Advantages of Using Same Species Enzyme for Replacement Therapy in a Feline Model of Mucopolysaccharidosis Type VI*

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In a feline model of mucopolysaccharidosis type VI (MPS VI), recombinant feline N-acetylgalactosamine-4-sulfatase (rf4S) administered at a dose of 1 mg/kg of body weight, altered the clinical course of the disease in two affected cats treated from birth. After 170 days of therapy, both cats were physically indistinguishable from normal cats with the exception of mild corneal clouding. Feline N-acetylgalactosamine-4-sulfatase was effective in reducing urinary glycosaminoglycan levels and lysosomal storage in all cell types examined except for corneal keratocytes and cartilage chondrocytes. In addition, skeletal pathology was nearly normalized as assessed by radiographic evidence and bone morphometric analysis. Comparison of results with a previous study in which recombinant human 4S (rh4S) was used at an equivalent dose and one 5 times higher indicated that rf4S had a more pronounced effect on reducing pathology than the same dose of rh4S, and in some instances such as bone pathology and lysosomal storage in aorta smooth muscle cells, it was as good as, or better than, the higher dose of rh4S. We conclude that in the feline MPS VI model the use of native or same species enzyme for enzyme replacement therapy has significant benefits.

The lysosomal storage disorders are a group of inherited metabolic diseases included among which are the mucopolysaccharidoses (MPS).† Each disorder results from the primary deficiency of a single gene product, which, in each of the MPS, is a specific enzyme involved in the degradation of glycosaminoglycans (GAG). In MPS VI (Maroteaux-Lamy syndrome) the enzyme deficient is N-acetylgalactosamine-4-sulfatase (4S; EC 3.1.6.12) (1). 4S hydrolyzes the 4-sulfated ester bond on nonreducing terminal N-acetylgalactosamine residues and hence is involved in the degradation of the GAG dermatan sulfate (DS) and chondroitin-4-sulfate. Failure to degrade these GAGs results in the storage of partially catabolized DS and chondroitin-4-sulfate in lysosomes, leading to progressive organ dysfunction and clinical symptoms such as skeletal abnormalities including epiphyseal dysplasia, dwarfism, and facial dysmorphism, as well as widespread soft tissue pathology (e.g., corneal clouding, hepatosplenomegaly, joint stiffness, and heart valve thickening). Patients with a severe MPS VI phenotype generally die from cardiac and respiratory failure in the first to second decade of life. Unlike many of the MPS, there is no obvious central nervous system involvement in MPS VI.

Present treatment of MPS patients is mostly limited to medical and surgical intervention aimed at alleviating symptoms when they occur. Bone marrow transplantation has been extensively evaluated for some MPS types and is currently considered of clinical benefit for MPS I and VI if treatment is commenced early (2, 3). However, it is clearly not totally effective. The fact that bone marrow transplantation is generally administered after diagnosis of clinical symptoms may provide one explanation for its limited effectiveness in modifying bone and central nervous system pathology.

Besides bone marrow transplantation, enzyme replacement therapy (ERT) is the only current therapeutic treatment for lysosomal storage disorders. Its therapeutic success has been exemplified in the treatment of nonneuronopathic Gaucher disease, where it has transformed the management of this disorder (4). A crucial requirement for ERT is the availability of relatively large amounts of enzyme. This has been made possible by the application of recombinant DNA technology. Recombinant enzymes produced in mammalian expression systems have the mannose 6-phosphate recognition signal required for targeting to the lysosome via the mannose 6-phosphate receptor (5). This receptor is present on the plasma and endosomal membranes of most cell types. Exogenous enzyme is rapidly endocytosed to lysosomes by cultured lysosomal storage disorder cells and is effective in removing stored substrate (6–10).

A number of inherited lysosomal enzyme deficiencies have been reported in animals, and these have been exploited to explore possible therapeutic strategies. ERT, for example, has been shown to be effective in several of these animal models of lysosomal storage disorders including the MPS VII mouse (11), the MPS I dog (12, 13), and the MPS VI cat (14–16). The MPS VI cat is an excellent large animal model for studying the somatic pathology associated with the MPS and particularly for the skeletal abnormalities that are a predominant feature of this disorder. In the published studies of ERT in MPS VI cats (14–16), recombinant human 4S (rh4S) was used. A correlation between earlier onset of therapy and a trend toward normal-
ization of bone mineral volume in vertebral bone samples was observed, and in general, the cats appeared more mobile and in general, improved quality of life. MPS VI kittens treated for 6 months from birth with various doses of rh4S ranging from 0.2 to 5 mg/kg body weight showed a marked reduction in skeletal pathology, which was dose-dependent. Urinary GAG levels and lysosomal storage in most organs were nearly normalized. However, no discernible reduction in lysosomal vacuolation in cartilage, cornea, or leukocytes was apparent.

In this paper, we present an evaluation of the efficacy of species-specific ERT in an MPS VI cat model and compare this with a previous study that utilized the human equivalent of this enzyme. Our results indicate that treatment of MPS VI cats with recombinant feline 4S (rf4S) is significantly more efficacious than treatment with rh4S at the same dose.

