Overexpression of *N*-acetylgalactosamine-4-sulphatase induces a multiple sulphatase deficiency in mucopolysaccharidosis-type-VI fibroblasts

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High-titre stocks of an amphotropic retrovirus, constructed so as to express a full-length cDNA encoding the human lysosomal enzyme *N*-acetylgalactosamine-4-sulphatase (4-sulphatase) from the cytomegalovirus immediate early promoter, were used to infect skin fibroblasts from a clinically severe mucopolysaccharidosis type VI (MPS VI) patient. The infected MPS VI cells showed correction of the enzymic defect with the enzyme being expressed at high levels and in the correct subcellular compartment. Surprisingly this did not result in correction of glycosaminoglycan turnover as measured by accumulation of ^{35}S in metabolically labelled cells. We demonstrate that this is apparently caused by an induced reduction of the activities of other lysosomal sulphatases, presumably due to competition for a sulphatase-specific processing mechanism by the over-expressed 4-sulphatase. The level of steroid sulphatase, which is a microsomal sulphatase, was also reduced. Infection of skin fibroblasts from a second, clinically mildly affected, MPS VI patient with the same virus also resulted in no significant change in the level of glycosaminoglycan storage. However, in this case the cause of the observed phenomenon was less clear. These results are of obvious practical importance when considering gene therapy for a sulphatase deficiency such as MPS VI and also provide possible new avenues for exploration of the processes involved in sulphatase synthesis and genetically determined multiple sulphatase deficiency.

INTRODUCTION

Multiple sulphatase deficiency (MSD) is a lysosomal storage disorder (LSD) characterized by the simultaneous deficiency of at least eight known sulphatase activities. Seven of these are lysosomal, whereas one, steroid sulphatase, is microsomal. MSD is inherited as an autosomal recessive disorder, and more than 40 cases have been identified (Kolodny, 1989). As with all of the LSD the disease is both clinically and biochemically heterogeneous. However, MSD patients have been broadly classified into two groups. Group I patients have been defined as those having less than 10% residual activity for all sulphatases and demonstrating a neonatal, clinically severe phenotype. Group II patients are those showing higher residual enzyme activities and have a correspondingly less severe clinical phenotype (Steckel et al., 1985). The significance of these divisions in terms of the biochemical defect is unclear. The biochemical defect in MSD has proved to be difficult to characterize, especially as cultured fibroblasts from MSD patients show variable expression of the phenotype over time and with differing culture conditions. Recently it has been demonstrated that sulphatases can apparently be synthesized in MSD cells with the same efficiency as in normal fibroblasts, but that the enzymes made have greatly reduced specific activities (Rommerskirch and von Figura, 1992). Normal folding and maturation of sulphatases expressed in MSD cells are strongly implied by the appearance of similar levels of fully mature enzyme in both MSD and normal fibroblasts and by the similarity of the half-life of sulphatases in MSD and normal fibroblasts (Taylor et al., 1990; Rommerskirch and von Figura, 1992). These results imply that a co- or post-translational processing step that is essential for the synthesis of enzymically active sulphatases is defective in MSD cells. However, the exact nature of this processing step is still unknown.

Mucopolysaccharidosis type VI (MPS VI), which is caused by a deficiency of *N*-acetylgalactosamine-4-sulphatase (4-sulphatase) activity, is a good model for studying the somatic defects of the mucopolysaccharidoses in particular and in LSDs in general as it typically shows development of most of the major somatic symptoms associated with these diseases (Neufeld and Muenzer, 1989). These include stiff joints, hepatomegaly, splenomegaly, nerve entrapment syndromes, cardiac valve thickening and corneal clouding. Direct involvement of the central nervous system has not been shown in MPS VI (Neufeld and Muenzer, 1989). The availability of a feline MPS VI model (Jezyk et al., 1977) also has obvious advantages in the development and evaluation of gene therapy for treatment of the symptoms associated with MPS VI and other LSDs. Such evaluation is necessary, as it is not clear how efficiently bone-marrow-derived cells will be able to deliver enzyme to all sites of pathology. As a first step towards gene therapy for MPS VI, a recombinant retroviral construct that expresses 4-sulphatase at high levels was made and introduced into fibroblasts from both a clinically severe and a clinically mild MPS VI patient. Although this results in correction of the enzymic defect, the phenotypic defect (accumulation of unmetabolized glycosaminoglycans) is unaffected. We show that this phenomenon is apparently due to an induced deficiency of other lysosomal sulphatases.

