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Accumulation of mannosyl-$\beta(1 \rightarrow 4)$-*N*-acetylglucosamine in fibroblasts and leukocytes of patients with a deficiency of β-mannosidase

Johannes van Pelt [1,*], Cornelis H. Hokke [1,2], Lambertus Dorland [1], Marinus Duran [1], Johannis P. Kamerling [2] and Johannes F.G. Vliegenthart [2]

[1] *University Children's Hospital "Het Wilhelmina Kinderziekenhuis", Utrecht (The Netherlands) and* [2] *Department of Bio-Organic Chemistry, Utrecht University, Utrecht (The Netherlands)*

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Summary

Cultured fibroblasts from two brothers with β-mannosidosis were shown to accumulate the disaccharide mannosyl-$\beta(1 \rightarrow 4)$-*N*-acetylglucosamine in amounts of 52 and 68 nmol/mg protein. Structural identification of the Man$\beta(1 \rightarrow 4)$GlcNAc, isolated by HPLC, was performed by 500-MHz ^{1}H-NMR spectroscopy and sugar composition analysis. Control fibroblasts did not contain a detectable amount of Man$\beta(1 \rightarrow 4)$GlcNAc. The disaccharide was also present in leukocytes from these patients, but not in controls, as could be demonstrated by thin-layer chromatography and sugar composition analysis. However, the amounts in leukocytes (5 and 6 nmol/mg protein) were much smaller than those in fibroblasts.

Introduction

β-Mannosidosis is an inherited disorder of glycoprotein catabolism caused by a deficiency of the enzyme β-mannosidase (EC 3.2.1.25). It has been described in

Correspondence to: L. Dorland, University Children's Hospital "Het Wilhelmina Kinderziekenhuis", Nieuwe Gracht 137, NL-3512 LK Utrecht, The Netherlands.

* Present address: De Wever Ziekenhuis, Department of Clinical Chemistry, H. Dunantstraat 5, NL-6419 PC Heerlen, The Netherlands.

goats [1] in which the deficiency is expressed in all investigated tissues. Recently five humans were discovered with β-mannosidosis [2–4].

Goats with β-mannosidase deficiency suffer from severe neurological deficits accompanied by the accumulation in tissues of a trisaccharide Manβ(1 → 4)GlcNAcβ(1 → 4)GlcNAc together with smaller amounts of a disaccharide Manβ(1 → 4)GlcNAc [1,5]. The same oligosaccharides are excreted into the urine in large amounts [6]. Human β-mannosidase deficiency can be demonstrated in leukocytes, fibroblasts and plasma. Affected patients show an excessive urinary excretion of the disaccharide Manβ(1 → 4)GlcNAc [2–4]. The present work shows the accumulation of the same disaccharide in cultured human fibroblasts and to a lesser extent in leukocytes.

Materials and methods

Fibroblasts

Fibroblasts were obtained from two brothers (AB and HB) with β-mannosidase deficiency [4] and five controls. The cells (2 Falcon flasks of 75 cm^2 for each subject) were cultured in HAM's F12 medium supplemented with 5% fetal calf serum, 1% Ultroser, 27 mM-glutamine and 2 mM-penicillin/streptomycin. The cells were harvested at confluence by trypsinization, washed several times in phosphate buffered (0.01 M, pH 7.4) saline (0.15 M) and disrupted in distilled water by sonication (two bursts of 10 s at 0°C). After centrifugation for 10 min at 23°C and $4000 \times g$, the supernatants were collected and the pellets were washed twice with distilled water. Portions of the supernatants were taken for determination of protein and for measurements of α- and β-mannosidase activities. The protein content was determined according to Lowry et al. [7] using bovine serum albumin as a standard. β-Mannosidase activity was measured by the method of Panday et al. [8], modified by taking 30 μl supernatant, 10 μl 2 mM 4-methylumbelliferyl-β-mannoside (Sigma, St. Louis, MO, U.S.A.) in 0.05 M sodium acetate buffer (pH 4.7), and 60 μl 0.3 M phosphate buffer (pH 4.0) as incubation mixture. α-Mannosidase activity was measured according to Galjaard [9]. The remaining part of the supernatants was lyophilized and redissolved in 0.2 ml of acetonitrile/water (4 : 1, v/v).