**EXPERIMENTAL PROCEDURES**

**Enzyme Production and Purification**—Cloning of the 4S gene and its expression in Chinese hamster ovary cells are detailed by Yogalingam et al. (17). For large scale production of rh4S, cells were grown to confluence on Cytodex 2 microcarriers (Amersham Pharmacia Biotech, Uppsala, Sweden) at 3 g/liter in 12 liters of 10% (w/v) fetal calf serum/Dulbecco’s modified Eagle’s medium/COON’s F12 medium (CSSL Ltd., Parkville, Victoria, Australia). Once confluence was attained, the culture medium was changed by rapid perfusion to Dulbecco’s modified Eagle’s medium/COON’s F12 12 medium supplemented with 0.5 mM butyric acid and 5 mM NH4Cl to enhance enzyme production (18, 19). The production phase of this fermentation was run as a 12-liter stirred continuous culture with perfusion carried out at approximately 0.2 volumes/106 cells/ml/day. The culture dissolved oxygen concentration (measured in situ with an Ingold oxygen probe) was monitored and controlled with FC4 software (Real Time Engineering, Sydney, Australia) and maintained at approximately 40% saturation with sparged oxygen. Culture pH, maintained at 7.4 with sparged CO2, was also measured in situ with an Aquadyn pH probe (Hantzsch: Pty, Ltd., Sydney, Australia) and monitored by the above software. The addition of alkali or antifoam was unnecessary. Collected culture supernatant was stored at 4 °C, with the addition of 0.1% (w/v) sodium azide, until use. The rf4S was purified from culture medium by immunoaffinity chromatography using Affi-Gel 10 linked to monoclonal antibody F58.3, which recognizes both human and feline 4S (20). The enzyme was assessed for purity by SDSPAGE (12% (w/v) acrylamide) according to the method of Laemmli (21), its activity determined with the fluorogenic substrate 4-methylumbelliferyl sulfate as described previously (22) with the only modification being that 0.05% (v/v) Tween 20 was included in the diluent. Protein concentration was determined using the Lowry method (23). The enzyme for the trial was produced in several batches, which were pooled and then stored in aliquots in PBS under sterile conditions and at 4 °C until use. The isoelectric point (pI) of rh4S and rf4S was determined on 5% (w/v) acrylamide gels with 2% (v/v) ampholytes (BioLyte® 3/10 amphotolytes, Bio-Rad) at 1300 V for 4 h using a Bio-Phoresis Horizontal Electrophoresis Cell (Bio-Rad) according to the manufacturer’s instructions and utilizing reagents supplied with the PNGase F. Duplicate samples were treated in a similar manner but without the addition of PNGase F. The concentration of PNGase F used was 500 units/10 μg of 4S, and reaction mixtures were incubated at 37 °C for 4 h. All samples were then analyzed by SDS-PAGE according to the method of Laemmli (21).

**Experimental Animals**—Two male MPS VI kittens born 1 week apart, the result of heterozygote matings from a naturally occurring feline model of MPS VI (24), were designated cat 249 and cat 250. They were diagnosed at birth by lack of stained eosinophil inclusions on blood films (15). Cat genotype status was later confirmed by polymerase chain reaction-based mutation analysis from blood spots (17).

**ERT Protocol**—The protocol followed in this study is essentially documented in Crawley et al. (15). Briefly, therapy was commenced within 24 h of birth by intravenous injection of 1 mg of rh4S/kg of body weight on a weekly basis for approximately 170 days. Throughout the course of therapy, the volume of enzyme injected ranged from 0.06 ml to 1.9 ml, and this was generally administered over 5–10 min. To maintain control over the antihostibody response, cats were subjected to a rigorous clinical, neurological, and radiological examination as described in Crawley et al. (15). Blood was collected in heparinized tubes for determination of antibody titer to rf4S in plasma using an enzyme-linked immunosorbent assay as follows. The wells of a 96-well microtiter plate were coated with 200 ng of rf4S/well (37 °C for 2 h and then overnight at 4 °C), after which blocking of any unreactive sites was done by the addition of 1% (w/v) bovine serum albumin in 20 mM Tris-HCl, pH 7.0 (blocking buffer) (2 h at 20 °C). After washing the plate with Tris/NaCl buffer, cat plasma was serially diluted in blocking buffer and allowed to incubate on the plate for 4 h at 20 °C followed by peroxidase-labeled goat anti-cat Ig (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 1 h at 20 °C. The assay was developed using peroxidase substrate solution (Bio-Rad ABTS substrate kit), and the color reaction was quantified by measuring the absorbance at 405 nm on a microplate reader (Ceres 900 HDi, Bio-Tek Instruments Inc.). As a control measure, all cat plasma was analyzed for nonspecific reactivity to wells coated with bovine serum albumin only. Immunoblotting of enzyme after electrophoresis onto nitrocellulose membranes (Bio-Rad) and detection with cat plasma samples followed by peroxidase-labeled goat anti-cat Ig and peroxidase substrate were performed as described previously (25).