Abbreviations used: BME, basal medium (Eagle's); CHO-K1, Chinese-hamster ovary-K1; cfu, colony-forming unit; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal-calf serum; GalNAc4S-GlcA-GalitolNAc4S, *O*-(β-D-4-sulpho-2-acetamido-2-deoxygalactosyl)-(1-4)-*o*-β-D-glucuronosyl-(1-3)-*o*-β-D-6-sulpho-2-acetamido-2-deoxy-[1-^{3}H]galactitol; G418, G418 sulphate (Geneticin; Gibco); Ham's F10, F10 Nutrient Mixture (Ham); MPS, mucopolysaccharidosis; MSD, multiple sulphatase deficiency; Polybrene, hexadimethrine bromide; 4-sulphatase, *N*-acetylgalactosamine-4-sulphatase; LSD, lysosomal storage disorder.

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MATERIALS AND METHODS

Recombinant DNA techniques and Southern blotting

All DNA manipulation used standard techniques and procedures (Ausubel et al., 1989). All plasmids were grown in *Escherichia coli* DH5 cells (*F⁻ supE44 hsdR17 recA1 gyrA96 endA1 thi-1 relA1 deoR* λ^-). Plasmids for transfection into mammalian cells were purified by precipitation with poly(ethylene glycol)/NaCl and subsequent CsCl isopynic centrifugation.

Cells and cell culture

BALB 3T3 (obtained from J. Beale, Department of Biochemistry, University of Adelaide) and PA317 (Miller and Buttimore, 1986) cells were grown in high-glucose DMEM/10 % (foetal-calf serum (FCS). ψCRE (Danos and Mulligan, 1988) cells were grown in high-glucose DMEM/10 % (v/v) newborn-calf serum, except when being used for the transient expression of viral constructs, in which case they were grown in 10 % (v/v) FCS. Skin fibroblast cultures were established from skin biopsies in basal medium (Eagle's) (BME)/10 % (v/v) FCS. Skin fibroblasts were grown in Ham's F10/20 % (v/v) FCS for retroviral infection and G418 selection. G418-resistant cells were then expanded and maintained in BME/10 % (v/v) FCS unless otherwise stated. All medium was supplemented with a combination of the following antibiotics: penicillin (100 units/ml), streptomycin sulphate (100 μg/ml) and kanamycin sulphate (120 μg/ml) unless otherwise stated.

Cell line SF3168 has minimal residual 4-sulphatase activity and was derived from an MPS VI patient with a clinically severe phenotype. The second MPS VI cell line used, SF2984, contains detectable levels of 4-sulphatase activity (Table 1 below) and was derived from a MPS VI patient with a less severe clinical phenotype.

High-titre virus

To produce high-titre amphotropic virus stocks, recombinant virus produced by transient expression of plasmid DNA in ψCRE (Danos and Mulligan, 1988) cells was infected into PA317 (Miller and Buttimore, 1986) cells. At 24 h after infection, the PA317 cells were subcultured 1:10 into medium containing 1 mg/ml G418. After 9 or 10 days, individual colonies were picked using cloning rings, expanded and used for production of virus stocks. These were prepared by growing cells to confluency in 100 mm dishes then collecting the virus by the conditioning of fresh medium (5 ml) for 12–16 h. Usually several cycles of virus collection were made. Viral stocks were filtered through a 0.22 μm filter before use. All viral infections included 8 μg/ml Polybrene.

Virus titre

Virus titre was assayed by infection of BALB 3T3 cells at 60–80 % confluency with suitable dilution of viral stocks made in DMEM/10 % FCS. At 24 h after infection, the cells were subcultured 1:10 into medium containing 1 mg/ml G418; this was reduced to 0.4 mg/ml after non-infected cells had been killed (3–5 days). Colonies were scored after 9–10 days after fixation with 70 % ethanol and staining with Trypan Blue.

Helper virus

Helper virus was assayed by measuring production of G418-resistant colony-forming units (cfu) from virus-infected BALB 3T3 cells. Briefly, $> 10^6$ cfu were infected into BALB 3T3 cells and the cells propagated in 1 mg/ml G418 for 22 days. The resulting cultures (two 100 mm dishes) were then assayed for the production of G418-resistant cfu, again on BALB 3T3 cells.

Infection of skin fibroblasts

Sub-confluent cultures were infected with high-titre virus for 12–16 h, then fed with fresh medium and reinfected after a further 24 h. At 16 h after the second infection, infected cells were selected with 0.15–0.2 mg/ml G418.

