

A mouse model for mucopolysaccharidosis type III A (Sanfilippo syndrome)

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Mucopolysaccharidosis type III A (MPS III A, Sanfilippo syndrome) is a rare, autosomal recessive, lysosomal storage disease characterized by accumulation of heparan sulfate secondary to defective function of the lysosomal enzyme heparan *N*-sulfatase (sulfamidase). Here we describe a spontaneous mouse mutant that replicates many of the features found in MPS III A in children. Brain sections revealed neurons with distended lysosomes filled with membranous and floccular materials with some having a classical zebra body morphology. Storage materials were also present in lysosomes of cells of many other tissues, and these often stained positively with periodic-acid Schiff reagent. Affected mice usually died at 7–10 months of age exhibiting a distended bladder and hepatosplenomegaly. Heparan sulfate isolated from urine and brain had non-reducing end glucosamine-*N*-sulfate residues that were digested with recombinant human sulfamidase. Enzyme assays of liver and brain extracts revealed a dramatic reduction in sulfamidase activity. Other lysosomal hydrolases that degrade heparan sulfate or other glycans and glycosaminoglycans were either normal, or were somewhat increased in specific activity. The MPS III A mouse provides an excellent model for evaluating pathogenic mechanisms of disease and for testing treatment strategies, including enzyme or cell replacement and gene therapy.

Key words: MPS III A/mouse/pathogenesis/Sanfilippo syndrome

Introduction

Lysosomal storage diseases are rare, autosomal recessive diseases that arise from a reduction in activity of one or more of

the lysosomal hydrolases responsible for the catabolism of a wide variety of lipids, glycans, or proteins (Neufeld, 1991). The resultant disruption in specific catabolic pathways leads to the accumulation of undegraded materials within lysosomes which causes lysosomal engorgement and cell swelling. The medical consequences can be devastating as many lysosomal storage diseases lead to severe neurological impairment and to major organ dysfunction. Death is common at an early age.

The mucopolysaccharide (MPS) storage diseases represent one broad category of lysosomal disorder in which enzymes needed to degrade glycosaminoglycans are deficient. Proteolytic cleavage of cellular proteoglycans generates glycosaminoglycans (dermatan sulfate, heparan sulfate, keratan sulfate, and chondroitin sulfate) which are normally catabolized by 10 different lysosomal enzymes (Neufeld and Muenzer, 1995). Numerous types of MPS disease are recognized on the basis of specific enzyme deficiencies and storage of one or more glycosaminoglycans. Sanfilippo syndrome or mucopolysaccharidosis type III is the most common form of MPS. Estimates of incidence range from 1:24,000 in The Netherlands (van de Kamp, 1981), to 1:66,000 in Australia (Meikle *et al.*, 1999) to approximately 1:324,000 in British Columbia (Lowry *et al.*, 1990). There are four subtypes of MPS III that result from deficiencies in different enzymes required to degrade heparan sulfate in the lysosome: glucosamine-*N*-sulfamidase in MPS III A, α -*N*-acetylglucosaminidase in MPS III B, acetyl-CoA acetyltransferase in MPS III C, and *N*-acetylglucosamine-6-sulfatase in MPS III D. MPS III A is the most common subtype in Northern Europe, whereas MPS III B is more prevalent in Italy and Greece (Betris, 1986; Michelakakis *et al.*, 1995). The genes coding for MPS III A, III B, and III D have been cloned (Robertson *et al.*, 1992; Scott *et al.*, 1995; Karageorgos *et al.*, 1996; Weber *et al.*, 1996; Zhao *et al.*, 1996), and mutations causing MPS III A in humans have been described previously (Blanch *et al.*, 1997; Bunge *et al.*, 1997; Weber *et al.*, 1997; Di Natale *et al.*, 1998).

All subtypes of MPS III result from defective degradation and subsequent storage of heparan sulfate in the lysosome (Neufeld and Muenzer, 1995). After a short period of normal development, affected individuals exhibit a range of symptoms that may include loss of social skills with aggressive behavior and hyperactivity, mental retardation, disturbed sleep, coarse facies, hirsutism, and diarrhea. In profoundly affected children, hearing loss and delayed speech development are often present at 2 years of age. Skeletal pathology, typical for other types of MPS disease, is relatively mild and often develops after the clinical diagnosis is established. However, there has been considerable variation reported in the age of onset and the severity of clinical phenotypes observed for MPS III patients.