Urine samples were collected at the specified times and stored at 20 °C without preservative until assayed for GAG and uronic acid concentration. Total urinary GAG was measured using a modified Alcian Blue spectrophotometric method (26). To determine uronic acid content of GAG, urine was adjusted with 50 mM sodium acetate buffer to pH 5 and chromatographed over an anion exchanger (DEAE Sephadex A-25, Amersham Pharmacia Biotech) equilibrated in 50 mM sodium acetate buffer, pH 5. Under these conditions, sulfated GAG bound to the matrix and were subsequently eluted with 2 M NaCl in acetate buffer. After desalting on Sephadex G-10 (Amersham Pharmacia Biotech) equilibrated in 0.1 M ammonium formate buffer, pH 6, the concentration of uronic acid in these charged GAG species was estimated using the dihydroxynaphthalen method (27). GAG from urine samples processed in this manner was used to generate PAGES (30–40% gradient of acrylamide) loading approximately 1 μg of uronic acid/fane (28). Standards comprising tetra-, hexa-, and octasaccharides prepared from bovine lung heparin were a generous gift from Dr. J. Turnbull (University of Birmingham, United Kingdom). The gel was stained with silver nitrate, and particular bands as indicated in Fig. 3 were analyzed by densitometry using a Molecular Dynamics Personal Densitometer with associated software. Image analysis of bone levels was measured using an autoanalyzer method (Synchron CX® System, Beckman Instruments, Inc., Fullerton, CA). To estimate relative proportions of DS in total urinary GAG, High Resolution Electrophoresis of cetlypyridinium chloride-precipitated GAG on cellulose acetate strips was performed, and the results were quantified by densitometry (29).

**Bone Morphometric Analysis**—To label newly mineralized bone, animals were injected subcutaneously with the fluorochrome dye calcein at a dose of 15 mg/kg on days 12 and 13 prior to euthanasia and with oxytetracycline on days 2 and 3 before euthanasia. At autopsy, the fifth lumbar vertebra (L5) was removed, Wrightained, wrapped in 0.9% (w/v) NaCl-soaked gauze, and stored at −80 °C until morphometric analysis. After thawing, the L5 vertebra was fixed in 10% (v/v) buffered formalin and dehydrated in increasing concentrations of acetone (70%–100%) before embedding in methylmethacrylate resin. 5-μm undecalcified tissue sections were cut and stained with von Kossa stain to visualize mineralized bone. An automated image analysis system was used to quantify trabecular bone in the primary center of ossification. Static parameters of bone formation including bone mineral volume (BV/TV), bone surface (BS/TV), trabecular thickness (TbTh), trabecular number (TbN), and trabecular separation (TsSp) were calculated using in situ morphometric formulae (30, 31). Dynamic parameters of bone formation (mineral apposition rate (MAR) and bone formation rate (BFR/BS)) were determined from unstained L5 vertebral sections by measuring the distance between fluorochrome dyes (calcein (green) and oxytetracycline (orange)) under UV light (420 nm) using a routine manual point counting technique. Standard formulae were then applied.
to calculate MAR and BFR/BS (30, 31), and results were compared with data from a previous study in which animals were injected with rh4S (16).

**Radiological Examination**—Standard radiographs (14) were taken at 90 and 150 days and before euthanasia (170 days) to visualize bone growth and remodeling. Measurements of L5 vertebrae of male cats were made from radiographs taken on the 150th day of treatment rather than from the final radiographs at 170 days in order to allow comparison with data from the cats treated at 5 mg of rh4S/kg (therapy in 5% of these cats was terminated at 150 days, (15)). Vertebral width (W) was measured with vernier callipers at the narrowest point of the vertebra. At the midway point of this measurement and perpendicular to it, the distance between the epiphyses was designated the vertebral length (L). Ratios of L/W were calculated from these values.

**Pathology**—Following a clinical, neurological, and radiological examination, cats were killed with an overdose of intravenous barbiturate 4 days after the final injection of rf4S. On autopsy, gross examination of tissues and joints was conducted, and samples of these tissues for electron microscopy and light microscopy were fixed and processed as described previously (15). To determine the degree of vacuolation in the cells of tissues, 1-μm sections were stained with toluidine blue, assessed at × 100–400 magnification, and graded subjectively as follows: 3+, severe degree of lysosomal storage; 2+, moderate; 1+, mild; 0, no vacuolation. The spinal cord was exposed and examined for compression by removal of dorsal vertebral arches between the fourth cervical and fourth lumbar vertebra.