4-Sulphatase activity

Cells were harvested by trypsin treatment, washed by resuspension in PBS, lysed by six cycles of freeze–thaw in 20 mM Tris/HCl (pH 7.5)/0.5 M NaCl, then clarified by microcentrifugation (12000 *g*, 4 °C, 5 min). Enzyme activity was then determined, either using the fluorogenic substrate 4-methylumbelliferyl sulphate (Gibson et al., 1987) after immune capture with the monoclonal antibody ASB4.1 (Brooks et al., 1991), or directly using the specific radiolabelled trisaccharide substrate GalNAc4S-GlcA-GalitolNAc4S (defined in the footnote) (Hopwood et al., 1986). Enzyme activity was normalized to total protein, as measured by the Bio-Rad assay kit, using BSA as a standard.

Steroid sulphatase activity

Steroid sulphatase was assayed by resuspending the pellet from the clarification of the freeze–thaw lysate (see 4-sulphatase activity) in 100 μl of 0.1 % Triton X-100 containing proteinase inhibitors (chymostatin, pepstatin, leupeptin and antipain), sonicating for 10 s and then assaying steroid sulphatase activity using the radiolabelled substrate [^{3}H]dehydroepiandrosterone sulphate, essentially as described by Eto et al. (1974). Enzyme activity was normalized to total protein in the extract, assayed by the method of Lowry et al. (1951), with BSA used as a standard.

Other enzymes

β-Hexosaminidase (*N*-acetyl-β-D-glucosaminidase) activity was measured with the fluorogenic substrate 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (Leaback and Walker, 1961). Acid phosphatase activity was assayed with the fluorogenic substrate 4-methylumbelliferyl phosphate (Kolodny and Mumford, 1976). β-Glucuronidase activity was assayed using the fluorogenic substrate 4-methylumbelliferyl β-glucuronide (Glaser and Sly, 1973). The activities of lysosomal sulphatases were assayed after dialysing the cell lysate into 5 mM sodium acetate, pH 4.0. Iduronate-2-sulphatase was assayed using the disaccharide substrate L-*O*-(α-iduronic acid 2-sulphate)-(1 → 4)-D-*O*-2,5-anhydro[1-^{3}H]mannitol 6-sulphate, as previously described (Bielicki et al., 1990). Sulphamidase (heparin-*N*-sulphatase) was assayed essentially as described by Hopwood and Elliott (1982), using 1–6 μg of cell extract and 420 pmol of substrate. *N*-Acetylglucosamine-6-sulphatase was assayed as described by Freeman and Hopwood (1989), and galactose-6-sulphatase as described by Hopwood and Elliott (1983) using the chondroitin 6-sulphate trisaccharide with 1–6 μg of cell extract and 500 pmol of substrate in 40 mM sodium acetate, pH 4.8, in a 15 μl reaction volume. All activities were standardized to mg of protein present in the cell extract, either as measured with the Bio-Rad protein assay reagent (undialysed cell extracts) or as described by Lowry et al. (1951) (dialysed cell extracts).

Analysis of glycosaminoglycan storage

Skin fibroblast cultures were grown to confluency in BME/10% (v/v) FCS then grown in Ham's F12/10% (v/v) FCS (with penicillin as the only antibiotic) for 24 h. The medium was replaced with Ham's F12/10% (v/v) FCS/10 μCi/ml $Na_2{}^{35}SO_4$, again with penicillin as the only antibiotic, for 72 h. This medium was removed, the cells washed once with PBS and fed with BME/10% (v/v) FCS. After 24 h the cells were trypsin-treated, washed in PBS by centrifugation then replated in the same medium and, after a further 72 h, harvested by trypsin treatment. Cell lysates were prepared as described above and ^{35}S measured by liquid-scintillation counting in LKB Optiphase Hisafe 3 under conditions of constant quenching. Results were standardized to total protein (Bio-Rad assay) as described for enzyme activity.

Subcellular fractionation

Cells were fractionated on Percoll gradients exactly as described previously (Anson et al., 1992a). Fractions were collected by bottom puncture and analysed for 4-sulphatase, β-hexosaminidase and acid phosphatase activity.

Molecular-mass analysis of stored material

^{35}S-labelled cell lysates were fractionated on a Sepharose CL6B column in 2 M guanidinium chloride/50 mM sodium acetate/100 mM sodium sulphate, pH 7.5 (Wasteson, 1971). Fractions were collected and analysed for ^{35}S radioactivity.