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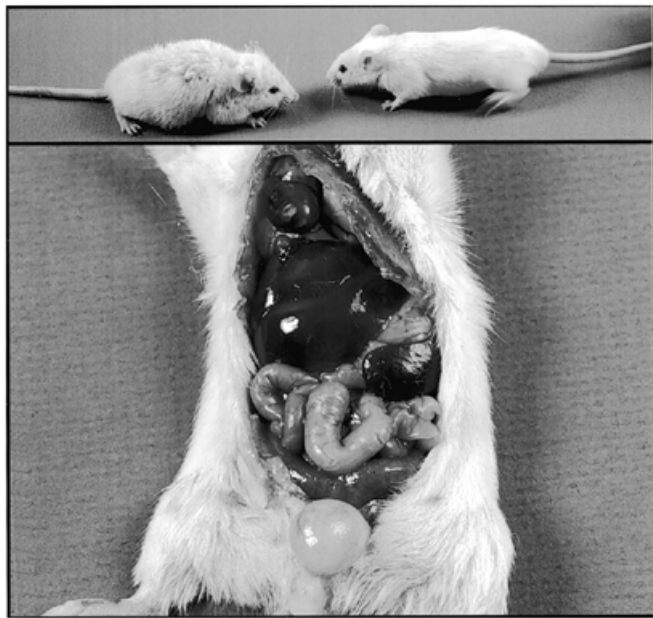


Fig. 1. (a) Photograph of an adult mouse (age 9 months) affected by MPS III A (left) and a normal littermate (right). (b) Affected adult mouse showing distended bladder and enlarged liver and spleen.

There have been four animal models described for MPS III. A Nubian goat model for MPS III D (Jones *et al.*, 1998) has provided valuable clinical, biochemical and morphological detail to assist comparison with human MPS III D; MPS III B has been described in emu (Giger, 1997) and in a mouse with a targeted mutation (Li *et al.*, 1998); MPS III A has been described in dog (Fischer *et al.*, 1998). We report here the discovery of a murine model of MPS III A that exhibits a profound deficiency of lysosomal sulfamidase (EC 3.10.1.1) activity, and many of the biochemical, pathological, and clinical features found in children with this disease.

Results

Characteristics of affected mice

During the course of breeding mice generated from an embryonic stem cell clone WW6.186 that transmitted a targeted mutation in the *Mgat3* gene to CD1 mice (Bhaumik *et al.*, 1998), a 14 month male homozygous for the *Mgat3*^{-/-} mutation was observed to be walking in circles and to be scruffy and ill in appearance. Sections prepared from the brain of this mouse showed dramatic alterations in lysosomal morphology and numerous zebra bodies characteristic of lysosomal storage disease. When additional *Mgat3*^{-/-} mice were examined, only about a third were found to be affected. Subsequently, the same brain lesions were discovered in two *Mgat3*^{+/-} mice. From the latter wild type mice, a colony that has produced ~150 affected mice was established. These mice are of mixed genetic background including predominantly 129SvJ and CD1 with some C57Bl/6 and SJL strain contributions.

Mice with lysosomal storage disease were routinely identified by light microscopy of muscle biopsy sections. Fibroblasts of affected mice had vacuolated and enlarged cytoplasm. When complete litters were biopsied, affected mice represented about 25% of progeny, indicating autosomal recessive inheritance. Consistent with this was the fact that some biopsy negative mice (presumed wild-type) did not produce any biopsy positive progeny in a complete litter when mated to a biopsy positive mouse, whereas others (presumed heterozygotes) produced about 50% affected progeny from a biopsy positive mating.

At birth, affected pups were indistinguishable from littermates. No significant differences in growth rate or appearance were observed until 6–7 months when affected mice were noted to be less active. At this time, the coats of affected animals appeared scruffy and the mice had a hunched posture and abdominal distension (Figure 1A). Males or females caged together (up to 5 per cage) did not show any overtly aggressive behavior. At ~7 months of age the clinical onset of corneal opacity was often noted in affected mice. By about 7–10 months affected mice died. Among 30 male and female mice the average age of death was 7.2 months (range 3 to 10.5 months). A few mice lived from 12–14 months. At death, mice invariably exhibited a grossly distended bladder filled with 1–2 ml of turbid urine, and they also had hepatosplenomegaly (Figure 1B).