**Residual 4S Activity in Liver**—Liver samples were frozen at −20 °C before processing. Samples of liver (1–2 g) were thawed, minced, and then homogenized for approximately 1–2 min on ice in twice their volume of 20 mM Tris-HCl, 0.25 M NaCl buffer, pH 7, using an Ultra T25-Turrax homogenizer (Janke & Kunkel, IKA®Labotechnik, Staufen, Germany). The homogenate was freeze/thawed six times and clarified by centrifugation at 13,000 × g for 10 min at 4 °C, and the resulting supernatant was dialyzed overnight at 4 °C against 50 mM sodium formate buffer, pH 3.5, before being assayed using radiolabeled trisaccharide substrate (22). Randomly selected cat livers were treated in the same way. Cat status, age at euthanasia, and the time in storage of the liver sample at −20 °C (shown in parentheses) were as follows: normal cat, 1 day old (8 months), two untreated MPS VI cats, 6 months (23 months) and 6 months (28 months), two MPS VI cats treated with 1 mg of rh4S/kg, 6 months (32 months) and 6 months (38 months), and two MPS VI cats treated with 5 mg of rh4S/kg, 5 months (33 months) and 5 months (55 months). Livers from cats 249 and 250 were stored at −20 °C for 6 months before assaying for 4S activity. Results of assays were normalized to total cell protein as estimated by the Lowry method (23).

To assess treated cats for hepatomegaly, wet weights of liver expressed as a percentage of total body weight at autopsy were compared with those of normal, untreated MPS VI and MPS VI rh4S-treated age- and sex-matched animals (two male MPS VI cats treated with 5 mg of rh4S/kg and euthanized at 5 months were included in this group). The specific activity of rf4S purified for this trial was approximately 62,500 nmol/min/mg using 4-methylumbelliferyl sulfate. Enzyme activity was stable at 4 °C in PBS with no evidence of loss of activity throughout the trial. IEF indicated seven major isoforms of rf4S and more than 10 of rh4S with pI ranging from 6.5 to 7.5 for rf4S and a much broader range of 4.45–7.0 for rh4S (Fig. 1A). Digestion of both enzymes with neuraminidase resulted in a change in pI of certain isomers of rf4S; however, no obvious changes were observed for rh4S. PNGase F digestion of rf4S resulted in a decrease in M₉ of both the precursor and mature forms by 14 and 11%, respectively. A higher degree of glycosylation was observed for rh4S, since the change in M₉ was greater than for rf4S (22% for the precursor form and 20% for the mature form) (Fig. 1B).

**RESULTS**

**Enzyme Characterization**

The specific activity of rf4S purified for this trial was approximately 62,500 nmol/min/mg using 4-methylumbelliferyl sulfate. Enzyme activity was stable at 4 °C in PBS with no evidence of loss of activity throughout the trial. IEF indicated seven major isoforms of rf4S and more than 10 of rh4S with pI ranging from 6.5 to 7.5 for rf4S and a much broader range of 4.45–7.0 for rh4S (Fig. 1A). Digestion of both enzymes with neuraminidase resulted in a change in pI of certain isomers of rf4S; however, no obvious changes were observed for rh4S. PNGase F digestion of rf4S resulted in a decrease in M₉ of both the precursor and mature forms by 14 and 11%, respectively. A higher degree of glycosylation was observed for rh4S, since the change in M₉ was greater than for rf4S (22% for the precursor form and 20% for the mature form) (Fig. 1B).

**Enzyme Replacement Therapy in Feline MPS VI**

**Clinical Examination**—Injections of enzyme were tolerated well by both cats, and neither showed any adverse reactions typical of anaphylaxis at any stage of therapy. Throughout the course of therapy, they progressively gained weight, cat 249 being at the top end of the range for normal cats and cat 250 at the lower end (Table 1). The two cats were very active and playful; however, mild gait changes were observed in cat 250 in the last 2 weeks of therapy. No hind limb neurological deficits were detected in either cat. After approximately 3 months of age, untreated MPS VI cats exhibited decreased levels of activity and had a high incidence of hind limb neurological deficits. Mild corneal clouding was present in both, and cat 250 had very mild kyphosis of the thoracolumbar spine. Both cats were otherwise difficult to distinguish physically from age-matched normal controls.

**Urinary GAG**—A rapid decrease in total urinary GAG concentration as estimated by the Alcian Blue test was seen in cats 249 and 250 were diagnosed at birth by the presence of nonstaining eosinophils on dried blood films. Diagnosis was confirmed by polymerase chain reaction analysis, which indicated both cats were homozygous for the severe L476P mutation (17). Throughout therapy, the persistence of heavy neutrophil granulation, nonstaining eosinophils, and vacuolated lymphocytes in blood was noted.

**Disease Assessment in MPS VI Cats Undergoing ERT**

**Diagnosis of MPS VI Status**
both animals with increasing age (Fig. 2). This reduction in GAG is a natural phenomenon and is observed in both normal and untreated MPS VI cats. However, the absolute level of urinary GAG in untreated MPS VI cats remains elevated from birth to 6 months by about 4-fold compared with normal cats. Within 25 days of treatment with rf4S, the concentration of urinary GAG was approximately halved compared with untreated MPS VI controls; however, this is still double the level found in normal control cats. Between 25 and 45 days of therapy, the concentration of GAG in cats treated with rf4S dropped rapidly to 1.5 times normal or 28% of MPS VI levels. Between 90 and 170 days of therapy, it stabilized at 41% of MPS VI levels or 1.7 times normal levels.