Enzyme correction of MPS VI fibroblasts

Gene-corrected MPS VI fibroblasts secrete significant amounts of 4-sulphatase. Secreted enzyme was collected by conditioning 3 ml BME/10% heat-inactivated (56 °C, 3 h) NuSerum (Collaborative Research) for 48 h. The conditioned medium was filtered through a 0.22 μm-pore-size membrane to remove cells and assayed for 4-sulphatase activity. MPS VI (SF3168) fibroblasts that had previously been metabolically labelled with ^{35}S as described in Anson et al. (1992a) were then exposed to 2500 pmol of this preparation of secreted 4-sulphatase/min per ml. The experiment was duplicated with 5 mM mannose 6-phosphate also being added to the medium. Purified human recombinant 4-sulphatase made in Chinese-hamster-ovary-K1 (CHO-K1) cells (Anson et al., 1992a) was used as a control. After the 6 h exposure to enzyme, the cells were rinsed and fed with fresh medium. After a further 24 h the cells were trypsin-treated, replated and cultured for a further 72 h, after which time they were harvested and freeze–thaw cell lysates prepared and assayed for 4-sulphatase activity and ^{35}S radioactivity, as described above.

RESULTS

Vector construction

A full-length cDNA encoding human 4-sulphatase (Anson et al., 1992a) was excised as an *Eco*RI/*Stu*I restriction fragment, made blunt-ended and cloned into the *Stu*I site of pLNCX (Miller and Rosman, 1989). Recombinants containing inserts with the correct orientation were identified by restriction-enzyme analysis. One such recombinant, designated pLNC4S (Figure 1), was chosen and used for all subsequent experiments.

High-titre virus

Plasmids were introduced into PA317 cells (as described in the Materials and methods section) and individual clones isolated and assayed for viral titre, for synthesis of human 4-sulphatase (by immune capture), for absence of helper-virus production and for the presence of a single provirus of the correct structure by Southern blotting. Two clones giving the highest titre virus in combination with the other required characteristics (expression of human enzyme, absence of helper virus, presence of a single, intact, provirus) were chosen and designated PA317/LCN4S2 and PA317/LCN4S12. These lines produced titres of 7×10^5 and 4×10^5 cfu respectively.

Infection of skin fibroblasts

Two MPS VI fibroblast lines, SF3168 and SF2984, were separately infected with virus from both PA317/LNC4S2 or PA317/LNC4S12 and G418-resistant cell populations were selected and expanded for analysis as described in the Materials and methods section.

Correction of the enzymic deficiency and glycosaminoglycan storage in gene-corrected MPS VI fibroblasts

Flasks (25 cm^2) of confluent cells were metabolically labelled and cell lysates were prepared. Cell lysates were then assayed for 4-sulphatase activity and glycosaminoglycan storage (^{35}S radioactivity). In both the MPS VI cell lines used, infection with either virus stock resulted in overexpression of 4-sulphatase at levels approx. 100 times normal (Table 1). However, this overexpression of 4-sulphatase did not result in correction of the glycos-

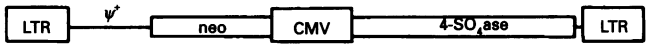

Figure 1 LNC4S retroviral construct

A diagrammatic representation of the LNC4S recombinant retroviral construct is shown. LTR indicates the retroviral long terminal repeats, ψ^+ the extended viral packaging signal, neo is the neomycin-resistance-gene sequence, CMV the cytomegalovirus immediate early promoter and 4-SO_4ase the 4-sulphatase cDNA.

Table 1 4-Sulphatase activity and [^{35}S]glycosaminoglycan accumulation in normal, MPS VI and gene-corrected MPS VI fibroblasts

The results of two individual experiments are shown. In the first (a) the results are shown for an experiment using the SF3168 cell line derived from a clinically severely affected MPS VI patient. SF3168 represents uninfected cells and SF3168/4S2 and SF3168/4S12 cells infected with virus from PA317/LNC4S2 and PA317/LNC4S12 cells respectively. A normal control SF3394, is included for comparison. In (b) the results of a similar experiment with a second MPS VI cell line, SF2984, derived from a clinically mildly affected patient, are shown. Again, a normal control (SF3521) is included for comparison. All results have been standardized to mg of total cell protein in the cell extract.