Microscopic evidence of lysosomal storage

Light microscopic analysis of brain and other tissues revealed widespread and variable intracellular storage in a variety of cell types (Figure 2). The overall degree of intracellular storage in brain varied with age, with the oldest animals exhibiting the greatest extent of accumulated material. Neurons within the cerebral and cerebellar cortices, the deep cerebellar nuclei, and other brain areas exhibited cytoplasmic distension with vacuoles containing material that often stained positively with PAS (Figure 2A). Toluidine blue staining of 2 μ m plastic sections taken from the cerebral cortex revealed different types and degrees of storage in different brain cells (Figure 2B). With light microscopy, cortical neurons characteristically exhibited dense inclusions which stained positively with toluidine blue, whereas adjacent glial cells typically exhibited a vesiculated appearance. Immunocytochemical staining of brain tissue using antibodies to LAMP1 revealed that storage predominated within the lysosomal system. In addition, storage material in many types of cells also frequently stained with antibodies to GM2 ganglioside (data not shown).

Electron microscopic (EM) analysis was necessary to identify the nature of the inclusions. EM of cerebral cortex revealed that neurons contained typical “zebra body” type storage material or inclusions with a more floccular characteristic. Most characteristically, combinations of these inclusions were found mixed within individual neurons (Figure 3A). Other cells in the brain parenchyma resembling microglia and perineuronal satellite cells contained clear, electron-lucent inclusions (Figure 3B).

Mesenchymal cells of the leptomeninges, perivascular spaces and endothelium were remarkable for exhibiting vesiculated cytoplasm which also gave positive staining with PAS. Many of these same cells, particularly those in the pial areas, also stained with antibodies to F4/80 indicating a monocytic lineage (data not shown). Microvesiculated cytoplasmic dis-

