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Analysis of N-Acetylgalactosamine-4-Sulfatase Protein and Kinetics in Mucopolysaccharidosis Type VI Patients

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Summary

A sensitive and specific, monoclonal antibody–based immunoquantification assay has facilitated determination of the N-acetylgalactosamine-4-sulfatase (4-sulfatase) protein content in cultured fibroblasts from normal controls and mucopolysaccharidosis type VI (MPS VI) patients. The assay enabled the quantification of 4-sulfatase protein by using a panel of seven monoclonal antibodies and has shown that fibroblasts from 16 MPS VI patients contained ≤5% of the level determined for normal controls. Fibroblasts from the most severely affected patients contained the lowest levels of 4-sulfatase protein, usually with few epitopes detected, while fibroblasts from mildly affected patients had higher levels of 4-sulfatase protein, with all seven epitopes detected. The pattern of epitope expression is proposed to reflect the conformational changes in the 4-sulfatase protein that arise from different mutations in the 4-sulfatase gene. Immunoquantification in combination with a specific and highly sensitive 4-sulfated trisaccharide–based assay of enzyme activity in these MPS VI patient fibroblasts enabled the determination of residual 4-sulfatase catalytic efficiency (k_{cat}/K_m). The capacity of fibroblasts to degrade substrate (catalytic capacity) was calculated as the product of 4-sulfatase catalytic efficiency and the content of 4-sulfatase in fibroblasts. One patient, 2357, with no clinical signs of MPS VI but with reduced 4-sulfatase activity and protein (both 5% of normal) and dermatansulfaturia, had 5% of normal catalytic capacity. The other 15 MPS VI patient fibroblasts had 0%–1.4% of the catalytic capacity of fibroblasts from normal controls and were representative of the spectrum of MPS VI clinical phenotypes, from severe to mild. It is proposed that an enzyme-replacement therapy achieving a correction of >5% of normal catalytic capacity is required to avoid the onset of MPS VI clinical phenotype. Moreover, it is suggested that the restored catalytic capacity must be correctly localized within the lysosomal compartment to enable effective treatment of MPS VI.

Introduction

Mucopolysaccharidosis type VI (MPS VI), or Maroteaux-Lamy syndrome (McKusick 25330), is an autosomal recessive disorder which is characterized by the intralysosomal accumulation and urinary excretion of partially degraded dermatan sulfate glycosaminoglycan (Matalon et al. 1974). Biochemical analysis has revealed that the lysosomal enzyme N-acetylgalactosamine-4-sulfatase (4-sulfatase, arylsulfatase B; E.C.3.1.6.1) is deficient in MPS VI patients and prevents the normal sequential degradation of dermatan sulfate and chondroitin sulfate. Clinically, the classical form of this disorder is characterized by early onset (at age ≤2 years), severe progression, and symptoms which include short stature, coarse facies, dystosis multiplex, joint stiffness and functional loss, hepatosplenomegaly, corneal clouding, and increased respiratory infection (Neufeld and Muenzer 1989). In general, MPS VI patients display variable clinical symptoms, representing a complex mixture of the latter features with gradations of severity of individual symptoms. It is now recognized that there is a broad

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spectrum of MPS VI clinical phenotypes which for individual patients may vary between the two extremes of near normal and severely affected.

Initially, the diagnosis of MPS VI was on the basis of clinical presentation and, later, by analyzing the presence of partially degraded dermatan sulfate in urine. With the advent of enzyme analysis using synthetic or natural substrates, the precise nature of the biochemical defect in MPS VI was defined as a deficiency in the activity of the lysosomal enzyme 4-sulfatase, permitting improved diagnosis of MPS VI (Delvin et al. 1976; Chang et al. 1981; Hopwood et al. 1986). The analysis of residual 4-sulfatase enzyme activity indicated <5% of 4-sulfatase activity in MPS VI patient samples when compared with normal controls (Hopwood et al. 1986). However, no relationship between the level of 4-sulfatase activity in MPS VI patient samples and the observed clinical phenotype was apparent. Thus, by itself, the measurement of enzyme activity has not been a suitable parameter for the prediction of clinical phenotype. Clearly, additional markers were required to dissect the complex picture of clinical phenotype. The molecular basis of this diversity is thought to be due to different mutations in the 4-sulfatase gene and to be complicated by the possible frequent double heterozygous nature of MPS VI patients. Now that the full cDNA sequence is available for 4-sulfatase (Peters et al. 1990; Schuchman et al. 1990), it is theoretically possible to directly relate each clinical phenotype with an exact genotype. With the prospect that in the near future, gene-product and gene-replacement therapies will be used for lysosomal storage disorders, the ability to predict clinical phenotype by the biochemical analysis of patient samples will become important. In the present paper we report the development of a precise immunochemical technique coupled with enzyme kinetic analysis for the characterization of residual 4-sulfatase in fibroblasts from MPS VI patients. The analysis of a panel of 16 MPS VI patients suggests that this procedure may have the capacity to predict MPS VI clinical phenotype. The techniques developed in the present paper have the potential to establish a clear relationship between the molecular biology of 4-sulfatase, the cell biology of the protein 4-sulfatase, and the pathophysiology of MPS VI patients.