Expt.	Cell line	4-Sulphatase activity (nmol/min per mg)	$10^{-3}\times$ [^{35}S]Glycosaminoglycan radioactivity (c.p.m./mg)
(a)	SF3394 ($n^*=3$)	nd†	4.88±0.68
	SF3168 ($n=3$)	0.0034±0.0015	237±10
	SF3168/4S2 ($n=3$)	48.0±6.7	296±35
	SF3168/4S12 ($n=3$)	78.2±4.4	247±46
(b)	SF3521 ($n=4$)	0.659±0.063	5.43±1.25
	SF2984 ($n=4$)	0.012±0.004	24.9±2.1
	SF2984/4S2 ($n=4$)	107±6	22.3±3.8
	SF2984/4S12 ($n=4$)	98±5	19.3±1.1

* n, number of individual cell samples harvested for analysis.
† nd, not done.

aminoglycan storage phenotype. After infection with either virus stock, both cell lines accumulated ^{35}S label to approximately the same level as uncorrected cells. Normal fibroblasts accumulated label only at very low levels (Table 1).

4-Sulphatase from gene-corrected MPS VI cells is active *in vivo*

To demonstrate that the 4-sulphatase from gene-corrected cells was active *in vivo*, enzyme secreted by SF3168/LNC4S2 cells was collected and added back to ^{35}S-labelled (uncorrected) SF3168 cells, as described in the Materials and methods section. Enzyme purified from a CHO cell line expressing human 4-sulphatase which has been shown to have normal kinetic parameters towards both the non-specific substrate 4-methylumbelliferyl sulphate and the trisaccharide substrate GalNAc4S-GlcA-GalitolNAc4S (Anson et al., 1992a) was used as a control. The results (Table 2) show that the enzyme from gene-corrected cells is efficient in initiating turnover of stored ^{35}S glycosaminoglycan. In addition, uptake of the enzyme from gene-corrected cells is 75% inhibited by 5 mM mannose 6-phosphate (Table 2). The CHO-cell enzyme gave essentially identical results for uptake, clearance of substrate and inhibition of uptake by mannose 6-phosphate (results not shown).

Subcellular localization of 4-sulphatase in gene-corrected MPS VI cells

Cell lysates of normal fibroblasts (SF3422), and gene-corrected MPS VI fibroblasts (SF3168) infected with PA317/LNC4S2 and PA317/LNC4S12 virus, were fractionated on Percoll gradients as described in the Materials and methods section. Fractions were collected and assayed for β-hexosaminidase, 4-sulphatase and acid phosphatase. The results (Figure 2) show that the subcellular localization of 4-sulphatase in gene-corrected cells is normal, the overexpression of enzyme measured in cell lysates being reflected by the presence of large amounts of 4-sulphatase activity in the dense fractions of the gradient, co-sedimenting with the activities of β-hexosaminidase (Figure 2) and acid phosphatase (results not shown). In addition, the distribution of 4-sulphatase, β-hexosaminidase and acid phosphatase is the same as that seen for normal, uninfected cells (results not shown).

Size fractionation of stored ^{35}S glycosaminoglycan from gene-corrected MPS VI cells

The size distribution of stored glycosaminoglycan in MPS VI (SF3168) and gene-corrected (SF3168/LNC4S2 and SF3168/LNC4S12) cells was analysed by Sepharose CL6B column chromatography as described above. The MPS VI cells showed a single peak of ^{35}S-labelled material with an estimated molecular mass of 17 kDa (Figure 3). The size distribution of ^{35}S-labelled material in gene-corrected cells was obviously different with the majority of material fractionating at a larger molecular weight. This was associated with the appearance of a small peak of material at a lower molecular mass (Figure 3).

Expression of other lysosomal enzymes in gene-corrected MPS VI cells

To determine if the over-expression of 4-sulphatase in the gene-corrected MPS VI fibroblasts was altering the levels of other lysosomal enzymes, cell lysates of these cells were assayed for

Table 2 Correction of MPS VI cells by 4-sulphatase secreted by gene-corrected cells

SF3168 cells were metabolically labelled with $^{35}SO_4$ and then exposed to enzyme secreted by SF3168 cells containing the LNC4S2 virus in the absence (SF3168/4S2) or presence (SF3168/4S2/M6P) of 5 mM mannose 6-phosphate as described in the Materials and methods section. Cell lysates were then assayed for 4-sulphatase activity and ^{35}S radioactivity.