Leukocytes

Leukocytes of the two brothers with β-mannosidosis and control leukocytes were isolated from 10 ml freshly drawn blood according to the method of Roos and Loos [10]. Cell homogenates were prepared in water by sonication. After centrifugation for 10 min at 23°C and $4000 \times g$, portions of the supernatants were taken for protein determination. The remaining part of the supernatants was lyophilized and redissolved in acetonitrile/water (1 : 1, v/v).

Chromatographic procedures

High-performance liquid chromatography was carried out with a Partisil 10 SAX column (250 × 4.6 mm, Whatman Inc., Clifton, NJ, USA). The compounds were eluted with a mixture of acetonitrile/water (4 : 1, v/v) at a flow rate of 2.0 ml/min.

The eluate was monitored at 200 nm. Calibration curves were made using various monosaccharides and Manβ(1 → 4)GlcNAc isolated from the patients' urine [4,11]. Thin-layer chromatography was performed on silica sheets (10 × 10 cm, Alufolien Merck, Darmstadt, FRG) using the system *n*-propanol/acetic acid/water (85 : 1 : 15, v/v) [4]. The chromatograms were stained by heating at 105°C for 10 min after dipping in a mixture of 40 mg orcinol in 4 ml concentrated sulphuric acid and 80 ml acetone.

Structural analysis

Carbohydrate composition analysis was performed by gas-liquid chromatography. The trimethylsilylated methyl glycosides were analyzed on a capillary CP-Sil 5 WCOT fused silica column (25 m × 0.32 mm, film thickness 0.11 μm, Chrompack, Middelburg, The Netherlands) after methanolysis, *N*-reacetylation and trimethylsilylation [12]. Helium was used as carrier gas at an inlet pressure of 0.7×10^5 Pa. Oven temperature program: 1 min at 125°C, 125 to 240°C at 5°C/min, 10 min at 240°C. The injection-port temperature was 220°C, and the detector temperature 310°C. 500-MHz ^{1}H-NMR spectroscopy was carried out on a Bruker AM-500 spectrometer equipped with a Bruker Aspect-3000 computer (Bruker GmbH, Rheinstetten, FRG), at probe temperatures of 300 and 310 K. Before analysis the disaccharide fraction was repeatedly exchanged in ^2H_2O (99.96 atom % ^{2}H, Aldrich, Milwaukee, WI, USA) with intermediate lyophilization [13].

Results and discussion

The results of the measurement of α- and β-mannosidase activities, as shown in Table I, indicate a complete deficiency of β-mannosidase in the fibroblasts. α-Mannosidase activities of patients AB and HB were in the normal range, similar to the plasma values reported earlier [4]. These data open the possibility for prenatal diagnosis, although this would require the analysis of cells from obligate heterozygotes.

The lyophilized supernatants were redissolved in the HPLC eluent and separated on a Partisil 10 SAX column in four runs. A chromatographic profile is given in Fig. 1. The elution volume of the carbohydrate positive peak corresponded exactly with that of the reference compound Manβ(1 → 4)GlcNAc, isolated previously from

TABLE I

β-Mannosidase and α-mannosidase activities in cultured fibroblasts (nmol·h^{-1} (mg protein)$^{-1}$)

	β-Mannosidase	α-Mannosidase
AB	n.d.	88
HB	n.d.	60
Controls (n = 5)	77–163	61–201

n.d.: not detectable.