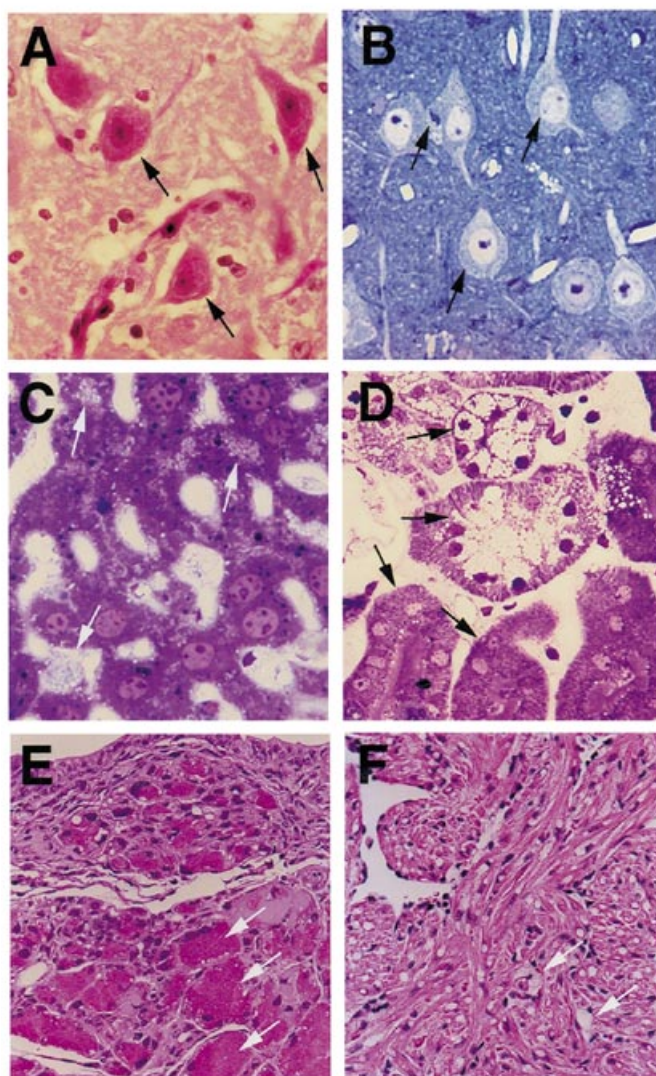


Fig. 2. Light microscopic analysis of storage in cells of various tissues. (A) PAS stain of a paraffin section showing neurons in a deep cerebellar nucleus. Arrows indicate PAS-positive material within neurons (125 \times). (B) Toluidine blue stained 2 μ m thick plastic section of cerebral cortex. Upper right and lower arrows indicate neurons with cytoplasmic storage; upper left arrow illustrates the presence of a typical perineuronal glial cell with vesiculated cytoplasm. (80 \times). (C) Toluidine blue stained 2 μ m thick plastic section of liver. Kupffer cells demonstrate the greatest degree of storage. Arrows show vesiculation (80 \times). (D) Toluidine blue stained 2 μ m thick plastic section of kidney showing storage in tubules. Distal tubules (upper arrows) showed substantially more storage than proximal tubules (lower arrows) (80 \times). (E) PAS stain of paraffin section showing storage within cells infiltrating the submucosa of the urinary bladder (arrows) (80 \times). (F) H&E stain of paraffin section of cardiac muscle illustrating massive infiltration of cells with foamy cytoplasm and disruption of normal tissue architecture (arrows). (80 \times).

tension of endoneurial fibroblasts, endothelial cells and scattered Schwann cells were prominent in dorsal root ganglia and peripheral nerves. Neurons of the dorsal root ganglia, like those of the CNS, exhibited fibrillogranular and zebra body type inclusions. Neither axonal or myelin alterations were observed in peripheral nerves. In the eye, rare corneal substantia propria fibroblasts had microvesiculated cytoplasm. Retinal

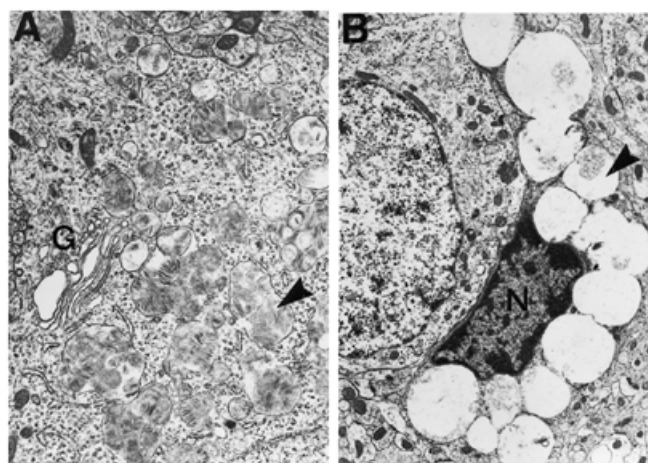


Fig. 3. Electron microscopy of cells in cerebral cortex. (A) Electron micrograph of typical storage within a cortical neuron like those shown in Figure 2B. Storage vacuoles ranged from those containing floccular material to others with stacks of membrane (arrowhead) sometimes resembling zebra bodies. (7920 \times). (B) Electron micrograph of a perineuronal glial cell showing characteristic vesiculated cytoplasm (arrowhead). (4680 \times). G, Golgi apparatus; N, nucleus.

pigment epithelial cells, ciliary epithelium and scleral fibroblasts often were identified with vacuolated cytoplasmic material. Cells with microvesiculated cytoplasmic distension randomly infiltrated the ear, affecting the osseous labyrinth and fibrovascular stalk of the tympanic membrane.

Lysosomal storage was dramatically evident in the liver (Figure 2C) and to a lesser extent in the spleen (not shown). Kupffer cells of the hepatic sinusoids were swollen with cytoplasmic vacuoles and randomly clustered together. In advanced cases the hepatocytes tended to develop microvesiculated cytoplasm. The splenic parenchyma contained microvesiculated cells in the dense connective tissue trabeculae and sinuses and in perivascular locations.

Terminally the mice developed urinary bladder distension (Figure 1B), often accompanied by unilateral or bilateral hydronephrosis. The kidney was altered by accumulation of storage material primarily in the cortical regions (Figure 2D). Epithelial podocytes of the glomerular tuft exhibited microvesiculated cytoplasm. Cytoplasm of distal convoluted tubules was microvesiculated, whereas proximal convoluted tubules appeared unaffected, or at most only mildly so. Interstitial cells were diffusely affected, characterized by microvesiculated cytoplasm. Epithelial cells lining collecting tubules had microvesiculated cytoplasm in the thick ascending limb, collecting ducts, thin descending limb and medullary thick ascending limb. The wall of the urinary bladder was thickened grossly, and microscopically the submucosa was distorted and expanded by infiltration of fibroblasts and macrophages with abundant cytoplasm containing PAS positive material (Figure 2E).

