Comparison of the Urinary Glycoconjugates Excreted by Patients with Type I and Type II Fucosidosis

N. M. K. Ng Ying Kin

Glycoconjugates were isolated, by repeated chromatography on Biogel P-4, from urine of two patients with different phenotypes of fucosidosis, type I (acute) and type II (chronic). Studies of the isolated compounds with thin-layer chromatography, chromatography on Biogel P-4, gas-liquid chromatography, and $^1$H- and $^{13}$C-nuclear magnetic resonance spectroscopy showed differences in the excretion patterns of fucosyl glycoconjugates in the urine of these patients. The amount of the diglycosylasparagine, $\text{fuc(1-6)glcNAC-Asn}$, excreted was significantly lower for the type I than for the type II patient. On the other hand, the reducing hexaosaccharide $\text{gal(1-4)(fuc(1-3)glcNAC-β1-2manα1-3}6\text{manβ1-}4\text{galcNAC}$ was present in much greater quantities in urine from the type I patient. The differences in the excretion patterns of these glycoconjugates may be attributed to different substrate specificites for the residual $\alpha$-L-fucosidases in the two forms of the disease. I propose that such differences may be exploited for the early laboratory diagnosis of the type II form of the disease, particularly by thin-layer chromatography.

Additional Keyphrases: heritable neurological diseases · synthetic substrates · oligosaccharides · glycopeptides · chromatography · pediatric chemistry · NMR · screening

Fucosidosis is an inherited neurological disorder, with autosomal recessive transmission. At least two clinical subtypes of this condition have been described (1).

In the type I cases, symptoms—notably muscle weakness and hypotonia—begin in early infancy, and the disease progresses rapidly, with neurological regression. Death usually occurs within five years.

In the less-severe (type II) form, the age of onset is much later, and the disease is less acute and more chronic, with psychomotor degeneration occurring later; patients may live into the early teens or even reach their twenties. The type II cases are further differentiated from type I by the presence of angiotensinogen corpus diffusum in most type II cases.

A deficiency of the enzyme $\alpha$-L-fucosidase (EC 3.2.1.51), as assessed with use of a synthetic substrate, can be demonstrated in various organs, cell cultures, and body fluids from the patients. This deficiency results in accumulation and urinary excretion of $\alpha$-fucosylated glycoconjugates (2-5).

But measurements of this enzyme alone cannot be used to differentiate the two phenotypes, there being little difference in the residual enzymatic activities in all cases of fucosidosis. Differentiation, therefore, is currently based only on clinical criteria.

Recently, during routine screening for fucosidosis by thin-layer chromatography (TLC), the urine of a patient with the type II form of the disease was found to exhibit a pattern different from that obtained for a clinically diagnosed type I patient (5, 6).1 A preliminary account of these studies has been reported in abstract form (7). The glycoconjugates from urine specimens from these patients were isolated by chromatography on Biogel P-4 (8) and their structures compared by using $^1$H- and $^{13}$C-nuclear magnetic resonance (NMR) spectroscopy and gas-liquid chromatography (GLC). Here, I give details of the findings for the two patients with characteristic clinical features of type I and type II phenotypes of fucosidosis.

Materials and Methods

Samples. Twenty-four-hour urine specimens from the patients were stored at $-20^\circ$C. The type I and type II cases were diagnosed clinically, and a deficiency of $\alpha$-L-fucosidase activity towards 4-methylumbelliferyl-$\alpha$-L-fucopyranoside was demonstrated in cultured fibroblasts, leukocytes, and sera of the two patients. A full report of the type I patient has already appeared (6).

Isolation of glycoconjugates. Glycoconjugates were isolated from the urine essentially as described earlier (8). Briefly, the procedure was as follows. Urine (up to 100 mL) was centrifuged (4000 × g, 20 min, 4°C). The supernatant fluid was then passed through a Sep-Pak C18 Cartridge (Waters Associates, Mississauga, Ontario, Canada) that previously had been conditioned with methanol and water. The effluent was concentrated 10-fold and precipitated in nine volumes of absolute ethanol at 4°C. The precipitate was then thoroughly dialyzed against water at 4°C. The diffusate was concentrated and subjected to repeated chromatography on a 45 × 25 cm column of Biogel P-4 (Bio-Rad Labs., Richmond, CA 94804). Fractions were monitored for carbohydrates by use of phenol–sulfuric acid reagent and by TLC with use of the solvent system n-butanol/acetic acid/water (2:1:1 by vol) and the orcinol–sulfuric acid spray reagent (9).

