Immortalization and characterization of a cell line exhibiting a severe multiple sulphatase deficiency phenotype

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Multiple sulphatase deficiency (MSD) is a rare genetic defect that causes a simultaneous deficiency of all known sulphatases. All available evidence suggests that the deficient gene product is normally responsible for the post-translational modification of a conserved cysteine residue to 2-amino-3-oxopropionic acid and that this modification is necessary for sulphatase activity. MSD often has an enzymically mild phenotype, with significant levels of residual sulphatase activity being detectable. Here we identify an MSD cell line in which the residual activity of the sulphatases assayed was generally very low. To characterize the phenotype of this cell line further, immortalized lines were established after transformation with simian virus 40 (SV40) T antigen. Immortalized cell lines representing normal and MSD phenotypes were then transduced with a retroviral vector carrying the gene

encoding human *N*-acetylgalactosamine-4-sulphatase. Analysis of *N*-acetylgalactosamine-4-sulphatase protein synthesis and enzyme activity showed that transduced cell lines expressed large amounts of enzyme and that the specific activity of this enzyme was approx. 0.5–1.5 % of normal, confirming that this cell line defines a severe phenotype for MSD. *N*-Acetylgalactosamine-4-sulphatase purified from a transduced MSD cell line seemed normal on denaturing PAGE. Kinetic analysis of the purified enzyme suggests that the residual activity is due to small amounts of normal enzyme rather than unmodified enzyme with low levels of residual activity. These cell lines and the availability of large amounts of inactive *N*-acetylgalactosamine-4-sulphatase from MSD cells should facilitate the further study of this disorder.

INTRODUCTION

Mammalian cells contain at least 12 sulphatase genes. Evolutionarily the sulphatases are well conserved in organisms as diverse as prokaryotes, lower eukaryotes and humans. The sulphatases that correspond to nine of the human sulphatase genes have been extremely well characterized. Eight of these sulphatases are localized in the lysosome and are involved in the degradation of glycosaminoglycans. The other, steroid sulphatase, is microsomal. Each of these enzymes demonstrates a highly restricted specificity for a corresponding natural substrate structure, although many have reduced specificities for artificial substrates such as 4-methylumbelliferyl sulphate (4-MUS, defining arylsulphatase activity), a commonly used fluorogenic substrate. The remaining three genes (arylsulphatases D, E and F) were recently cloned as candidate chondrodysplasia punctata genes [1] and identified as sulphatases via their sequence similarity to known sulphatases. The arylsulphatase E gene was shown to encode an arylsulphatase activity at neutral pH; however, the endogenous substrate is not known.

The vital role that these sulphatases have in human metabolism is exemplified by the corresponding genetic diseases caused by deficiencies of sulphatases, for example any one of the storage disorders corresponding to deficiency of a lysosomal sulphatase [2], X-linked ichthyosis [3] caused by a deficiency of steroid sulphatase, and chondrodysplasia punctata [1] which has been linked to mutations in the arylsulphatase E gene. There is also a rare disease, multiple sulphatase deficiency (MSD) [4], in which the activity of all sulphatases is reduced. Patients with

MSD have been classified into two groups on the basis of the level of residual sulphatase activities. Group I patients have less than 10 % of normal activity of all sulphatases, whereas group II patients have residual activities of up to 90 % of normal. Storage of ³⁵SO₄²⁻-labelled material has been demonstrated in MSD; the degree of storage reflects the severity of the enzymic defect [5]. It is now clear that the primary defect in MSD is in a gene product that is involved in the co-translational or post-translational modification of a conserved cysteine residue found in all mammalian sulphatases so far described [6]. This cysteine residue is normally modified to a 2-amino-3-oxopropionic acid residue [7]: it is this modification that is deficient in MSD cells. The lack of this modification seems to alter the specific activity of the enzyme rather than its stability, processing or compartmentalization within the cell [8].