Cardiac muscle was often markedly affected in chronic cases. Microvesiculated fibroblasts and macrophages commonly expanded the myocardial endomysium and perivascular spaces (Figure 2F). In advanced cases myocardiocytes underwent degenerative changes and were replaced by fibroblasts with foamy cytoplasm. Microvesiculated cells also infiltrated

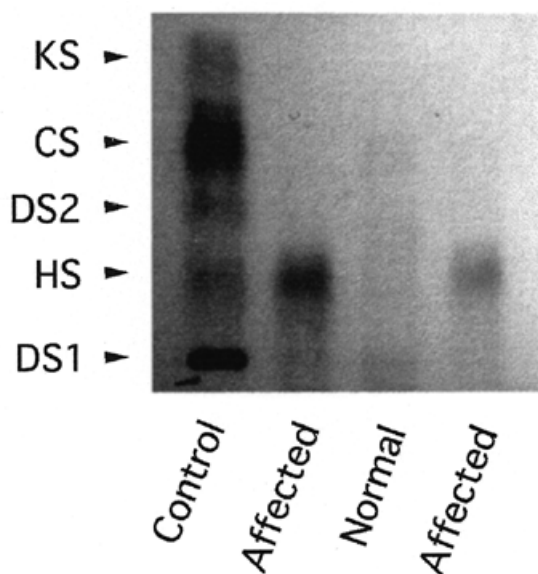


Fig. 4. High resolution electrophoresis of urine GAGs. Samples were prepared and analyzed as described in *Materials and methods*. Control contains GAGs from an equal volume mixture of urine from a patient with MPS II (deficient in iduronate-2-sulfatase) and a patient with MPS-IVA (deficient in *N*-acetylgalactosamine-6-sulfatase); Affected lanes contain GAGs from urine of an affected mouse at 1× and 0.5× dilution, respectively; Normal contains GAGs from a control, unaffected mouse at 1×. The mice were ~10 months of age.

the subendothelial connective tissue core of valvular cusps and arterial perivascular spaces.

Bone deformation is commonly reported in the mucopolysaccharidoses (Neufeld and Muenzer, 1995). The calvarium was abnormally thickened in all affected mice when compared to controls. Vertebral deformation was often the most severe lesion, and frequently cartilagenous matrix of particularly the thoracic vertebrae, proliferated within the spinal canal. Chondrocytes had microvesiculated cytoplasm, as did some periosteal cells.

Urine analysis identifies accumulation of heparan sulfate in affected mice

Urine glycosaminoglycans (GAGs) from affected and control mice were analyzed by high-resolution electrophoresis. Whereas control mouse urine had a mixture of mostly chondroitin sulfate, with heparan sulfate and dermatan sulfate, affected mice had predominantly heparan sulfate (Figure 4). The pattern in affected mice was typical of patients that have Sanfilippo syndrome (Hopwood and Harrison, 1982). The gradient gel electrophoresis pattern obtained for urine and brain GAGs from affected mice was also typical of patients with MPS III A (see below and Figure 5).

Lysosomal hydrolase activities in liver, brain, and kidney

The urinalysis data pointed strongly to Sanfilippo Syndrome as the basis of lysosomal storage in the affected mice. This syndrome can be caused by a deficiency in any one of four enzymes: glucosamine-*N*-sulfamidase (MPS III A), α -*N*-