GLC analyses of glycoconjugates. The ratio of non-reducing N-acetylgalactosamine residues to reducing ones was determined by treating the glycoconjugates with sodium borohydride overnight at 4°C before hydrolysis in 1 mol/L HCl at 100°C for 10 h (8). After the solvents were removed under reduced pressure at room temperature, the products were acetylated with acetic anhydride in the presence of sodium acetate and analyzed by GLC on a 3% ECNSS-M column on Gas Chrom Q (Chromatographic Specialties, Ontario, Canada) at 230°C. The non-reduced N-acetylgalactosamine pentacetate was eluted as a single peak, clearly separated from the earlier-eluting reduced hexaacetate (8).

1 Nonstandard abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; and NMR, nuclear magnetic resonance.
For total compositional analyses, the glycoconjugates were hydrolyzed directly and the resulting sugars separated by GLC as the borohydride-reduced and acetylated alditols (10). The alditol acetates were analyzed isothermally at 190 °C.

NMR spectroscopy. Both 1H- and 13C-NMR spectra were obtained with a Brucker WH 400 spectrometer, equipped with Fourier transform capabilities.

For 1H-NMR spectroscopy at 400 MHz, the glycoconjugates were exhaustively exchanged with D2O, and the spectra were recorded both at ambient temperature and at 70 °C. The instrument was first calibrated with a reference solution of sodium 4,4-dimethyl-4-silapentane-1-sulfonate in D2O.

For proton-decoupled 13C NMR spectroscopic determinations I used solutions of glycoconjugates in D2O at 100.62 MHz, at ambient temperature. The chemical shifts (ppm) were measured relative to internal methanol (49.0 ppm) and then referenced to external tetramethylsilane.

Results

TLC analyses

Figure 1 shows a typical thin-layer chromatogram obtained for 24-h urine specimens from the fucosidosis patients. Although most of the compounds present in the type I patient are also found in the type II patient, the relative amounts of compounds c and d differ in the two cases. In the type I patient, compound c, which was identified as the reducing hexasaccharide (Figure 2) in earlier studies (5), is the major compound, whereas compound d, which has a diglycosyl-asparagine structure (Figure 2) as determined by 13C- and 1H-NMR spectroscopy and GLC studies, is a relatively minor component. The reverse is true of the type II patient, compound d being the most prominent.

These TLC findings were reproducible for several 24-h urine specimens from each of the two patients.

Biogel P-4 chromatography. The glycoconjugates obtained after precipitation with ethanol and dialysis of the 24-h urine specimens from the type I and type II patients were fractionated on a Biogel P-4 column. The elution profiles (Figure 3, A and B) essentially reflect the findings described above. Thus, in Figure 3A (the type I patient) the peak corresponding to the fraction containing compound c was twice as great as that for compound d, the amounts being 16 vs 8 mg per 100 mL of urine. Conversely, in Figure 3B (type
the fraction containing compound c was smaller than that containing d: 15 vs 86 mg/100 mL of urine. Little difference was evident in the fractions eluting earlier.

The fractions containing compounds c and d were purified by repeated Biogel chromatography for NMR studies. In the type I patient, c was readily obtained, almost exclusively as the hexasaccharide. However, in the type II patient, this compound was contaminated with a glycopeptide having a slightly lower mobility on TLC (see Figure 1) and staining positively with ninhydrin. When the contribution of this contaminant is taken into account, the yield of the reducing hexasaccharide c in the type II patient is even lower than shown in Figure 3B.

The purified compounds c obtained from the type I and type II patients had superimposable 1H NMR spectra, identical to that published earlier for the hexasaccharide depicted in Figure 2.