Material and Methods

ABTS substrate kit was purchased from Bio-Rad Laboratories (Richmond, VA) and was used according to the manufacturer's instructions. Polyvinyl chloride plates (96 well) and culture flasks (75 cm^2) were obtained from Costar (Cambridge, MA). Peroxidase-labeled sheep anti–mouse immunoglobulin was purchased from Silenus Laboratories (Melbourne). Laboratory film (Parafilm "M") was from the American Can Company (Greenwich, CT).

The radiolabeled trisaccharide substrate N-acetylgalactosamine-4-sulfate-(β1-4)-glucuronate-(β1-3)-N-acetyl[1-^{3}H]galactosaminitol-4-sulfate (GalNAc4S-GlcA-GalitolNAc4S) was prepared according to a method described by Hopwood et al. (1986).

Monoclonal and Polyclonal Antibodies

The monoclonal antibodies 4-S F58, 4-S F17, 4-S F56, 4-S F18, and 4-S F22 were raised and characterized against immunopurified human liver 4-sulfatase according to a method reported elsewhere for the monoclonal antibodies 4-S 4.1 and 4-S 5.4 (Gibson et al. 1987; Brooks et al. 1990). A rabbit polyclonal anti-human 4-sulfatase was prepared against 4-S 4.1 immunopurified 4-sulfatase and was rendered specific by absorption against ovalbumin and BSA and by immunoaffinity chromatography on a 4-sulfatase antigen column (Gibson et al. 1987).

Immunoquantification Method

A "sandwich" ELISA was developed to quantify 4-sulfatase and to permit multiple epitope detection by using a panel of monoclonal antibodies. Polyclonal antiserum captured 4-sulfatase from complex protein mixtures and the 4-sulfatase was quantified using specific monoclonal antibodies in conjunction with second antibody–detection reagents.

One hundred microliters of a 1:16 dilution of polyclonal anti-4-sulfatase antiserum in 0.1 M $NaHCO_3$, pH 8.5, was added to each well of a polyvinyl chloride plate and incubated first for 2 h at 37°C and then overnight at 4°C. Unbound antiserum was aspirated from each well, and the remaining reactive sites were blocked by adding 200 μl of buffer A (0.02 M Tris HCl, pH 7.0, containing 0.25 M NaCl, 1% [w/v] ovalbumin) to each well and incubating for at least 4 h at 20°C. Test samples (30 μl/well) were incubated overnight at 4°C, and the well then was aspirated and washed three times with 200 μl of buffer A. Wells were then incubated with 100 μl of monoclonal antibody for 4 h at 20°C. Each well was aspirated and washed three times with 200 μl of buffer A, then incubated with 50–100 μl of a peroxidase-labeled anti–mouse immunoglobulin second antibody (1:1,000 dilution

[v/v] in buffer A) for 1 h at 20°C. The antibody was then aspirated, and each well was washed three times with 200 μl of 0.02 M Tris HCl, pH 7.0, containing 0.25 M NaCl. The wash buffer was aspirated, and 200 μl of peroxidase substrate (ABTS substrate kit) was added to each well, and the plate incubated for 30–70 min at 20°C. Peroxidase substrate color development was quantified by measuring absorbance at 414 nm on an automated ELISA reader (Titertek multiscan; Flow Laboratories, Sydney). All results were interpolated through a standard curve and were expressed as nanograms of 4-sulfatase protein per milligram of extracted cell protein. Samples were identified as not detectable within the range of 2 SDs either side of background for the assay.

Patient Diagnosis, Fibroblast Culture, and 4-Sulfatase Extraction

Human diploid fibroblasts were established from skin biopsies, either submitted to this hospital for diagnosis (Hopwood et al. 1982) or obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). MPS VI patients were classified according to both age at diagnosis and progression and severity of clinical symptoms (table 1). Patient 2357 showed no clinical signs of MPS VI but had mild dermatansulfaturia, inclusion bodies in peripheral blood leukocytes, and a deficiency of 4-sulfatase enzyme activity in fibroblasts. Fibroblast culture and harvesting and the preparation of cell extracts were according to methods described by Brooks et al. (1990). Protein levels of fibroblast extracts were determined by the method of Lowry et al. (1951).