High resolution electrophoresis of GAG when analyzed for DS indicated that although %DS fluctuated, overall treatment resulted in a reduction of DS compared with untreated MPS VI levels (Table II).

Analysis of uronic acid content of urinary GAG by gradient PAGE followed by silver nitrate staining and densitometric analysis indicated that the ratio of specific bands (A:B and C:D; Fig. 3 and Table III) was clearly altered in MPS VI samples in comparison with normal. Treatment with rf4S resulted in almost complete normalization of the banding pattern. The effect was definitely much more pronounced than 1 mg of rh4S/kg and only slightly different from the 5 mg of rh4S/kg dose. However, overall the concentration of charged saccharides in urine was still higher in each treatment group compared with that in normal cats.

Antibody Response—No significant antibody response to rf4S was detected in plasma from cat 249 either by enzyme-linked immunosorbent assay or Western blot, and a very low titer (1 in 5,100) was observed for cat 250 (results not shown). Western blot analysis of plasma from cat 250 showed a weak response, which did not change from day 90 to euthanasia (results not shown). Preimmune serum was not available to test for background reactivity. The protocol used for determining antibody titer was validated using plasma from cat A, which had a titer to rh4S of 1 in 512,000 (cat A was treated with rh4S from 7 months of age (14)). This titer was identical to that obtained by a previous method (25).

Macroscopic Pathology—Gross examination of soft tissues of cats 249 and 250 at autopsy revealed no abnormalities. Neither cat showed any sign of spinal cord compression. Examination of

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**TABLE I**

| Body weight (grams) of male MPS VI cats undergoing ERT compared with untreated male MPS VI and normal male control cats age 90, 150, and 170 days |
|---|---|---|
| 90 days | 150 days | 170 days |
| Normal | 1690 ± 38 (n = 13) | 2955 ± 220 (n = 5) | 3422 ± 215 (n = 15) |
| MPS VI (no ERT) | 1472 ± 223 (n = 10) | 2114 ± 259 (n = 10) | 2305 ± 292 (n = 9) |
| MPS VI (1 mg of rf4S/kg) | 1976 (2145, 1806) (n = 2) | 3068 (3403, 2732) (n = 2) | 3072 (3448, 2696) (n = 2) |
| MPS VI (5 mg of rh4S/kg) | 1524 ± 158 (n = 3) | 2744 ± 341 (n = 3) | 2740 (n = 1) |
| MPS VI (1 mg of rh4S/kg) | 1446 ± 271 (n = 3) | 2603 (2855, 2350) (n = 2) | 2836 (2941, 2731) (n = 2) |

**Fig. 2.** Urinary GAG excretion in MPS VI untreated (●), MPS VI treated with 1 mg of rf4S/kg (○), MPS VI treated with 5 mg of rh4S/kg (○), MPS VI treated with 1 mg of rh4S/kg (▲), and normal (●) cats from birth to 6 months. Error bars, 1 S.D. Points with no error bars indicate n < 3.
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TABLE II
Comparison of the percentage of DS in total urinary GAG of cats on ERT with untreated MPS VI cats at different ages

| Age (days) | MPS VI (no ERT) | MPS VI (1 mg of rh4S/kg) | MPS VI (1 mg of rh4S/kg) | MPS VI (5 mg of rh4S/kg) |
|-----------|----------------|--------------------------|--------------------------|--------------------------|
|           | %              | %                        | %                        | %                        |
| 23        | 40.8 ± 4.8     | 36.6 (37.2, 35.9)        | 37.8 ± 14.1              | 19.4 (19.1, 19.6)        |
| 49        | 52.4 ± 5.0     | 34.0                     | ND                       | ND                       |
| 90        | 61.9 ± 8.2     | 41.6 (39.2, 44.0)        | 37.8 ± 5.3               | 25.8 ± 7.1               |
| 150       | 58.9 ± 3.7     | 35.2 (40.2, 36.1)        | 41.9 (41.7, 42.0)        | 37.2 ± 12.9              |
| 170       | 62.8 ± 3.8     | 36.9 (35.4, 38.3)        | 59.3 (61.7, 56.9)        | ND                       |

*a: n = 1.
b: ND, not determined.

TABLE III
Comparison of ratios of sulphated uronic acid-containing GAG fragments isolated from urine of normal, untreated, and treated MPS VI cats after gradient PAGE

| Age | MPS VI (1 mg of rh4S/kg) | MPS VI (5 mg of rh4S/kg) |
|-----|--------------------------|--------------------------|
|     | A:B                      | C:D                      |
|     | OCT:HEX                  | TET:HEX                  |
| 250 | 63:37 (73:27)            | 68:32 (78:22)            |
| 249 | 64:36 (68:32)            | 70:30 (74:30)            |
| 250 | 60:40 (70:30)            | 67:33 (71:33)            |
| 249 | 64:36 (70:30)            | 77:23 (80:26)            |
| 250 | 46:54 (52:48)            | 52:48 (56:48)            |
| 249 | 44:56 (49:51)            | 52:48 (56:48)            |

*ND, not determined.