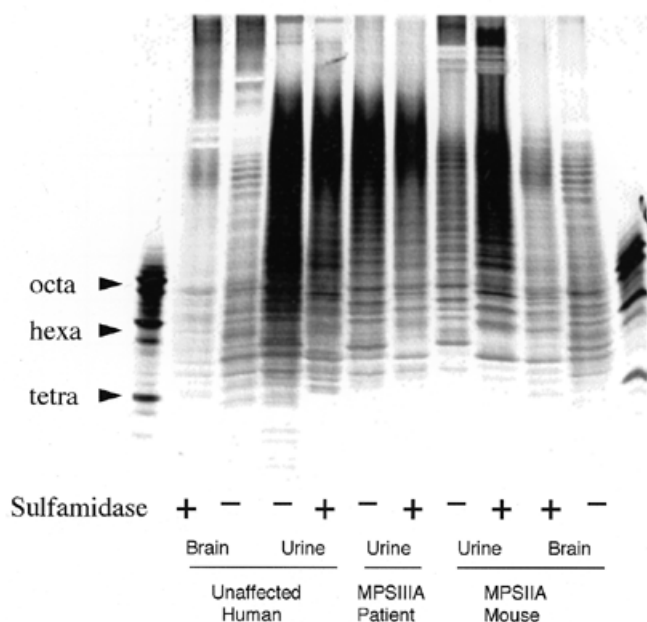


Fig. 5. Gradient gel electrophoresis of urine and brain GAGs. Samples were prepared and analyzed as described in *Materials and methods* from an unaffected human control, a MPS III A patient and an affected MPS III A mouse, age ~10 months. Standards were heparan sulfate octa-, hexa-, and tetra-oligosaccharides. Sulfamidase + or - denotes treatment (or not) with recombinant human sulfamidase prior to gel electrophoresis.

acetylglucosaminidase (MPS III B), acetyl-CoA acetyltransferase (MPS III C), or *N*-acetylglucosamine-6-sulfatase (MPS III D). Therefore, each of these enzymes, as well as iduronic acid-2-sulfatase, another sulfatase that is required for both heparan sulfate and dermatan sulfate degradation, was assayed in various tissue extracts. In addition, several other lysosomal enzymes were analyzed.

The data in Table I show significant elevation of β -hexosaminidase activity in liver and β -glucuronidase activity in brain. Specific activities of enzymes responsible for GAG degradation were also increased about 2-fold, with the notable exception of sulfamidase. Sulfamidase activity was markedly deficient (3–4% of the specific activity in control mice). Therefore, sulfamidase was the only hydrolase among those that give rise to heparan sulfate accumulation and Sanfilippo syndrome that was severely reduced in activity in affected mice.

Nature of nonreducing-end of glycosaminoglycans (GAGs) isolated from mouse MPS III A urine and brain

GAGs were isolated from urine and brain of an affected mouse and compared to similar preparations from a MPS III A patient and an unaffected human control. Complex banding patterns that showed differences between samples from normal and affected subjects were observed on gradient gel electrophoresis (Figure 5). A comparison particularly in the low molecular weight oligosaccharide region of the gel, clearly demonstrates the presence of similar GAG patterns between patient and mouse MPS III A samples. We have previously reported that

Table I. Activity of lysosomal hydrolases in tissues homogenates

| Lysosomal hydrolase (source) | Normal | Specific activity (nmol/mg protein/h) | |
|--|---------------------|---------------------------------------|------|
| | | Affected | %A/N |
| α-Mannosidase | | | |
| Liver | 120 \pm 17(3) | 59 \pm 5(2) | 49% |
| Kidney | 482 \pm 66(3) | 366 \pm 50(2) | 76% |
| Brain | 33 \pm 9(3) | 20 \pm 1(2) | 61% |
| β-Hexosaminidase | | | |
| Liver | 959 \pm 41(3) | 5040 \pm 405(2) | 526% |
| Kidney | 4964 \pm 608(3) | 9859 \pm 1059(2) | 199% |
| Brain | 2846 \pm 283(3) | 7242 \pm 2423(2) | 254% |
| β-Glucuronidase | | | |
| Liver | 212 \pm 23(3) | 409 \pm 10(2) | 193% |
| Kidney | 1319 \pm 438(3) | 1032 \pm 478(2) | 78% |
| Brain | 20 \pm 1(3) | 79 \pm 15(2) | 395% |
| β-Galactosidase | | | |
| Liver | 44 \pm 5(3) | 76 \pm 3(2) | 173% |
| Kidney | 677 \pm 27(3) | 746 \pm 133(2) | 110% |
| Brain | 41 \pm 2(3) | 68 \pm 3(2) | 166% |
| IdUA-sulfatase | | | |
| Liver | 0.16(1) | 0.28(1) | 175% |
| α-GlcNAc'ase | | | |
| Liver | 0.87 \pm 0.06(2) | 1.5 \pm 0.32(4) | 172% |
| AcCoA-N-acetyltransferase | | | |
| Liver | 1.35 \pm 0.07 | 2.8 \pm 1(4) | 207% |
| GlcNAc-6-sulfatase | | | |
| Liver | 0.024(1) | 0.06(1) | 250% |
| Sulfamidase | | | |
| Liver | 0.135 \pm 0.01(2) | 0.004 \pm 0.001(4) | 3% |
| Kidney | 0.19(2) | 0.007(2) | 3.7% |
| Brain | 0.054(2) | 0.002(2) | 3.9% |