Determination of 4-Sulfatase Enzyme Activity

Enzyme activity and kinetic data were obtained by a modification of the technique of Hopwood et al. (1986). Fibroblast lysates were dialyzed against 0.05 M sodium formate, pH 3.5, and 9 μl of extract was incubated with substrate (GalNAc4S-GlcA-GalitolNAc4S, 1 μl, 630 pmol), BSA (0.5 mg/ml) and β-hexosaminidase inhibitor (2-acetamido-2-deoxy-D-gluconolactone, 3 mM final concentration) in a total volume of 12 μl. The assay mixture was incubated

Table 1

Identifying Data on MPS VI Patients

Patient(s) (Fibroblast Number)	Age at Diagnosis	Clinical Phenotype	Urinary Dermatan Sulfate[a] (g/mol creatinine)	Residual 4-Sulfatase[b] (pmol/min/mg fibroblast protein)
MF (1022)	7 d	Severe	34	.6
LW (260)	3 years	Severe	NA	.5
EK (368)	2 years	Severe	49	.6
AO (2221)	3 years	Severe	62	2.4
PG (2259)	2 years	Severe	79	.7
TO (2424)	2 mo	Severe	127	1.5
GM 0519 (2468)	4 years	Severe	NA	NA
GM 3722 (2471)	4 mo	Severe	NA	NA
BM (1246)	16 mo	Severe	NA	.2
DR (839)	17 years	Mild	NA	.8
CH (1795)	25 years	Mild	2	.4
ET (913)	16 years	Mild	9	1.2
ST (912)	14 years	Mild	8	.8
CO (2724)	18 years	Mild	4	1.9
GM 2849 (2467)	35 years	Mild	NA	1.4
JW (2357)	44 years	Normal	1	.6
Controls	<2 mo	Normal	<4[c]	11.8–39.2 (n=73)
	2 mo–2 years	Normal	<2[c]	
	2–6 years	Normal	<1[c]	
	>6 years	Normal	<0.7[c]	

[a] Determined by the method of Hopwood and Harrison (1982). NA = not available.

[b] Determined by using a radiolabeled trisaccharide by the method of Hopwood et al. (1986). NA = not available; n = number of individuals.

[c] Urinary glycosaminoglycan ranges (for >1,000 individuals) were determined in the Department of Chemical Pathology, Adelaide Children's Hospital.

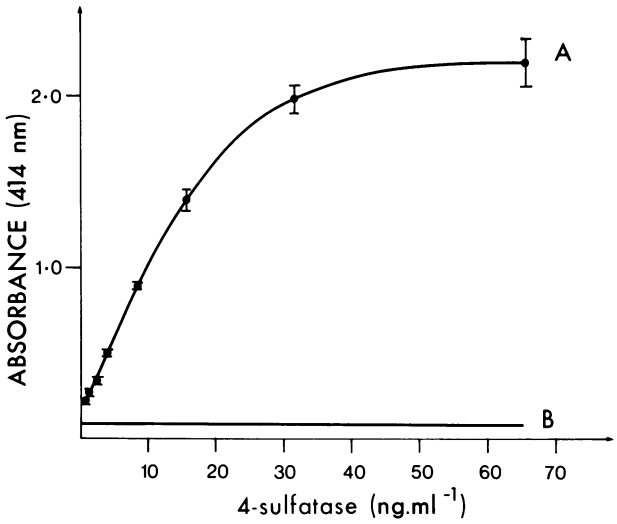

Figure 1 Standard curve for 4-sulfatase immunoquantification. A standard curve (A) was generated for the 4-sulfatase immunoquantification assay using a pool of normal control fibroblast cell extracts which had been serially diluted in 0.02 M-Tris-HCl, pH 7.0, containing 0.25 M-NaCl and 1% (w/v) ovalbumin. B represents the background reactivity of the assay. Control samples were assayed for 4-sulfatase activity by using radiolabeled substrate and were converted to nanograms of 4-sulfatase/ml by using the specific activity measure reported by Gibson et al. (1987). The immunoquantification data presented was a mean ± SD for triplicate samples detected by using the monoclonal antibody 4-S 4.1. Similar curves and SDs were generated for the other six monoclonal antibodies described in the Material and Methods section (data not shown).

for 48 h at 37°C in 20-μl wells of sealed Parafilm as described by Hosli (1977). Substrate and product were separated by high-voltage electrophoresis according to a method described elsewhere (Hopwood et al. 1986). K_m and V_{max} were determined from a double reciprocal Lineweaver-Burk plot by using linear regression analysis. Immunoquantified 4-sulfatase protein and enzyme activity were determined on the same fibroblast extract and were used to calculate turnover number (k_{cat}) as moles per minute per mole of 4-sulfatase. Catalytic efficiency was calculated as the ratio of k_{cat} to K_m, by using the 4-sulfatase M_r of 57,000 (Gibson et al. 1987) and is expressed as minutes per micromole. To define the effective amount of 4-sulfatase activity per fibroblast extract, the term "catalytic capacity" was generated. Catalytic capacity was calculated as the product of catalytic efficiency and the amount of 4-sulfatase protein per milligram of cell protein (nanograms per milligram per minute per micromole) and has been expressed as a percentage of the level in normal control fibroblasts.