Fig. 3. Gradient PAGE of GAG isolated from urine of normal, untreated, and treated MPS VI cats. Lanes 1 and 2, cat 249 and cat 250, respectively; lane 3, MPS VI untreated; lane 4, MPS VI treated with 1 mg of rh4S/kg; lane 5, MPS VI treated with 5 mg of rh4S/kg; lane 6, normal urine; lane 7, heparin oligosaccharide standards. A and B as well as C and D indicate pairs of bands for which densitometric ratios were obtained.

FIG. 4. Comparison of ratios of sulphated uronic acid-containing GAG fragments isolated from urine of normal, MPS VI-untreated and MPS VI-treated cats after gradient PAGE

A:B and C:D are ratios of pairs of bands (Fig. 3) analyzed by densitometry. For details, see “Experimental Procedures.”

Resultants were expressed as the mean ± S.D., where the number of cats *(n)* is ≥3. Where *n* = 2, the mean value is given with the individual values shown in parentheses.

| Age         | MPS VI (no ERT) | MPS VI (1 mg of rh4S/kg) | MPS VI (1 mg of rh4S/kg) | MPS VI (5 mg of rh4S/kg) |
|-------------|----------------|--------------------------|--------------------------|--------------------------|
|             | %              | %                        | %                        | %                        |
| 23          | 49.0 ± 4.8     | 36.6 ± 37.2, 35.9        | 37.8 ± 14.1              | 19.4 (19.1, 19.6)        |
| 49          | 50.4 ± 5.0     | 34.0                     | ND                       | ND                       |
| 90          | 61.9 ± 8.2     | 41.6 (39.2, 44.0)        | 37.8 ± 5.3               | 25.8 ± 7.1               |
| 150         | 58.9 ± 3.7     | 35.2 (40.2, 36.1)        | 41.9 (41.7, 42.0)        | 37.2 ± 12.9              |
| 170         | 62.8 ± 3.8     | 36.9 (35.4, 38.3)        | 59.3 (61.7, 56.9)        | ND                       |

*a: n = 1.
b: ND, not determined.

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Table V; Ref. 16). MAR, however, is not significantly different in normal and MPS VI trabecular bone (16), suggesting that MPS VI osteoblasts are capable of depositing a matrix with the potential for mineralization. As expected, MAR in all treatment groups was also similar to normal. Furthermore, although the

joints showed an overall decrease in articular cartilage thickness and improved subchondral bone quality; however, significant joint pathology similar to that observed in untreated MPS VI controls was still present. Abnormalities included superficial erosions of the articular cartilage on both femoral heads (cat 249 only) and fissures in the glenoid surfaces of both scapulae in cat 250.

Light and Electron Microscopy—The L5 vertebra was used for histomorphometric analysis of trabecular bone. MPS VI cats are severely osteopenic, as indicated by measurement of bone mineral volume, expressed as a percentage of tissue volume (BV/TV), the bone mineral volume of MPS VI cats being only 20% of normal (16). Treatment with 1 mg of r4S/kg resulted in an increase of bone volume to 77% of normal (Fig. 6, Table V). A similar trend was observed for bone surface density (BS/TV, 98% of normal) as well as TbTh (80% of normal) and Tbn and ThSp (both 98% of normal) upon treatment with 1 mg of r4S/kg. Treatment with the feline enzyme appeared to have a greater effect on reversing these static parameters of bone pathology compared with the same dose of human enzyme, the latter resulting in a BV/TV of 40% of normal; BS/TV, TbTh, and Thn approximately 60% of normal; and TbSp about twice normal. Indeed, the feline enzyme was more effective than a 5 times higher dose of human enzyme (BV/TV with 5 mg of rh4S/kg was 71% of normal; BS/TV, TbTh, and Thn were 85% of normal; and TbSp was 22% greater than normal (16)). Over-all, r4S-treated MPS VI animals have static bone histomorphometric parameters that all approach normal.

As previously reported (16), BFR/BS is also severely affected in MPS VI cats (14% of normal). This dynamic parameter also showed a markedly improved response to therapy with r4S in comparison with human 4S, being corrected to 75% of normal, 1.7 times better than either dose of human enzyme (Fig. 6, Table V; Ref. 16). MAR, however, is not significantly different in normal and MPS VI trabecular bone (16), suggesting that MPS VI osteoblasts are capable of depositing a matrix with the potential for mineralization. As expected, MAR in all treatment groups was also similar to normal. Furthermore, although the
number of osteoblasts was different in normal and MPS VI trabecular bone, when normalized to bone surface area, the difference was not significant (16), indicating that recruitment of osteoblasts from mesenchymal precursor cells is unaffected in MPS VI. These data suggest that other factors such as the rate of production of extracellular matrix, its organization, and/or its modification may be abnormal in MPS VI. In general, bone morphometric data reinforced the view that treatment with feline enzyme resulted in a trend toward normalization of bone quality that according to most parameters was better than the 5 times dose of the human enzyme.