Tissue homogenates were prepared by method 1 or 2 and assayed as described in Methods. The first four enzymes were assayed from extracts prepared by method 1 and the remainder from extracts prepared by method 2. The number of mice per group is given in parentheses. Average values are given \pm standard deviation. IdUA, iduronic acid; AcCoA, acetyl CoA.

the pattern of GAGs obtained using this method is different for each MPS type and therefore diagnostic (Byers *et al.*, 1998).

Digestion of isolated GAGs with recombinant human sulfamidase before gradient gel electrophoresis (Byers *et al.*, 1998), provided a definitive diagnosis of a deficiency of sulfamidase as the cause of heparan sulfate storage and the clinical phenotype in the mouse reported here. A shift in banding pattern to more rapidly migrating species was observed after sulfamidase digestion, reflecting a change in charged species due to the replacement of negatively charged, nonreducing end, glucosamine-*N*-sulfate residues by a positively charged glucosamine residue (Figure 5).

Discussion

In this paper we describe a new mouse model for Sanfilippo disease. The spontaneous mouse mutant we have identified is specifically deficient in sulfamidase activity, and has heparan sulfate-uria, making this a model for MPS III A. Like patients with MPS III A, the mice have little, if any, sulfamidase activity (Table I) and they accumulate heparan sulfate that has glucosamine-*N*-sulfate nonreducing ends (Figure 5), as expected if sulfamidase is inactive. Other lysosomal hydrolases are for the most part increased in activity, a common characteristic of many lysosomal storage diseases (Neufeld and Muenzer, 1995). In humans, MPS III A is caused by a variety of different inherited mutations that reduce the activity of glucosamine *N*-sulfamidase (Blanch *et al.*, 1997; Bunge *et al.*, 1997; Weber *et al.*, 1997; Di Natale *et al.*, 1998). The mouse sulfamidase gene sequence has not been reported. Using a human sulfamidase cDNA probe and 10 μ g poly(A)+ RNA that gave a strong signal with a control 3 kb probe, no mouse sulfamidase transcripts were detected by Northern analysis (R.Bhattacharyya, unpublished observations). However, the fact that residual activity was evident in tissue extracts from affected mice (Table I) suggests that the mouse mutation is likely to be a point mutation that reduces sulfamidase activity, similar to the mutations observed in humans with MPS III A.

Two cases of MPS III A have been reported in dogs (Fischer *et al.*, 1998). These animals exhibited pelvic limb ataxia as young adults which progressed over several years to severe cerebellar ataxia. Mild cerebral and cerebellar atrophy was found and neurons in many brain regions exhibited substantial intracellular storage. Purkinje cells of the cerebellum were particularly affected and widespread loss of these cells apparently contributed to the clinically-evident ataxia. In the MPS III A mice, there is less obvious motor system dysfunction and Purkinje cells are not lost in substantial numbers. Like the dog model, there is widespread neuronal storage, with the ultrastructure of the storage material being similar to that reported in the dog. In viscera of the dog model, fibroblasts, hepatocytes, and renal tubular cells were vacuolated. For the mice, only distal renal tubules were severely affected, rather than both proximal and distal as in the dog. However, in both mouse and dog the urinary bladder wall was conspicuously thickened. Changes in cardiac muscle observed in the MPS III A mice (Figure 2F) were not described in the dog model.

The MPS III A mouse should be useful for investigations of the cell biological and neurological consequences of all mucopolysaccharidoses, including MPS III A. A wide variety of functions have been suggested for different GAGs and for individual proteoglycans, particularly in the developing and aging brain (Small *et al.*, 1996). When added to cultured cortical neurons, for example, heparan sulfate induced the formation of long singular axons but few or no dendrites (Calvet *et al.*, 1998). In contrast, the addition of dermatan sulfate increased dendrite growth, possibly through changes in adhesion properties of the growing neurites. Heparan sulfate also constitutes the major GAG sidechain of proteoglycans like syndecan, glypican, and cerebroglycan, that have been implicated in a variety of functions, including modulation of growth factor-receptor interactions (Small *et al.*, 1996).