Results

Evaluation of the Method

The immunoquantification method has been optimized for the titer of the polyclonal capture antibody (1:16 dilution of antiserum with a titer of 1:3,000) to give the maximal binding of 4-sulfatase, measured by the ability to bind enzyme activity (Brooks et al., in press). The ELISA detection was then optimized by ensuring that the monoclonal antibody was present in saturating amounts, and the polyclonal peroxidase-labeled second antibody was titered to give maximal detection with minimal background activity. A standard curve for 4-sulfatase enzyme quantification has been generated by using the monoclonal antibody 4-S 4.1 (fig. 1) and demonstrates a lower limit of quantification of 10 pg of 4-sulfatase protein in a 30-μl sample (0.3 ng/ml). A single pool of extracts from normal fibroblasts was used to generate a standard curve for each assay performed, and an additional pool of normal fibroblast extracts was analyzed with every quantification in order to monitor interassay variability. The assay demonstrated low intra-assay variability for triplicate samples (fig. 1) and a mean of 58.1 ± 11.3 ng/mg when the quality-control fibroblast extract was quantified in 10 separate assays performed on different days.

Immunoquantification Using Multiple Antibody Analysis

A panel of seven monoclonal antibodies was used to immunoquantify the level of 4-sulfatase protein in fibroblast extracts from normal controls and MPS VI patients (fig. 2). For the monoclonal antibody 4-S 4.1 a range of 39–206 ng of 4-sulfatase protein/mg of cell protein, with a mean of 111 ng/mg, was observed for 10 normal controls. The normal control fibroblast extracts used to analyze the other six monoclonal antibodies were from different donors, but similar ranges of 4-sulfatase protein detection were observed. The difference between 4-sulfatase levels detected by using monoclonal antibody 4-S 4.1 and 4-S 5.2 reflects the small number of cell extracts analyzed with 4-S 5.2, and, although the mean values were different, the individual assays fall within the range detected with the 4-S 4.1 antibody. Moreover, the analysis of 4-S 4.1 and 4-S 5.2 on the same samples gave comparable but not identical results (data not shown).

In contrast, a panel of fibroblasts from 16 MPS VI patients all expressed <10% of the mean of the 4-sulfatase protein found in fibroblast extracts from normal controls, giving a range from none detected to 4.6 ng

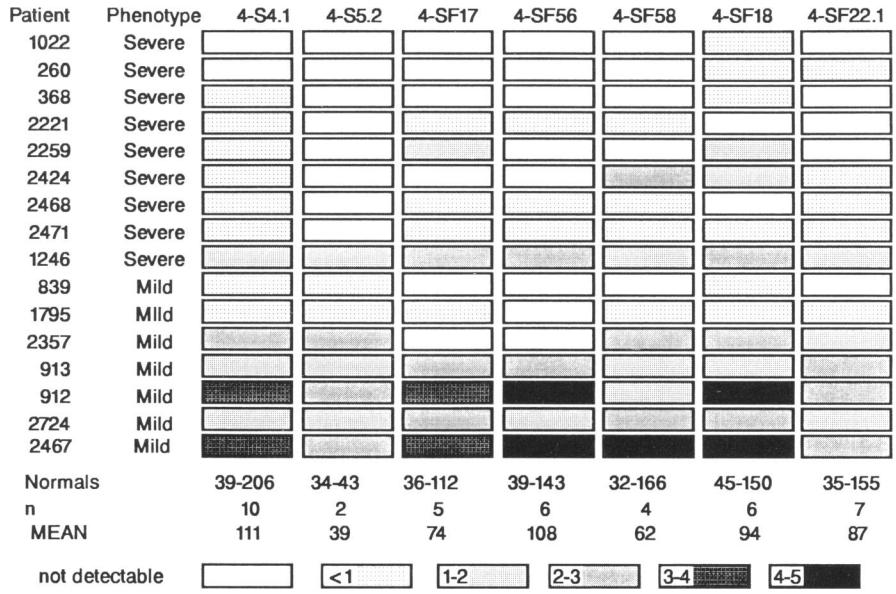

Figure 2 4-Sulfatase protein levels (numbers shown below columns), expressed as nanograms of 4-sulfatase protein per milligram of cell protein in fibroblast extracts and measured in immunoquantification assay using monoclonal antibodies 4-S 4.1, 4-S 5.2, 4-S F17, 4-S F56, 4-S F58, 4-S F18, and 4-S F22.1. Results for MPS VI patients (MEAN) were calculated from the mean of triplicate samples. n = Number of samples tested to determine the mean 4-sulfatase protein levels for normal controls.