In this paper we describe the immortalization and characterization of a severe MSD cell line. This cell line represents group I MSD and has little detectable residual sulphatase activity. The lysosomal sulphatase *N*-acetylgalactosamine-4-sulphatase (Gal4S) was overexpressed in one of the immortalized cell lines generated and purified in microgram amounts. Purified Gal4S from the overexpressing cell line was characterized by physical and kinetic analysis.

MATERIALS AND METHODS

Materials

All media were purchased from Gibco-BRL. Foetal calf serum (FCS), trypsin/versene and antibiotics were from CSL Ltd. BSA,

Abbreviations used: CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; Gal4S, *N*-acetylgalactosamine-4-sulphatase; Gal6S, *N*-acetylgalactosamine-6-sulphatase; Glc6S, *N*-acetylglucosamine-6-sulphatase; IDS, iduronate-2-sulphatase; 4-MUS, 4-methylumbelliferyl sulphate; MSD, multiple sulphatase deficiency; SV40, simian virus 40; VNTR, variable number tandem repeat.

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human serum albumin, Polybrene and 4-MUS were from Sigma. *Taq* polymerase and dNTP species were from Boehringer Mannheim. $^{35}\text{SO}_4^{2-}$ was from NEN–DuPont, and horseradish peroxidase-conjugated sheep anti-(mouse IgG) antisera were from Silenus.

Cell culture, transfection/transduction and immortalization

SF4007 (normal control) and SF3931 (MSD) cells were from patient samples submitted to the Women's and Children's Hospital (North Adelaide, South Australia) for diagnosis. Cells were grown in either basal modified Eagle's medium/10% (v/v) FCS containing penicillin, or high-glucose Dulbecco's modified Eagle's medium (DMEM)/10% (v/v) FCS containing penicillin and streptomycin. Skin fibroblasts were transfected with the construct pRSVT, which expresses the SV40 large T and small T antigens under the transcriptional control of the Rous sarcoma virus long terminal repeat [9], by using Lipofectin (Gibco Life Sciences). Morphologically transformed clones were isolated after 3–4 weeks by using cloning rings, and expanded.

Immortalized cell lines were transduced with the recombinant retrovirus LNC4S (which expresses Gal4S from the cytomegalovirus immediate early promoter) [10] as follows. Cells were exposed to three cycles of supernatant (0.2 μm filtered) from the PA317/LNC4S.12 amphotropic retrovirus producer cell line in the presence of 8 $\mu\text{g}/\text{ml}$ Polybrene. Mass cultures of transduced cells were selected with 100–200 $\mu\text{g}/\text{ml}$ G418. Secretion of Gal4S was assessed by growing the cells to confluency in T25 cm^2 flasks (triplicate cultures for each cell line) and then replacing the medium with 5 ml of fresh medium. Samples of 0.5 ml were collected every 24 h for 4 days and stored at 4 °C until assayed. Samples were microcentrifuged (12000 g at 4 °C for 5 min) immediately before analysis to remove cell debris and were assayed for Gal4S protein by ELISA [11] and total arylsulphatase activity with the 4-MUS substrate [12]. Clonal cell lines were subsequently isolated (KD3/LNC4S cells only) after serial dilution in 100 mm dishes and isolation of individual colonies with cloning rings. To assay for secretion of Gal4S, clones were grown to confluency in T25 cm^2 flasks; the medium was aspirated and replaced with 5 ml of fresh medium. After 72 h the medium was collected, filtered through a 0.2 μm membrane and stored at 4 °C until analysed for Gal4S by ELISA or enzyme (4-MUS) assay.

PCR variable number tandem repeat (VNTR) analysis

Cells were typed for the apolipoprotein B 3' hypervariable region as described by Boerwinkle et al. [13].

Cytogenetic analysis

Metaphase cells were examined with standard cytogenetic techniques.