**Radiological Examination—**Analysis of radiographs demonstrated reduced skeletal pathology in rf4S-treated cats compared with untreated MPS VI controls. Changes included increased length and improved shape of vertebrae, more uniform bone density, and smooth subchondral bone surfaces in long bone epiphyses. Improvements in skeletal appearance in rf4S-treated cats based on L5 vertebral L:W ratios were not substantially different from those observed in rh4S-treated cats (Table VI). Cats on rf4S therapy showed an improvement in L:W ratio up to 81% of normal with rf4S, indicating that remodeling of vertebrae had occurred. However, although the radiological appearance was not that different among all treated groups, the quality of bone did appear to be markedly

![Image](image-url)
improved in rf4S-treated cats and those treated with 5 mg of rh4S/kg (Fig. 6, Table V; Ref. 16).

Residual Enzyme Activity in Liver—4S activity as determined by assay with a specific radiolabeled substrate was detected in liver homogenates of treated MPS VI cats compared with untreated MPS VI cats, which had less than 1% of normal activity (Table VII). Interestingly, the mean level of activity of 4S in cats treated with f4S, although higher than that seen in a normal cat, was nevertheless lower than the equivalent dose of human enzyme, which in turn was about 4 times lower than the 5 mg of rh4S/kg dose regimen.

Statistical analysis of liver wet weight data (Table VIII) indicated that male MPS VI cats have livers that are 24% larger than normal age- and sex-matched controls and that this difference is statistically significant. Furthermore, all treatments (1 and 5 mg of rh4S/kg and 1 mg of rf4S/kg) in male cats resulted in a decrease in liver size to a value not statistically different from normal male cats but significantly different from untreated MPS VI male cats. Despite the high degree of storage in liver Kupffer cells and in contrast to human MPS VI, hepatomegaly does not present as an obvious clinical symptom in the MPS VI cat. However, these results indicate that MPS VI cats do have significantly enlarged livers, and treatment by enzyme replacement has resulted in the complete elimination of storage with a concomitant decrease in liver size. This is not unexpected, since distribution studies using radiolabeled rh4S...
TABLE VII

Residual 4S activity in liver homogenates of normal, untreated, and treated MPS VI age-matched male cats

Results are normalized to total cell protein; the mean of two measurements is shown; individual results for each liver homogenate are given in parentheses.

| Treatment          | 4S activity (pmol/min/mg total protein) |
|--------------------|----------------------------------------|
| Normal             | 7.37                                   |
| MPS VI (1 mg of rf4S/kg) | 12.81 (7.43, 18.18)                   |
| MPS VI (1 mg of rh4S/kg) | 24.08 (29.84, 18.32)                  |
| MPS VI (5 mg of rh4S/kg) | 92.10 (120.92, 63.28)                 |
| MPS VI (no ERT)     | 0.057 (0.06, 0.053)                   |

TABLE VIII

Comparison of liver size of normal and MPS VI treated and untreated male cats

For details, see "Experimental Procedures."

Results are expressed as mean ± S.D. n represents the number of cats in each group.

| Condition                  | Liver size as a percentage of total body weight |
|----------------------------|-----------------------------------------------|
| Normal (n = 5)             | 3.01 ± 0.597                                  |
| MPS VI, no ERT (n = 9)     | 3.850 ± 0.767                                 |
| Treated MPS VI (n = 8)     | 2.998 ± 0.496                                 |

a Significant difference at p < 0.05, normal vs. MPS VI untreated.
b Significant difference at p < 0.05, MPS VI treated vs. MPS VI untreated.

and rf4S (14) demonstrate that over 50% of injected enzyme is localized to liver after 4 h. Furthermore, results of residual 4S activity in liver after therapy indicated elevated levels of enzyme activity, which in the case of rh4S was approximately proportional to dose.

DISCUSSION

In this study, we have shown that treatment of MPS VI cats with rf4S at 1 mg/kg over approximately 170 days of life resulted in a dramatic alteration to the clinical course of the disease. Treated cats showed a reduction in total urinary GAG and in particular DS excretion to levels well below those of untreated MPS VI cats. They also demonstrated skeletal improvement and correction of lysosomal storage to varying extents in all tissues examined with the exception of cartilage, cornea, and white blood cells. Comparison of results obtained from a similar study treating MPS VI cats with rh4S at 1 and 5 mg/kg (Ref. 15 and this study) showed that in terms of most pathologies, a dose of 1 mg of rf4S/kg was clearly more efficacious than the same dose of human enzyme and, in many instances, as good as or better than the 5 mg/kg dose of human enzyme. The most outstanding demonstration of efficacy of therapy was seen in the almost complete elimination of storage in aorta, where 1 mg of rf4S/kg was clearly better than 5 mg of rh4S/kg, with 1 mg of rh4S/kg having the least effect. Normalization of the structure of the elastic fibers in the aorta, however, was not observed. One possible explanation for this is that irreversible change has already occurred in utero. Whether long term therapy can reverse this abnormality or, indeed, whether it compromises long term survival has not been established. Furthermore, there was marked improvement in storage in heart valve with rf4S compared with the equivalent dose of h4S. This is of particular relevance to therapy of MPS VI patients who generally die from heart valve failure. Most parameters of bone pathology also reflected the improved outcome of therapy with rf4S as compared with 5 mg of rh4S/kg. In addition, the reduction in total urinary GAG excretion by treatment with 5 mg of rh4S/kg was only marginally better than with 1 mg of rf4S/kg as was the decrease in lyosomal vacuolation in skin, heart valve, and dura mater fibroblasts. With all treatment strategies, clearance of storage in cartilage chondrocytes and corneal keratocytes was unaffected. This correlates with the fact that corneal clouding and joint pathology appear to be most refractory to ERT.