Cell line	4-Sulphatase activity (nmol/min per mg)	$10^{-3} \times {}^{35}S$ radioactivity (c.p.m./mg)
SF3168 (n* = 3)	0.007 ± 0.002	170 ± 8
SF3168/4S2 (n = 3)	1.33 ± 0.09	12.7 ± 2.5
SF3168/4S2/M6P (n = 3)	0.30 ± 0.01	37.9 ± 13.6

* *n*, number of individual samples of cells harvested and analysed.

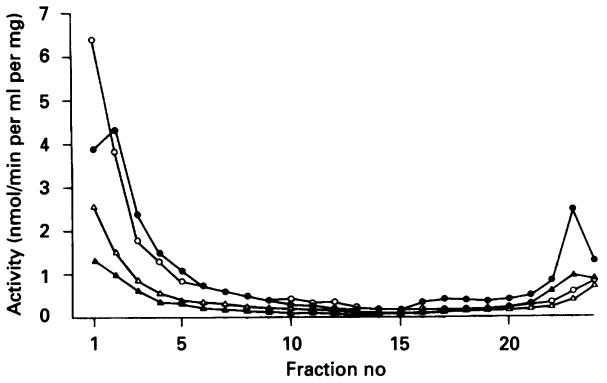

Figure 2 Subcellular fractionation of fibroblasts overexpressing 4-sulphatase

SF3168 cells infected with virus from either PA317/LNC4S2 (LNC4S2) or PA317/LNC4S12 (LNC4S12) were lysed and post-nuclear extracts fractionated on Percoll gradients as described in the Materials and methods section. Fractions were assayed for 4-sulphatase (○, LNC4S2; ●, LNC4S12) and β-hexosaminidase (△, LNC4S2; ▲, LNC4S12) activity and the results corrected for the amount of cell extract loaded on to the gradient. Fraction 1 represents the bottom of the gradient, fraction 24 being at the top.

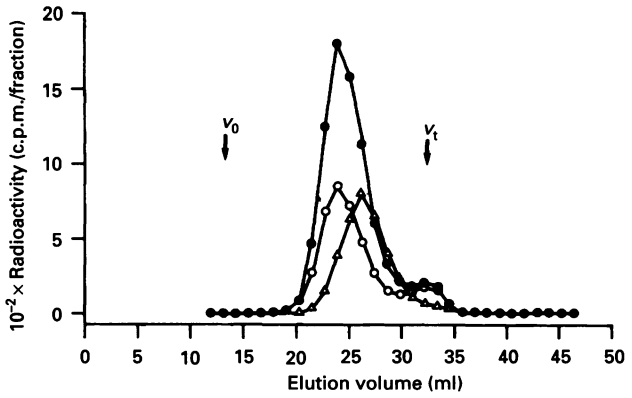

Figure 3 Sepharose CL6B chromatography of ^{35}S-labelled material from gene corrected and uncorrected MPS VI cells

Cell lysates from uncorrected MPS VI cells (SF3168, △) and gene-corrected MPS VI cells (○, SF3168/4S2; and ●, SF3168/4S12) were fractionated on Sepharose CL6B as described in the Materials and methods section. V_0 indicates the column void volume, and V_t indicates the column total volume.

Table 3 Expression of other enzymes

Cell extracts were assayed for β-hexosaminidase (β-Hex), β-glucuronidase (β-Gluc), acid phosphatase (AcPh), iduronate-2-sulphatase (I2S), galactosamine-6-sulphatase (Gal-6-Sase), glucosamine-6-sulphatase (Gluc-6-Sase), sulphamidase (Sulph) and steroid sulphatase (STS) as described in the Materials and methods section. The units for β-hexosaminidase, β-glucuronidase and acid phosphatase activities are given as nmol/min per mg of total cell protein, all other enzymes are pmol/min per mg. *n* Refers to the number of individual samples of cells used for analysis. The range and mean of values for normal fibroblasts is also given along with the number of individual normal lines (NL) assayed.