Analysis of enzyme expression in cell lysates

Cell lysates were prepared by six cycles of freezing and thawing in 0.25 M NaCl/20 mM Tris/HCl (pH 7.0) followed by microcentrifugation (12000 g at 4 °C for 5 min) to remove cell debris. Gal4S activity was assayed with the trisaccharide substrate as described by Hopwood et al. [14], iduronate-2-sulphatase (IDS) activity as described by Bielicki et al. [15], *N*-acetylgalactosamine-6-sulphatase (Gal6S) as described by Bielicki et al. [16] and sulphamidase as described by Bielicki et al. [17]. *N*-Acetylglucosamine-6-sulphatase (Glc6S) was assayed with the radiolabelled disaccharide substrate *O*-(α -*N*-acetylglucosamine-

6-sulphate)-(1 $\rightarrow$ 3)-L-[6- ^3H]idonic acid [18] with the following reagent mixture: 1 μl of disaccharide substrate (to give a final concentration of 17 μM), 3 μl of 0.1 M 3,3-dimethylglutaric acid, pH 6.0, 1 μl of 6 mg/ml BSA and 1 μl of cell lysate that had been dialysed against 5 mM sodium acetate, pH 4.0. β -Hexosaminidase was assayed as described by Leaback and Walker [19]. The activity of each enzyme was corrected to total cell protein as measured with the Lowry assay [20] and human serum albumin as a standard.

Analysis of $^{35}\text{SO}_4^{2-}$ -labelled glycosaminoglycan storage

Cells were grown to confluency in triplicate T25 cm^2 flasks. The medium was then aspirated from the flask, the cells rinsed with PBS and the medium changed to Ham's F12/10% (v/v) FCS (without antibiotics). After 4 h the medium was exchanged for Ham's F12/10% (v/v) FCS/5 $\mu\text{Ci}/\text{ml}$ $^{35}\text{SO}_4^{2-}$ (3 ml per flask) and the cells were incubated for 65 h. The medium was then aspirated; the cells were rinsed with DMEM/10% (v/v) FCS and then re-fed with DMEM/10% (v/v) FCS. After 32 h the cells were harvested and cell lysates were prepared by freeze/thaw (see above) and analysed for ^{35}S c.p.m., total cell protein (Bio-Rad assay with BSA as a standard), total arylsulphatase (4-MUS) activity [12] and β -hexosaminidase activity [19].

Western blot analysis of SV40 large T expression

Cells were harvested from confluent T25 cm^2 flasks, washed in PBS by resuspension/centrifugation and finally resuspended in 50 μl of water and lysed by sonication. The total protein content of each lysate was determined (Bio-Rad assay, BSA standard) and a 50 μg portion was subjected to electrophoresis on an SDS/PAGE [21] minigel [12% (w/v) gel] under denaturing and reducing conditions. Prestained molecular mass markers (Bio-Rad low molecular mass) were included. After electrophoresis the gel was electroblotted on nitrocellulose membrane (Bio-Rad Transfer-blot, 0.45 μm pore size) in 25 mM Tris base/192 mM glycine/20% (v/v) methanol.

After transfer the membrane was blocked with 1% (w/v) non-fat milk in 10 mM Tris/HCl (pH 7.0)/0.125 M NaCl at room temperature for 2 h and then incubated with 10 $\mu\text{g}/\text{ml}$ anti-(SV40 T antigen) monoclonal Ab-1 (Oncogene Science) in the same buffer at room temperature for 2 h. The membrane was subsequently washed three times in 10 mM Tris/HCl (pH 7.0)/0.125 M NaCl before being incubated in 1% (v/v) horseradish peroxidase-conjugated sheep anti-mouse polyclonal antisera (Silenus) in 1% (w/v) non-fat milk in 10 mM Tris/HCl (pH 7.0)/0.125 M NaCl for 2 h. The membrane was then washed three times in 10 mM Tris/HCl (pH 7.0)/0.125 M NaCl and developed with 4-chloro-1-naphthol substrate (Bio-Rad) in accordance with the manufacturer's instructions.