of 4-sulfatase/mg of cell protein (fig. 2). The analysis of MPS VI patients by a panel of monoclonal antibodies suggests three categories of reactivity: (1) those expressing little or no detectable 4-sulfatase protein and in whom the majority of epitopes were not detected (patients [fibroblast numbers] 1022, 260, 368, 2221, and 2259), (2) those with variable antibody reactivity with some epitopes undetected (patients 2424, 2468, 2471, 1795, 2357, and 839), and finally (3) those expressing 4-sulfatase protein and in whom all of the epitopes were detected (patients 913, 912, 2724, 2467, and 1246). Except for one case (patient 1246), patients expressing higher levels of 4-sulfatase protein and in whom all epitopes were detected exhibited mild clinical phenotypes; and those expressing little or no detectable 4-sulfatase protein and in whom most epitopes were missing were associated with severe clinical phenotypes. The intermediate group contained both severely affected and mildly affected patients. All of the patients tested expressed at least one epitope, albeit at a much reduced level compared with that of the normal controls. The monoclonal antibody 4-S 5.2 did not react with the 4-sulfatase protein from eight of nine severely affected MPS VI patients, but it did react with 4-sulfatase protein from all seven of the clinically mildly affected MPS VI patients tested.

Immunoquantification Analysis of Non-MPS VI Patients

To determine both the specificity of the quantification method and the possible inhibitory affects of dermatan sulfate storage product on the assay, MPS I and MPS II patients were analyzed for 4-sulfatase protein (table 2). Both MPS I and MPS II fibroblasts contained elevated levels of 4-sulfatase protein. Similarly, another lysosomal enzyme, β-hexosaminidase, involved in dermatan sulfate degradation was shown to be elevated in MPS I and MPS II patients. In contrast, α-L-iduronidase protein (the activity of which was deficient in MPS I patients) was present at similar levels in MPS VI and MPS II patients, when compared with the levels in normal controls.

Enzyme Kinetics of 4-Sulfatase

The very low 4-sulfatase activity present in fibroblast extracts from patients with MPS VI required an assay of both high specificity and high sensitivity, to reliably determine enzyme kinetics. The high purity of the trisaccharide substrate (i.e., absence of contamination with either 6-sulfated nonreducing terminal N-acetylgalactosamine or substrates for other sulfatases) was demonstrated by its total resistance to digestion by both pure N-acetylgalactosamine-6-sulfatase (a gift

Table 2

Protein and Enzyme Analysis of MPS Patient Fibroblasts

Fibroblast Source	Range, Mean ± SD		
	4-Sulfatase Protein[a] (ng/mg)	α-L-Iduronidase Protein[b] (ng/mg)	β-Hexosaminidase Activity[c] (nmol/min/mg)
Normal	39–206, 103 ± 52 (n = 13)	17–54, 30 ± 9 (n = 12)	54–139, 94 ± 31 (n = 14)
Control:			
MPS I	42–488, 208 ± 123 (n = 12)	ND–2, .21 ± .45 (n = 22)	88–630, 347 ± 177 (n = 12)
MPS II	74–756, 236 ± 221 (n = 12)	21–55, 38 ± 13 (n = 6)	130–768, 347 ± 203 (n = 9)
MPS VI	ND–4, 1.0 ± 1.1 (n = 25)	25–66, 41 ± 14 (n = 8)	25–497, 168 ± 96 (n = 25)

Note.—All results have been normalized for cell protein (mg).

[a] Determined by the immunoquantification assay. ND = not detectable.

[b] Determined using a polyclonal antibody against α-L-iduronidase and the monoclonal antibody Id1A (Clements et al. 1989), by using a protocol similar to that described for the quantification of 4-sulfatase (L. J. Ashton and D. A. Brooks, unpublished observations). ND = not detectable.

[c] Determined by a standard enzyme assay protocol (Leaback and Walker 1961).

from J. Bielicki; data not shown) and some MPS VI (e.g., patient 2471) fibroblast extracts which were shown to contain normal or higher than normal levels of the lysosomal enzymes N-acetylgalactosamine-6-sulfatase, β-hexosaminidase, α-mannosidase, and α-L-iduronidase (unpublished observations). Improved sensitivity of the assay was achieved by increasing the incubation time to 48 h. This was facilitated by use of very-small-volume sealed Parafilm wells and demonstrated linear substrate degradation for 72 h when either MPS VI or normal fibroblast extracts were used (fig. 3). These techniques enabled the determination of 4-sulfatase enzyme kinetics from fibroblasts of most MPS VI patients. In some, however, the residual activity was either undetectable or too low (<0.05% of that seen in control fibroblasts) to produce reliable kinetic data.