		Enzyme activity							
Cell line	*n*	β-Hex	β-Gluc	AcPh	I2S	Gal-6-Sase	Gluc-6-Sase	Sulph	STS
SF3168	4	211±72	12.2±1.7	29.5±14	227±14	82.2±6.6	11.0±0.3	128±4	119±24
SF3168/4S2	4	88.4±15.5	7.50±0.74	25.8±2.3	30.1±9.9	23.8±5.4	2.90±0.53	9.43±0.78	5.55±0.60
SF3168/4S12	4	127±16	7.23±0.72	27.9±0.6	39.2±4.3	44.4±4.0	6.58±1.24	27.1±3.5	7.20±1.07
SF2984	4	72.3±1.6	2.69±0.17	29.0±1.0	106±12	57.6±16.8	8.10±0.72	70.4±6.7	142±45
SF2984/4S2	4	80.1±5.5	2.16±0.17	27.1±2.3	65.4±5.5	49.0±3.5	7.48±0.87	48.5±7.5	158±28
SF2984/4S12	4	69.7±7.2	1.84±0.21	25.6±1.7	61.7±3.6	60.6±17.5	7.88±0.96	48.5±2.0	128±20
Normal range...		60.2–160	6.22–11.5	19.8–38.7	125–256	88–209	7.5–16.7	58–250	41.3–110
Normal mean...		96.2±21.8	7.92±2.06	29.2±6.1	185±45	151±61	12.1±4.0	148±91	69.1±36.2
NL assayed...		*n*=7	*n*=5	*n*=6	*n*=7	*n*=5	*n*=6	*n*=5	*n*=3

various lysosomal enzymes (Table 3). In SF3168 cells, infection of the LNC4S virus results in a significant decrease of β-hexosaminidase (approx. 2-fold) and β-glucuronidase (approx. 1.6-fold); however, owing to the higher-than-normal expression of these enzymes in uninfected SF3168 cells, this results in normal levels of these enzymes. In SF2984 the level of β-hexosaminidase is not affected upon infection with the LNC4S virus, whereas the level of β-glucuronidase is slightly reduced. The levels of acid phosphatase are not significantly affected in any of the cells upon infection with the LNC4S virus.

By contrast, infection of the LNC4S virus into SF3168 cells (clinically severe MPS VI) causes a moderate to severe reduction in the level of activity of other sulphatases (Table 3). The microsomal steroid sulphatase is most affected, being reduced more than 16-fold, to levels of less than 10% of the mean normal value (compared with 172% of the normal mean in control uninfected SF3168 cells). Sulphamidase and iduronate-2-sulphatase are also very significantly reduced. The levels of sulphamidase are reduced 5–14-fold (to 18 and 7% of the normal mean value) compared with uninfected SF3168 cells, and iduronate-2-sulphatase 6–7-fold (to 21 and 16% of the normal mean value). The levels of all three of these enzymes in LNC4S-infected SF3168 cells is reduced below the normal range. The levels of galactosamine-6-sulphatase and glucosamine-6-sulphatase are less affected, being reduced 2–3-fold and 1.5–4-fold respectively. However, the levels seen in the LNC4S-infected SF3168 cells are again below the range of values seen in the normal cells assayed.

In SF2984 (clinically mild MPS VI) a less pronounced and less consistent reduction in the level of sulphatases is seen upon infection with LNC4S virus (Table 3). Only iduronate-2-sulphatase and sulphamidase are consistently and obviously reduced, the levels of reduction being 1.5-fold compared with uninfected cells.

DISCUSSION

We have demonstrated that infection of MPS VI cells with recombinant retrovirus designed to express high levels of 4-sulphatase, the enzyme deficient in MPS VI, results in over-expression of 4-sulphatase and hence correction of the enzymic defect. The enzyme is correctly targeted to the lysosome (Figure 2) and is active towards both the fluorogenic and trisaccharide substrates *in vitro*. This confirms the data, presented by Peters et al. (1991), showing enzyme correction of gene transfected MPS VI fibroblasts. However, we have demonstrated that the over-expression of 4-sulphatase does not lead to correction of the storage phenotype exhibited by MPS VI cells. The 4-sulphatase made by the gene-corrected cells was shown to be active *in vivo* by the observation that conditioned medium from these cells, which contains significant amounts of 4-sulphatase, is able to correct both the enzymic defect and to initiate turnover of stored glycosaminoglycan in MPS VI cells (Table 2). The uptake of enzyme in this experiment was also inhibited by 5 mM mannose 6-phosphate to the same extent as control enzyme, indicating that the enzyme has the correct mannose 6-phosphate residues for lysosomal targeting.