Large-scale production and purification of Gal4S

KD3/LNC4S.12 cells were inoculated into a two-layer (1500 cm^2) cell factory (Nunc) and grown to confluency. Once the culture had reached confluency it was maintained in DMEM/1% (v/v) heat-inactivated FCS. The medium (approx. 250 ml) was exchanged every 3 days. Harvested medium was centrifuged (2000 g for 10 min at 4 °C) and filtered through a sterile filter (0.22 μm pore size) to remove cell debris. Sodium azide was added to 0.02% and the medium stored at 4 °C until processing. Immunoaffinity purification of Gal4S on an (anti-Gal4S) 58.3 monoclonal antibody column was essentially as described by Crawley et al. [22], with a 2 ml column. After elution the recovered enzyme was concentrated with an Amicon Centricon

10 concentrator and dialysed into 50 mM sodium acetate (pH 5.6)/125 mM NaCl. Enzyme was then quantified with the Lowry assay [20] with human serum albumin as a standard.

SDS/PAGE and kinetic analysis of purified MSD Gal4S

Purified MSD Gal4S was analysed under denaturing and reducing conditions by SDS/PAGE [21] with a 12% polyacrylamide minigel. Low-molecular-mass markers (Pharmacia) were included. The gel was stained with Coomassie Blue R250.

Kinetic data (K_m , V_{max} and pH optimum) for recombinant human Gal4S and MSD Gal4S with the 4-MUS and trisaccharide substrates were obtained as described previously [12,14] with substrate concentrations of 0.5–5 mM for 4-MUS and 7.5–53 μ M for the trisaccharide. K_m and V_{max} were calculated from Lineweaver–Burk plots.

RESULTS

Immortalization of SF4007 and SF3931

Transfection of both SF3931 (MSD) and SF4007 (normal control) with pRSVT resulted in the isolation of a large number

of morphologically transformed clones from each cell line. Eight SF3931-derived clones and five SF4007-derived clones were expanded. All clones reached crisis before immortalization occurred. Five SF3931 clones immortalized and have been successfully grown for 100 generations. These cell lines have been designated KD1, KD2, KD3, KD5 and KD7. KD3 seemed the most vigorous of these cell lines. Expansion of SF4007 transformed clones was severely limited by the onset of crisis: only one line became immortalized. This line has been designated KD8 and has also been successfully grown for 100 generations.

Western blot analysis of SV40 T antigen expression

Western blot analysis of SF3931, SF4007, KD1, KD2, KD3, KD5, KD7 and KD8 (Figure 1) demonstrated that all the immortalized (KD) cell lines expressed both large and small SV40 T antigen. Small T antigen consistently migrated as a doublet of equal-intensity bands of approx. 20–21 kDa. In KD1, 2 and 3, large T antigen migrated predominantly as a single band of approx. 94 kDa, although smaller minor bands were also visible. In KD5, 7 and 8 large T antigen migrated predominantly as a doublet. T antigen was not detectable in the untransformed lines SF3931 and SF4007 from which the immortalized cell lines were derived.

Genetic and cytogenetic characterization of immortalized clones

VNTR analysis with the apolipoprotein B 3' hypervariable region was used to analyse the genetic relationship between parental and immortalized cell lines. This analysis showed that SF3931 and KD1, 2, 3, 5 and 7 carried identical alleles (results not shown), confirming the origin of the immortalized (KD) MSD cell lines. Similarly SF4007 and KD8 were shown to carry identical alleles, again confirming the origin of the immortalized cell line.

Cytogenetic analysis of the cell lines showed that SF3931 and SF4007 had normal male and female karyotypes respectively. All of the immortalized cell lines showed variable chromosome numbers and multiple chromosomal rearrangements. The chromosome number in KD1, 2, 5 and 7 was approximately 2N, whereas in KD3 and 8 it was approximately 3N.

Enzymic characterization of immortalized clones

SF3931, SF4007 and KD1, 2, 3, 5, 7 and 8 were analysed for expression of β -hexosaminidase, Gal4S, Gal6S, sulphamidase,

Figure 1 Western blot analysis of SV40 T antigen

Total cell lysates were analysed by Western blot analysis with a monoclonal antibody that recognizes both SV40 large and small T antigen as described in the Materials and methods section. Lane 1, molecular mass markers (values shown in kDa at the left); lane 2, SF3931 (untransformed primary MSD cell line); lane 3, KD1 (MSD immortalized cell line); lane 4, KD2 (MSD immortalized cell line); lane 5, KD3 (MSD immortalized cell line); lane 6, KD5 (MSD immortalized cell line); lane 7, KD7 (MSD immortalized cell line); lane 8, SF4007 (untransformed primary normal control cell line); lane 9, KD8 (normal control immortalized cell line).