4-Sulfatase from most MPS VI fibroblasts had a K_m close to the normal range (fig. 4). Fibroblasts from one MPS VI patient (368) had a high K_m, and those from one other patient (260) had a low K_m; clinically, both were severely affected. Immunoquantification of 4-sulfatase, in conjunction with determination of 4-sulfatase enzyme activity, allowed the calculation of 4-sulfatase catalytic efficiency and catalytic capacity. Fibroblasts from several patients show reduced catalytic efficiency, but there appeared to be no correlation with clinical severity (fig. 4). With one exception (patient 2357), fibroblasts from all MPS VI patients had <1.5% of the catalytic capacity observed for fibroblasts from normal controls (fig. 4). Fibroblasts from mildly affected patients all had significant catalytic capacity, whereas, except for patients 260, 2259, and 1246, fibroblasts from the severely affected patients had undetectable catalytic capacity. Fibroblasts from patient 2357 had 5% of the catalytic capacity seen in fibroblasts from normal controls.

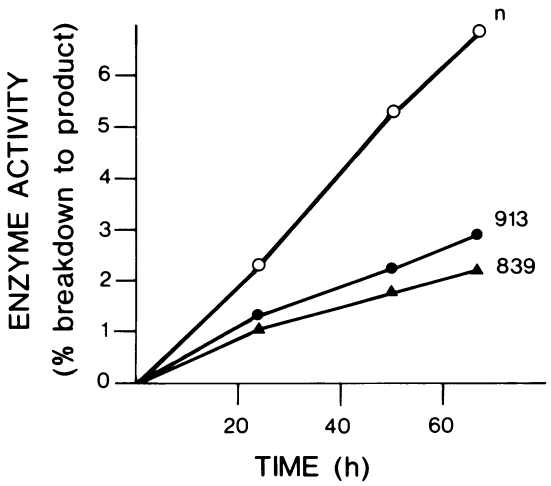

Figure 3 Time course of 4-sulfatase substrate digestion. Lysates from normal fibroblasts (n) diluted 1:90 with 0.05 M-sodium formate buffer, pH 3.5, containing 0.5 mg BSA/ml or lysates of fibroblasts from MPS VI patients (dialyzed against the latter buffer; 913 and 839) were assayed for 4-sulfatase activity by using the radiolabeled trisaccharide substrate (GalNAc4S-GlcA-Galitol-NAc4S) as described in the Material and Methods section. Loss of nonreducing terminal 4-sulfate was determined by high-voltage electrophoresis and was expressed as the percentage of substrate converted to product with time of incubation, after subtraction of background reactivity (<1% breakdown).