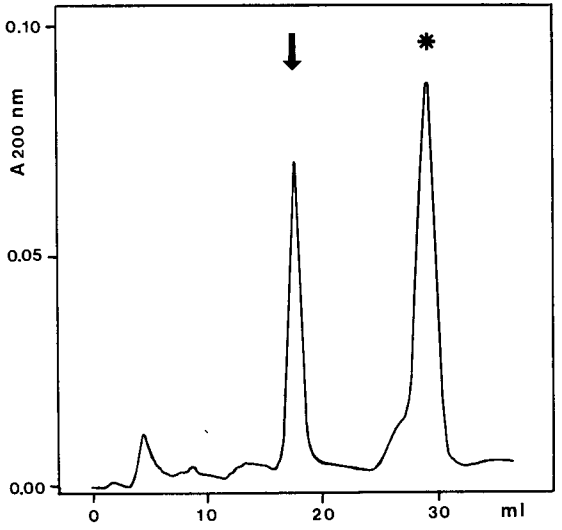

Fig. 1. HPLC elution profile of the supernatant of β-mannosidosis fibroblasts of patient HB. The supernatant was lyophilized, redissolved in 0.2 ml acetonitrile/water (4:1, v/v) and separated on a Partisil 10 SAX column, with the same solvent as eluent. The arrow shows the elution position of Manβ(1 → 4)GlcNAc; the peak marked with an asterisk did not contain carbohydrate material.

β-mannosidosis urine [4,11]. A supernatant of control fibroblasts did not reveal any peak in this region. The fractions of the four different runs from one supernatant were pooled and a small part of the sample was used for sugar composition analysis, showing the presence of Man and GlcNAc. The remaining portion of the sample was used for 500 MHz ^{1}H-NMR spectroscopy. The ^{1}H-NMR spectrum of the unknown carbohydrate turned out to be completely identical to that of urinary reference Manβ(1 → 4)GlcNAc [11].

Accumulated Manβ(1 → 4)GlcNAc was quantitated from the HPLC peak areas, after calibration with the reference compound. The cultured fibroblasts of AB and HB contained 52 and 68 nmol/mg protein, respectively. The accumulation of oligosaccharides in fibroblasts has also been demonstrated in various other lysosomal storage diseases, such as human α-mannosidosis [14], caprine β-mannosidosis [15], and human sialidosis and galactosialidosis [16,17]. In the latter two disorders the content of sialyloligosaccharides accumulating in fibroblasts was 50–100 nmol/mg protein, equivalent to the amount of disaccharide observed in β-mannosidosis fibroblasts. Therefore, it seems that Manβ(1 → 4)GlcNAc, although being a relatively small and neutral molecule, cannot diffuse across the lysosomal membrane and accumulates in β-mannosidosis fibroblasts in the same proportion as the much larger and negatively charged sialyloligosaccharides in sialidase deficient cells.

The supernatants of the β-mannosidosis leukocytes of AB and HB showed a complete absence of β-mannosidase activity and a normal α-mannosidase activity, as published before [4]. HPLC of the supernatants on Partisil 10 SAX resulted in both cases in a small peak in the same position as that of the reference disaccharide

TABLE II

Total monosaccharide content in nmol (after methanolysis) of the 'HPLC-disaccharide' fraction of leukocytes, isolated from 10 ml blood

	Control	AB	HB
Man	8	16	19
Glc	27	19	21
GlcNAc	–	6	12

Manβ(1 → 4)GlcNAc. Control leukocytes did not give rise to a peak in that region. For each of the leukocyte-supernatants, the HPLC fractions corresponding with the disaccharide elution volume, were combined and lyophilized. After redissolving in water, one half of the samples were used for TLC. The β-mannosidosis samples contained a compound migrating with the same mobility as reference compound Manβ(1 → 4)GlcNAc.

The sample obtained from control leukocytes did not show a band coinciding with Manβ(1 → 4)GlcNAc. All samples revealed also a carbohydrate positive band at the position of the monosaccharides glucose and mannose. The TLC results were confirmed by sugar composition analysis, performed on the other half of the samples (Table II). GlcNAc was identified solely in the β-mannosidosis samples, and Man and Glc in all samples. Calculation of the amounts of Manβ(1 → 4)GlcNAc in leukocytes of AB and HB gave 5 and 6 nmol/mg protein, respectively. These figures are much lower than those observed in the fibroblasts. The reason for this is unclear but it may reflect a different total turnover of glycoproteins in the lysosomes of the two different cell types.

Up to now the clinical phenotype of β-mannosidosis in goats seems to be more severe than that in man. The reason for this is unclear but is probably not caused by different amounts of storage material in the two species. The major storage products in β-mannosidosis in goats and humans differ in size, being a tri- and a disaccharide, respectively. Humans probably possess a different set of lysosomal enzymes, which prevents the accumulation of the trisaccharide. The significance of the size of the accumulating storage product for the development of clinical symptoms remains to be established.

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