Table 1 Enzymic analysis of cell lines

All enzyme activities are in pmol/min per mg of total cell protein with the exception of β -hexosaminidase (β -Hex), which is in nmol/min per mg of total cell protein. Results are means $\pm$ S.D. for three individual experiments.

Cell line	β -Hex activity (nmol/min per mg)	Enzyme activity (pmol/min per mg)				
		Gal4S	Gal6S	Sulphamidase	IDS	Glc6S
SF3931	78.7 $\pm$ 5.34	0.89 $\pm$ 1.44	0.16 $\pm$ 0.28	2.86 $\pm$ 0.34	4.86 $\pm$ 0.20	0.18 $\pm$ 0.32
KD1	65.6 $\pm$ 0.77	0.42 $\pm$ 0.10	1.10 $\pm$ 0.19	4.89 $\pm$ 0.13	1.67 $\pm$ 1.19	0.11 $\pm$ 0.05
KD2	40.1 $\pm$ 1.53	0.15 $\pm$ 0.17	3.12 $\pm$ 0.24	0.85 $\pm$ 0.02	1.56 $\pm$ 0.10	0.11 $\pm$ 0.06
KD3	83.1 $\pm$ 1.98	0.09 $\pm$ 0.08	0.25 $\pm$ 0.03	0.37 $\pm$ 0.03	0.75 $\pm$ 0.35	0.16 $\pm$ 0.02
KD5	52 $\pm$ 0.46	0.18 $\pm$ 0.31	1.07 $\pm$ 0.14	0.04 $\pm$ 0.05	0.83 $\pm$ 0.21	0.05 $\pm$ 0.01
KD7	49.6 $\pm$ 1.73	0.20 $\pm$ 0.18	1.04 $\pm$ 0.18	0.27 $\pm$ 0.06	1.56 $\pm$ 0.09	0.07 $\pm$ 0.01
SF4007	47.3 $\pm$ 1.86	344 $\pm$ 21.1	399 $\pm$ 28	354 $\pm$ 44	24.1 $\pm$ 0.57	16.9 $\pm$ 0.18
KD8	53.2 $\pm$ 2.32	53.2 $\pm$ 4.0	505 $\pm$ 29	160 $\pm$ 34	18.3 $\pm$ 0.57	6.79 $\pm$ 0.49

Table 2 Storage of $^{35}\text{SO}_4^{2-}$ -labelled material in cell lines

Results are means $\pm$ S.D. for three individual experiments. Abbreviations: n.d., not detected; β -Hex, β -hexosaminidase.

Cell line	^{35}S (c.p.m./mg)	Enzyme activity (nmol/min per mg)	
		β -Hex	4-MUS
SF4007	$(3.98 \pm 0.22) \times 10^4$	57.0 ± 4.5	1.34 ± 0.34
SF3931	$(8.98 \pm 0.40) \times 10^5$	78.5 ± 12.8	n.d.
KD8	$(1.45 \pm 0.28) \times 10^4$	41.4 ± 4.9	0.209 ± 0.022
KD3	$(4.34 \pm 0.20) \times 10^5$	105.6 ± 19.3	n.d.

IDS and Glc6S (Table 1). The results show that in SF3931 (MSD) and the immortalized cell lines derived from SF3931 (KD1, 2, 3, 5 and 7) the level of each sulphatase, with the exception of IDS, is decreased to 1%, or less, of that in the normal control (SF4007 and KD8). The level of IDS in the MSD cell lines is somewhat higher at approx. 3–5% of normal, although this in part represents a level of IDS in SF4007 (24.1 pmol/min per mg) and KD8 (18.3 pmol/min per mg) that is at the lower end of the normal range (14–153 pmol/min per mg). These results clearly show that SF3931 displays a severe type I MSD phenotype and that the immortalization of this cell line does not alter the phenotype displayed. The expression of a non-sulphatase lysosomal enzyme, β -hexosaminidase, was elevated in SF3931 in comparison with SF4007 and in some of the immortalized MSD cell lines (KD1 and 3) in comparison with the normal immortalized cell line (KD8) but does not seem to be significantly affected by the immortalization process. Of the enzymes assayed only Gal4S seems to be significantly altered by the immortalization process, being decreased to approximately one-seventh in KD8 in comparison with SF4007.