In the case of the gene-corrected SF3168 cells from a clinically severely affected MPS VI patient, it is clear that the over-expression of 4-sulphatase from the LNC4S virus induces a deficiency of the other sulphatases that were assayed. Although different sulphatases are affected to various extents, the effect is both obvious and consistent (Table 3). The level of acid phosphatase is unaltered in infected cells; however, the levels of β-hexosaminidase and β-glucuronidase, which are elevated above normal levels in SF3168 cells, are reduced to levels within the normal range in the LNC4S-infected SF3168 cells. The reduction in β-hexosaminidase and β-glucuronidase can be explained by competition for the mannose 6-phosphate lysosomal-transport pathway. We have observed a similar effect when expressing high levels of α-L-iduronidase in MPS I cells (Anson et al., 1992b). Acid phosphatase is not affected by overexpression of mannose-6-phosphate targeted enzymes, as it has an alternative and independent lysosomal targeting signal. However, the reduced levels of sulphatases in LNC4S-infected cells cannot be explained by competition for the mannose 6-phosphate pathway; the reduction in sulphatase levels seen is much greater than seen for β-hexosaminidase and β-glucuronidase, and steroid sulphatase, which is a microsomal, as opposed to a lysosomal sulphatase, is also affected. Analysis of the size distribution of ^{35}S-labelled glycosaminoglycan in SF3168 and LNC4S infected SF3168 cells by Sepharose CL6B chromatography (Figure 3) strongly suggests that the storage product seen in the gene-corrected cells is not the same as in the uninfected control SF3168 cells. These results indicate that the failure to correct the storage phenotype in LNC4S-infected SF3168 cells is most probably due to an induced

deficiency of lysosomal sulphatases, other than 4-sulphatase, rather than a lack of effective 4-sulphatase activity within the lysosome.

The situation in SF2984 cells (derived from a clinically mildly affected MPS VI patient) is less clear; again infection of the LNC4S virus results in high level expression of 4-sulphatase, with no correction of glycosaminoglycan storage levels. However, of the sulphatases assayed, only iduronate-2-sulphatase and sulphamidase are significantly reduced, both being reduced about 1.5-fold in cells containing the LNC4S virus compared with uninfected SF2984 cells. In addition, β-glucuronidase is also significantly reduced in LNC4S-infected cells. It is noteworthy that uninfected SF2984 cells contain levels of all the lysosomal enzymes assayed, with the exception of acid phosphatase, that are near or below the lower limit of the range of activities seen in normal cells. These cells may therefore be more susceptible to a further small decrease in enzyme levels. It is apparent from studies of genetic MSD that multiple enzyme deficiencies need not be extreme to cause clinical and biochemical disease (Kolodny, 1989).

These results would appear to indicate that the maturation of sulphatases involves a biosynthetic step that is sulphatase-specific and is of a limited capacity such that overexpression of one of these sulphatases (4-sulphatase) can, at least in some cells, severely reduce the levels of other sulphatases. It has previously been proposed that such factors exist and that deficiencies of such factors are responsible for some, if not all, cases of MSD. We are currently examining the observed phenomenon in more detail and in a variety of cell types. Initial results show that, although overexpression of 4-sulphatase from the LNC4S virus in normal skin fibroblasts significantly reduces the levels of other sulphatases, it does not lead to the generation of a detectable storage phenotype. Other factors, presumably related to the disease phenotype of the MPS VI cells, are therefore likely to be involved in generating the results we have observed. Expression of α-L-iduronidase from analogous retroviral constructs in MPS I cells (Anson et al., 1992b) also resulted in a reduction in the level of a lysosomal sulphatase (iduronate-2-sulphatase). However, this did not appear to be a specific effect, being paralleled by reductions in similar magnitude of other, non-sulphatase, lysosomal enzymes. This effect is most easily explained by the saturation of the mannose 6-phosphate-dependent lysosomal-transport system. Further experiments will hopefully allow us to explain more completely, and to characterize, the relationship between MPS disease and the effects of 4-sulphatase overexpression on glycosaminoglycan storage and activities of other sulphatases.

The results described here also have clear implications for the treatment of MPS VI and other lysosomal sulphatase deficiencies in particular, and all metabolic diseases in general. It is clear that correction of the enzymic defect does not result implicitly in correction of the associated phenotype and that constructs will have to be designed so that enzyme expression is controlled between certain defined limits. While some degree of overexpression is probably required to ensure efficient delivery of enzyme, via the circulation, to cells that have not received the gene construct, too high a degree of overexpression is likely to result in a new storage disease in the cells receiving the construct.

We thank Ms. Sophie Lazenkas and Ms. Peta Knapman for expert assistance with word processing of the manuscript, Dr, Doug Brooks for the monoclonal antibody ASB4.1, and Mrs. Kathy Nelson for assistance with cell culture. This work was supported by an NH and MRC program grant.

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Received 11 January 1993/2 April 1993; accepted 27 April 1993