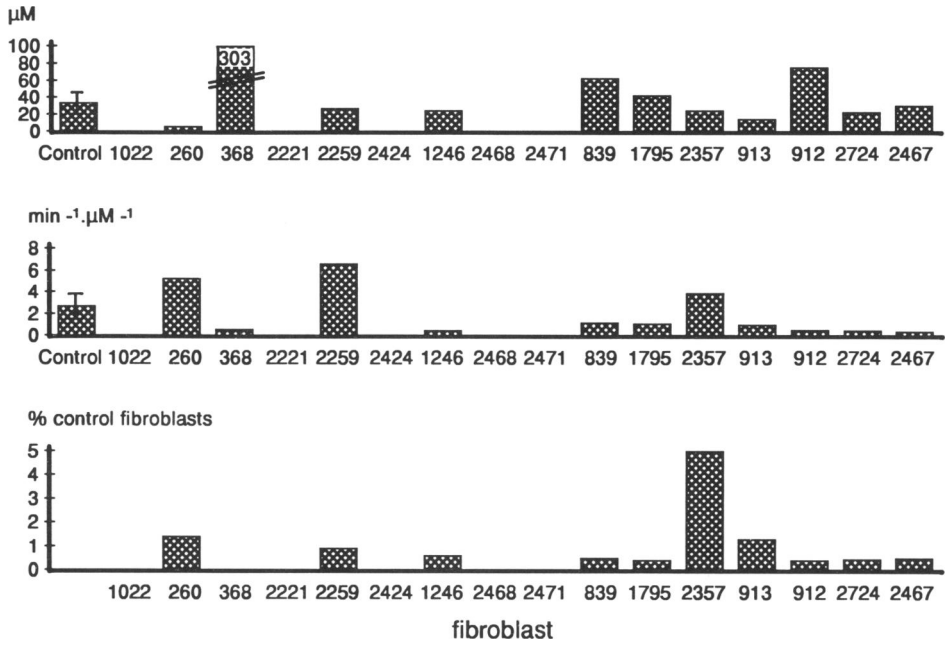

Figure 4 Enzyme kinetic analysis of patient samples. K_m (*top graph*) and V_{max} were determined by using radiolabeled trisaccharide substrate, and the catalytic efficiency (*middle graph*) was calculated, by using immunoquantified 4-sulfatase protein, as the ratio of K_{cat} (mol product/min/mol enzyme) to K_m (μM). The results presented for MPS VI K_m and V_{max} determinations were generated from double reciprocal Lineweaver-Burk plots which had correlation coefficients ≥.90. Control values represent the mean of five analyses of fibroblasts from three normal individuals, and the bars show the range of these values. The latter samples were all diluted to levels of enzyme activity expected for MPS VI patients. Catalytic capacity (*bottom graph*) was calculated as the product of catalytic efficiency and the amount of 4-sulfatase protein per milligram of cell protein and is expressed as a percentage of that in normal controls.

Discussion

In the present paper we have described the development of a sensitive and reliable immunoquantification assay which uses an affinity-purified polyclonal antibody to capture 4-sulfatase protein and a panel of specific monoclonal antibodies to detect this bound protein. The accurate immunoquantification of 4-sulfatase protein has enabled the calculation that normal control fibroblasts contain ~100,000 molecules of 4-sulfatase/cell. The detection limit of the immunoquantification assay was ~10 pg (0.3 ng/ml) or 10^8 molecules of 4-sulfatase/assay. This compares favorably with results of other reported assays for low-abundance lysosomal enzymes, including β-galactosidase and β-glucuronidase (2 ng and 10 ng detection limit, respectively; DeGroen et al. 1989) and is also 1,000 times more sensitive than a previously reported 4-sulfatase quantification, which used a monoclonal antibody–purification step (Brooks et al. 1990) instead of the polyclonal capture technique. Moreover, the method facilitates the detection of 4-sulfatase protein by a panel of monoclonal antibodies, providing a method for the comparative analysis of 4-sulfatase protein structure by using antibodies to different epitopes.

The immunoquantification assay was applied to the analysis of 4-sulfatase protein content in fibroblast cell extracts from 16 MPS VI patients. Detection with the monoclonal antibody 4-S 4.1 demonstrated that all of the patients tested had <5% of the levels of 4-sulfatase protein observed for normal controls. This low level of 4-sulfatase protein content in MPS VI fibroblasts was substantiated by analysis with six additional monoclonal antibodies and also indicated variable epitope detection for some MPS VI patients. There was a trend for severely affected MPS VI patients to have lower 4-sulfatase protein levels and most epitopes not detected, grading to mild patients having higher levels of 4-sulfatase protein and all epitopes detected. Either failure to detect a particular epitope or a lowered detection of individual epitopes may indicate that the 4-sulfatase protein is structurally modified. These modifications may include conformation changes in the protein which either mask certain epitopes or re-

sult from missing protein sequence. Thus, more missing epitopes suggests greater structural changes in the 4-sulfatase protein being analyzed. Monoclonal antibody 4-S 5.2 detected 4-sulfatase protein in mildly affected patients but not in the clinically severely affected MPS VI patients, with the exception of one patient sample (1246). The absence of 4-S 5.2 epitope detection in fibroblasts from clinically severely affected patients may reflect a particular 4-sulfatase structural modification which predisposes a patient to this clinical phenotype. Thus, epitope analysis with the panel of monoclonal antibodies described shows a correlation between 4-sulfatase content, structure/conformation, and the degree of severity of MPS VI clinical symptoms.

The lower reactivity of MPS VI fibroblast extracts in the quantification assay was not due to interference from stored dermatan sulfate, since the level of 4-sulfatase protein in MPS I and MPS II patient samples, which also contain high levels of partially degraded dermatan sulfate, was equal to or above that in normal controls. In some instances β-hexosaminidase activities were also elevated in MPS I, MPS II, and MPS VI fibroblasts and may reflect an increase in lysosomal enzymes involved in the degradation of the same substrate molecule. The measure of catalytic efficiency in MPS VI patients, which was similar to that of normal controls, was also consistent with MPS VI cell lines having low levels of 4-sulfatase protein. It was not consistent with the possibility of having high levels of 4-sulfatase protein, most of which eluded detection by the monoclonal antibody–quantification assay.