Storage of $^{35}\text{SO}_4^{2-}$ -labelled material in immortalized clones

To characterize the phenotype of SF3931 further, storage of $^{35}\text{SO}_4^{2-}$ -labelled material in SF3931, SF4007 and two representative immortalized lines, KD3 (MSD) and KD8 (normal) was analysed. The results (Table 2) show that the two MSD lines, SF3931 and KD3, each accumulated $^{35}\text{SO}_4^{2-}$ -labelled material at more than 20 times the relevant normal control (SF4007 and KD8 respectively). The MSD lines also showed an elevation in β -hexosaminidase activity compared with the controls, this being more pronounced with the immortalized lines than with the original cultures. As expected, total arylsulphatase activity was not detectable in SF3931 or KD3. The total arylsulphatase activity of KD8 was approximately 16% of SF4007, reflecting the decrease in Gal4S activity noted in KD8 (see above and Table 1).

Overexpression of Gal4S

To characterize the defect in SF3931 more accurately, the immortalized cell lines KD3 (MSD) and KD8 (normal control) were transduced with a retroviral construct, LNC4S [10], in which Gal4S expression is under the transcriptional control of the CMV immediate early promoter. Neomycin-resistant cells were isolated, and conditioned medium from triplicate cultures of untransduced and transduced KD3 and KD8 cells was assayed for Gal4S protein by ELISA and for total arylsulphatase activity as a measure of Gal4S activity. Mass cultures of both KD3 and KD8 cells transduced with LNC4S secreted high levels of Gal4S

Figure 2 Overexpression of Gal4S in KD3 and KD8

(a) Secretion of Gal4S protein assayed by ELISA into the medium of KD3 cells (■), KD3/LNC4S cells (▲), KD8 cells (□; obscured by KD3 (■) data points) and KD8/LNC4S cells (×). (b) Secretion of arylsulphatase activity into the medium of KD3/LNC4S cells (◆) and KD8/LNC4S cells (■). Results are means $\pm$ S.D. ($n = 3$).

protein as assayed by ELISA (Figure 2a); however, significant Gal4S (arylsulphatase) activity was detected only in conditioned medium from the transduced KD8 (immortalized normal control) culture (Figure 2b). The results show that Gal4S from the KD8/LNC4S culture has normal or near-normal specific activity and that the degree of overexpression of Gal4S resulting from transduction with the LNC4S virus has not exceeded the cells' ability to modify the enzyme correctly. An extremely small amount of activity was detectable in the KD3/LNC4S culture medium. Untransduced KD3 and KD8 cells did not secrete detectable amounts of arylsulphatase activity. Cell lysates were not tested for Gal4S activity. Isolation and characterization of individual KD3/LNC4S clones allowed the identification of an MSD cell line, KD3/LNC4S.12, that accumulated Gal4S at levels of approx. 1 mg/l in conditioned medium after 3 days.

Purification and characterization of Gal4S from MSD cells

MSD Gal4S from approx. 800 ml of conditioned medium from the KD3/LNC4S.12 cell line was purified on a 58.3 immunoaffinity column as described in the Materials and methods section.

Figure 3 SDS/PAGE analysis of purified MSD Gal4S

MSD Gal4S immunoaffinity purified from the KD3/LNC4S.12 cell line was analysed by SDS/PAGE under denaturing and reducing conditions as described in the Materials and methods section. Lane 1, molecular mass markers (values shown in kDa at the left); lane 2, 4 μ g of purified MSD Gal4S; lane 3, 8 μ g of MSD Gal4S.