The reasons for f4S eliciting an overall more effective therapeutic response compared with h4S are not clear. However, we consider the following parameters may be involved. First, it was observed that treatment with rh4S resulted in all cats showing some adverse effects to the administration of enzyme at some stage throughout therapy, despite antihistamine premedication. In contrast, those cats treated with f4S demonstrated no untoward signs whatever. The reasons for the observed reactions in the previous cohort of cats are not obvious. These cats had demonstrable antibody titers to rh4S, but the levels were not significantly different from those measured in untreated MPS VI and normal control cats (25). It is conceivable that the antibody titer detected in these cats, although low, may nevertheless be sufficient to elicit a transient immunogenic response such as anaphylaxis but not one that is sufficiently severe to necessitate termination of therapy. Confirmation of the presence of IgE antibodies in these cats was precluded by unavailability of anti-cat IgE antibodies. However, it is not clear that even if such a response were present that it would have a significant effect on efficacy. Indeed, results from other animal studies (13) and clinical trials (32) suggest that the presence of even a significant immune response is not necessarily detrimental to the therapeutic response. Therefore, we would suggest that an immune response to the human enzyme was, at the most, only a minor contributing factor to the observed differences between the efficacy of the human and feline enzymes.

Second, although rf4S and rh4S share similar physical and kinetic properties, two notable differences between the two enzymes are in relative pi values and degree of glycosylation. rf4S is significantly more basic and is composed of fewer isomeric forms than its human equivalent as determined from IEF. However, based on amino acid sequence alone, the relative pi value of both enzymes is almost identical (6.98 for f4S and 7.58 for h4S) (39). The observed difference on IEF must therefore be due to variation in secondary modifications such as glycosylation and/or phosphorylation. Based on results obtained from PNGase F treatment, it is obvious that rh4S is more highly glycosylated than rf4S. Results from neuraminidase digestion suggest that a significant proportion of the f4S has complex carbohydrate structures that terminate in sialic acids, whereas the degree of sialylation on rf4S was not clear from the IEF analysis due to the large number of isomeric forms. The similarity in plasma circulating half-life of both enzymes, namely 5.8 min for rf4S (data not shown) and 3.6 min for rh4S when administered at a dose of 1 mg/kg, however, implies that any difference in sialylation is not significantly altering the overall clearance rate of the enzyme. (The previously published half-life of 13.7 min (14) was incorrectly calculated.) The degree of sialylation of oligosaccharide chains of proteins has been shown to influence bioactivity such as increasing plasma circulating half-life (33, 34). While we cannot conclude at this stage that the differences in the pi and/or glycosylation of the two enzymes are responsible for the observed differences in the efficacy of therapy, it remains an intriguing possibility.

Third, it is clear that an enzyme could be more effective in its native cellular environment through conserved interactions with other proteins. Although there is no evidence that 4S...
participates in interactions with other proteins that affect its biological activity, this remains a possibility. Trafficking of both enzymes via the mannose 6-phosphate receptor appears similar, although binding constants for both enzymes to the feline receptor have not been determined. The analysis by gradient PAGE of urinary GAG from cats treated with h4S and h4S, however, suggests that treatment with the two enzymes results in quantitatively differences in the species of GAG secreted. This could reflect differences in substrate specificity for the two enzymes or differences in tissue uptake that result in the release of different GAG species. Although there is an extra cysteine residue in the feline enzyme, raising the possibility of dimerization, we have not detected homodimers of the enzyme as previously reported (35). Finally, it is possible that the use of same species enzyme may be important in terms of other less obvious interactions (e.g. with other factors involved in targeting and lysosomal functions). Although such interactions have not been described and little is known of the architecture of the lysosome, there is some suggestion of a physical association between some lysosomal enzymes to enable substrate tunneling (36–38).

In conclusion, we believe that treatment with same species enzyme from birth may be of major benefit to patients. Furthermore, this study has demonstrated that it is feasible to use lower doses of same-species enzyme to achieve the same efficacy of therapy as a higher dose of non-species-specific enzyme.

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