The product of catalytic efficiency and the cellular content of 4-sulfatase protein enabled a determination of the capacity of fibroblasts to degrade substrate. Fibroblasts from all MPS VI patients but one had extremely low catalytic capacity (<1.5% of that seen in normal control fibroblasts). The exception, patient 2357, although presenting with mild dermatansulfaturia, metachromatic inclusions in leukocytes, and a catalytic capacity for 4-sulfatase of only 5% of that seen in normal control fibroblasts, is a healthy 45-year-old with no clinical symptoms of MPS VI and can thus be considered to represent the lower limit of the normal range of catalytic capacity. The clinical variation between mildly affected patients (who, although showing some skeletal signs of the MPS VI syndrome, are all teenage or older and have relatively normal life-styles) and the remaining patients (who, in general, have a classical severe phenotype) is reflected in the range of 4-sulfatase catalytic capacity in their fibroblasts, from not detectable to only 1.4% of that seen in normal controls.

The clinical description of patient 1246, originally classified as severely affected and demonstrating anomalously high catalytic capacity, was reappraised and found to be intermediate between our arbitrary classification of severe and mild clinical phenotype. Studies of the biosynthesis of 4-sulfatase may explain the high catalytic capacity of fibroblast extracts from this patient and severely affected patient 2259. Taylor et al. (1990) have previously shown that cultured fibroblasts from eight MPS VI patients had levels of incorporation of radiolabel into immunodetectable 4-sulfatase that was <10% of that seen in normal control fibroblasts. However, although evidence for delayed maturation was a general observation, correct lysosomal targeting and maturation was inferred from the accumulation of mature 43-kDa species in most fibroblast cultures. Fibroblasts from MPS VI patient 2259 (i.e., patient PG in Taylor et al. 1990) showed both the accumulation of an apparent processing intermediate of 52 kDa and the absence of the mature 43-kDa species. Thus, this patient, although having fibroblasts with 4-sulfatase catalytic capacity similar to that seen in fibroblasts of the mildly affected patients, may have the severe clinical phenotype because the residual 4-sulfatase activity is ineffective because of incorrect subcellular location or processing. The investigation of enzyme biosynthesis in fibroblasts from patients 260 and 1246 may show defects in subcellular location or processing that are similar to those demonstrated for patient 2259. These observations are significant with regard to enzyme-replacement therapy, as they suggest that restoration of catalytic capacity alone is not sufficient to correct MPS VI—that, rather, this catalytic capacity must be correctly located within the recipient cell. Thus, when used in conjunction with cell location studies, the measure of catalytic capacity is capable of distinguishing between mild and severe MPS VI phenotypes.

The very low amounts of residual 4-sulfatase, the low level of radiolabel incorporated into 4-sulfatase in fibroblasts from all MPS VI patients examined, and the epitope detection of residual 4-sulfatase protein suggest that structurally altered enzyme is synthesized but rapidly degraded soon after insertion into the endoplasmic reticulum. Only very small amounts of enzyme, albeit with relatively normal catalytic efficiency, appear to reach the lysosome. The normal processes of protein export from the endoplasmic reticulum have been shown to be sensitive to mutations affecting pro-

tein structure or conformation and result in the rapid degradation of newly synthesized protein (Goldberg and St John 1976; Rose and Doms 1988). The apparent predominance of this type of mutation in MPS VI patients (Taylor et al. 1990) does not necessarily suggest that intracellular transport and degradation of 4-sulfatase is exquisitely sensitive to mutations affecting structure or conformation, but rather that only those mutations resulting in an almost complete depletion of the lysosomal enzyme (and thus, by inference, resulting in major structural changes) are seen in MPS VI patients. More moderate structural changes—and hence a lesser depletion of catalytic capacity—are probably not found, because, as seen in patient 2357, they do not result in an obvious MPS VI clinical phenotype.

We would predict that MPS VI patients showing near-normal or elevated levels of lysosomal 4-sulfatase protein may occur and represent structurally conservative mutations which do not alter protein conformation but which aberrate the enzyme's active site. Although analyses of fibroblasts from ~30 MPS VI patients (some data not shown) have been completed, no mutations of this type have been detected. We have, however, identified an MPS I patient who shows this type of mutation (unpublished observations). MPS VI patient 368 does, however, represent a mutation which alters substrate binding capacity—but which must also cause structural changes in the 4-sulfatase molecule, as only low levels of protein are seen in this patient.

The observation that patient 2357 has only 5% of normal 4-sulfatase catalytic capacity but no clinical signs of the MPS VI syndrome is very encouraging for the prospects of enzyme- or gene-replacement therapy. It would appear that it is necessary to increase the level of lysosomal 4-sulfatase to only 5% of that seen in normal controls to prevent the major clinical symptoms of this condition. However, as is evident from results for specific MPS VI patients (e.g., 2259), this replacement of catalytic capacity must be targeted to the correct cellular compartment. The immunoquantification method, in conjunction with the specific 4-sulfatase enzyme assay described, may, with further verification, be used to predict clinical severity in MPS VI patients, allowing the selection of individuals best suited for enzyme- and gene-replacement therapy.

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