Table 3 Kinetic analysis of purified MSD Gal4S

See the Materials and methods section for details. Abbreviation: rhGal4S, recombinant human Gal4S expressed in CHO cells [23].

Substrate	Enzyme	K_m (μ M)	V_{max} (nmol/min per mg)	Specific activity (nmol/min per mg)	pH optimum
Trisaccharide	rhGal4S	21.43	916	594	2.85
	MSD Gal4S	27.10	11.6	7.3	2.85
4MUS	rhGal4S	1600	63361	49759	5.6
	MSD Gal4S	4150	374	203.2	5.6

The MSD Gal4S was bound to and eluted from the matrix in a manner identical to that of normal [recombinant human enzyme from Chinese hamster ovary (CHO) cells] Gal4S. The final yield of Gal4S after purification, concentration and dialysis was approx. 600 μ g. SDS/PAGE analysis of the purified Gal4S (Figure 3) showed the presence of both precursor (66 kDa) and mature (43 and 8 kDa) bands. No other bands were detected, indicating that the material had been purified to homogeneity. Analysis of the activity of the purified enzyme towards both the 4-MUS fluorogenic substrate and the trisaccharide substrate showed that the enzyme had specific activities of 0.4% and 1.3% of normal towards these two substrates respectively (Table 3). Analysis of the K_m and pH optimum of the MSD enzyme (Table 3) towards both substrates showed these parameters to be not significantly different from those for recombinant human Gal4S from CHO cells [23]. The MSD Gal4S showed a decreased V_{max} towards both substrates exactly paralleling the decreased specific activity of the enzyme.

DISCUSSION

MSD has a spectrum of clinically and enzymically distinguishable phenotypes. The milder forms are defined by a later onset of clinical symptoms and relatively high levels of residual sulphatase activity, and correspondingly low levels of storage in cultured fibroblasts. The more severe, neonatal, form of MSD is charac-

terized by low or undetectable levels of residual sulphatase activity and high levels of storage. However, it is not clear whether or not neonatal MSD includes examples of a null phenotype.

In this paper a neonatal MSD cell line, SF3931, and a normal control cell line, SF4007, were immortalized after being transformed by expression of SV40 T antigen. SF3931 seemed to transform and immortalize in a manner indistinguishable from that of the normal control; five immortalized MSD cell lines and one immortalized normal cell line were isolated. All expressed SV40 large and small T antigen and showed karyotypes typical of immortalized human fibroblast cell lines. Enzyme analysis and VNTR analysis confirmed the phenotypic and genetic integrity of the immortalized cell lines with respect to the parental cell lines.

Enzymic analysis of a range of sulphatases suggested that SF3931 defines an exceptionally severe MSD phenotype. To define more accurately the MSD phenotype in these cells, Gal4S was overexpressed in KD3 cells and purified from the culture medium. The purified Gal4S was analysed by SDS/PAGE and found to have the same subunit structure as human Gal4S purified from liver or expressed in CHO cells. The MSD Gal4S had residual activities of approx. 0.4% and 1.3% of normal towards the 4-MUS and trisaccharide substrates, respectively. The apparently normal K_m values and pH optima of the purified MSD Gal4S suggest that this residual activity is due to a small amount of normal, properly modified Gal4S rather than residual activity in the unmodified Gal4S. This suggests that the modification of the conserved cysteine residue to 2-amino-3-oxopropionic acid in sulphatases is an absolute requirement for enzymic activity.

Co-culture of the KD3/LNC4S cell line with MPS VI fibroblasts and mixing experiments with MPS VI cell lysates and purified MSD Gal4S did not produce any convincing evidence for correction of the MSD phenotype as measured by Gal4S activity (results not shown).

In conclusion, the KD3/LNC4S.12 cell line should prove useful in further characterizing MSD, for example by allowing production of MSD Gal4S for three-dimensional structural analysis and as a possible substrate for developing an assay for the activity involved in producing the 2-amino-3-oxopropionic acid residue that is deficient in MSD cells.

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