After at least two repeated cycles of chromatography, fraction d could be obtained in sufficiently purified form in the case of the type I patient. A single such cycle sufficed for the urine from the type II patient, reflecting the presence of this compound in major quantities. 1H- and 13C-NMR spectroscopic and GLC analyses confirm that in both cases this compound has the diglycosyl asparagine structure shown in Figure 2. Thus, the 1H-NMR spectrum of compound d was identical to that published for this compound, with prominent absorances at 4.99 and 4.81 ppm, respectively representing the anomic protons of the N-acetylgalactosaminyl and fucosyl residues (11).

Unequivocal evidence for the 1–6 linkage between the two sugar residues was obtained from the 13C-NMR spectrum of compound d (Figure 4). All the peaks present can be assigned individually to each of the 18 carbon nuclei in the diglycosyl asparagine (11, 12), indicating the homogeneity of the compound. There is no evidence for the presence of a 1–3 linkage between the two sugar moieties.

GLC analyses readily confirmed the presence of fucose and N-acetylgalactosamine as the non-reducing components of compound d. Similarly, free reducing N-acetylgalactosamine, as well as non-reducing fucose, galactose, N-acetylgalactosamine, and mannose were shown to be present in compound c.

Discussion

The laboratory diagnosis of both the type I and type II forms of fucosidosis is usually made by demonstrating a deficiency of the enzyme α-L-fucosidase—as evaluated with the synthetic substrate 4-methylumbelliferyl-α-L-fucopyr-

anoiside—in cultured fibroblasts, leukocytes, and blood sera from the patients (13). But the two major forms of this disease can only be distinguished on the basis of certain well-established clinical criteria (1), because the residual enzyme activities towards the artificial substrate are about the same in most patients.

In this paper, different patterns of excretion of glycoconjugates by the type I and type II patients have been demonstrated. TLC of patients’ urine could thus offer a simple means of differentiating the two types. Of particular interest is the precocious diagnosis of the type II form of this disease, in which clinical signs are apparent only in later years. In our studies on the chemical structures of the various glycoconjugates excreted by fucosidosis patients, the free reducing hexasaccharide c was shown to be a major component in the type I patient, a minor one in the type II patient. The reverse was true of the glycoprotein d. Furthermore, NMR and GLC studies of the other larger-mass fractions eluting earlier from the Biogel column indicated that the type II patient excreted more glycopeptides than did the type I.

The differences in patterns of excretion of the glycoconjugates by patients with the two major phenotypes of fucosidosis may possibly be explained by the polymorphism exhibited by human α-L-fucosidase (14) and differences in substrate specificity of these isoenzymes. Indeed, in two recent reports (15, 16), residual enzyme activities as high as 34–64% and almost 100%, respectively, towards the synthetic substrate 4-methylumbelliferyl-α-L-fucopyranoside were found in fucosidosis patients diagnosed clinically and by TLC. Gordon et al. (16) further demonstrated in their patient a normal fucosidase activity towards the diglycosyl asparagine, d.

In the two cases described here, I speculate that the type I patient has a high residual fucosidase activity towards the glycosidic linkage of the fucal-6glicNAc type (present in compound d) but not the fucal-3glicNAc type (in compound c). Thus, removal of the fucosyl residue from the dichitobi-syl unit of compound I (Figure 2) gives rise to a substrate more accessible to endogluconaminidase attack, resulting in a less reducing oligosaccharide. Further cleavage by an endomannosidase (17) would give rise to the hexameric product. On the other hand, the type II patients are probably deficient in fucosidase activity towards both the fucal-3 and fucal-6 linkages, so that the glycopeptides I and II (Figure 2) are less accessible to the action of endogluconaminidase. In these patients, glycopeptides I and the resulting linear glycopeptides after endomannosidase attack would thus accumulate. The major source of the compound d is probably the incomplete sequential actions of exoglycosidases on the type of glycopeptides with structure II commonly found in mammalian tissues and body fluids.

Studies are now in progress in our laboratories to test fibroblast cultures from various fucosidosis patients for fucosidase activities towards some of the glycoconjugates described in this paper.

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References


