) Structures and functions of the sugar chains of glycoproteins. *Eur. J. Biochem.* **209**, 483-501.
11. Kato, S., Oda-Tamai, S. & Akamatsu, N. (1988) Postnatal changes in N-linked oligosaccharides of glycoproteins in rat liver. *Biochem. J.* **253**, 59-66.
12. Olea, M.T. & Nagata, T. (1991) Quantitative assessment of lysosomal size, number and enzyme activity in mouse kidney during maturational development. *Cell. Mol. Biol.* **37**, 679-685.
13. De Duve, C., Pressman, B.B., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955) Intracellular distribution patterns of enzymes in rat liver tissue. *Biochem. J.* **60**, 604-617.
14. Wójczyk, B., Hoja, D. & Lityńska, A. (1991) Purification of β-glucuronidase and structural assessment of the carbohydrate chains by lectin affinity immunoelectrophoresis. *Glycoconjugate J.* **8**, 340-349.
15. Chenais, F., Virella, G. & Patrick, C.C. (1977) Isolation of soluble immuno complex by affinity chromatography using staphylococcal protein A-Sepharose as substrate. *J. Immunol. Methods* **18**, 183-192.
16. *Affinity Chromatography: Principles and Methods* (1979) p.15, Pharmacia Fine Chemicals Uppsala.
17. Haselbeck, A., Schickaneder, E., von der Eltz, H. & Hosel, W. (1990) Structural characteriza-

tion of glycoprotein carbohydrate chains by using digoxigenin-labeled lectins on blot. *Anal. Biochem.* **191**, 25–30.

18. Towbin, H., Staehelin, T. & Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354.

19. Laemmli, U.K. (1970) Maturation of the head of bacteriophage T4. *Nature* **227**, 690–685.

20. Kaplan, H.A. & Jamieson, J.C. (1977) The effect of inflammation on the rat liver β-galactosidase and β-*N*-acetylglucosaminidase. *Life Sciences* **21**, 1311–1316.

21. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

22. O'Dowd, B.F. & Mahuran, D. (1984) Characterization by nuclear magnetic resonance of the concanavalin A binding oligosaccharides on the β_b chain of placental β-hexosaminidase B: Lectin binding to the separated polypeptide chains of hexosaminidases A and B. *Can. J. Biochem. Cell. Biol.* **63**, 723–729.

23. O'Dowd, B.F., Cummings, D.A., Gravel, R.A. & Mahuran, D. (1988) Oligosaccharide structure and amino acid sequence of the major glycopeptides of mature human β-hexosaminidase. *Biochemistry* **27**, 5216–5226.

24. Hasilik, A. & Neufeld, E.F. (1980) Biosynthesis of lysosomal enzymes in fibroblasts. *J. Biol. Chem.* **255**, 4937–4935.

25. Hasilik, A. & von Figura, K. (1981) Oligosaccharides in lysosomal enzymes. *Eur. J. Biochem.* **121**, 125–129.

26. Bianchi, R.A., Fogal, T., Bertini, F. & Sosa, M.A. (1995) Lysosomal enzyme activity in rat adrenal gland related to the postnatal development. *Mechanisms Aging Dev.* **84**, 151–156.

27. Oda-Tamai, S., Kato, S. & Akamatsu, N. (1991) Postnatal changes in sialylation of glycoproteins in rat liver. *Biochem. J.* **280**, 179–185.

28. Sosa, M.A., Belmonte, S. & Bertini, F. (1996) Changes in *N*-acetyl-β-D-glucosaminidase binding system in rat liver during growth. *Mechanisms Aging Dev.* **86**, 75–81.