In addition to storing GAGs, most forms of MPS disease are known to store gangliosides. Thus, not only does heparan sul-

fate accumulate in brain tissue of humans and dogs affected by MPS III A, but abnormal amounts of GM2 and GM3 gangliosides also occur (Constantopoulos *et al.*, 1980; Jones *et al.*, 1997; Fischer *et al.*, 1998). The MPS III A mice reported here are similar in that abnormal accumulation of GM2 ganglioside was detected in neurons of the cerebral cortex. It has been proposed that ganglioside storage in MPS disease may be due to secondary inhibition of ganglioside specific neuraminidases by accumulated sulfated GAGs (Baumkotter and Cantz, 1983; Jones *et al.*, 1997, 1998). The degree of ganglioside accumulation in these diseases often mimics that of the primary ganglioside storage disorders and the overabundance of particular gangliosides may be responsible for some aspects of brain dysfunction, including mental retardation (Walkley, 1995, 1998; Walkley *et al.*, 1995). Availability of the MPS III A model in mice will be useful for elucidating the relationship between the primary enzyme deficiency, secondarily-induced biochemical abnormalities, and neuronal dysfunction leading to clinical neurological disease.

Animal models of storage diseases are also useful for testing treatment strategies. In several animal models of lysosomal disorders the development of clinical disease can be ameliorated by both enzyme replacement therapy and bone marrow transplantation (see Walkley *et al.*, 1994; Crawley *et al.*, 1996; Walkley, 1998). In theory, many more lysosomal storage diseases could be treated by such therapies since lysosomal hydrolases with mannose-6-phosphate residues amongst their *N*-linked glycans, bind to cell surface mannose-6-phosphate receptors and are delivered to lysosomes following receptor-mediated endocytosis (Neufeld, 1980; Kornfeld, 1986). Similarly, lysosomal enzymes bearing terminal mannose or galactose residues can be efficiently endocytosed by certain cell types (Rattazzi and Dobrenis, 1991). The existence of these pathways to the lysosome provides the rationale for lysosomal hydrolase enzyme replacement therapy. Provided a lysosomal hydrolase is processed with the correct *N*-linked glycans, it can be targeted to lysosomes following injection into the bloodstream. Effective uptake and delivery to lysosomes can also be achieved through modifications such as the use of the C fragment of tetanus toxin to specifically enhance targeting to neurons (Dobrenis *et al.*, 1992). Because of the blood-brain barrier, lysosomal enzymes are unlikely to enter the brain from the circulation. Therefore, alternate strategies must be developed to target lysosomal enzymes to sites of pathology in the brain. One potential means of delivery to brain is via bone marrow transplantation. In this case, donor bone marrow-derived monocytes are believed to enter the brain where they differentiate as microglia and serve as a potential source of missing lysosomal hydrolases (Walkley *et al.*, 1994; Krivit *et al.*, 1995; Walkley *et al.*, 1996). Differences in the secretion and/or stability of secreted enzymes by such cells is a likely explanation for the variable success of this technique in the treatment of a variety of storage diseases (Walkley and Dobrenis, 1995). Over the years numerous models of lysosomal hydrolase deficiency have been identified from spontaneous mutations in animal populations and, more recently, additional models have been generated by targeted gene mutation in the mouse (reviewed in Jolly and Walkley, 1997; Suzuki and Proia, 1998). The murine model of MPS III A described here will be a valuable tool to determine effective treatment strategies for this and related storage diseases.

Materials and methods

Experimental animals

A colony of mice with the sulfamidase deficiency was generated as described in *Results*. Affected mice were identified by microscopic examination of biopsies of the quadriceps femoris muscle taken under local anesthesia. Biopsies were immediately placed in 4% paraformaldehyde, left overnight at 4°C and subsequently embedded by routine methods in Epon for 2 µm sections. Affected animals were identified by light microscopy of toluidine blue stained sections on the basis of heavily vesiculated interstitial cells among muscle fibers. All studies using animals had the approval of the Institutional Care and Use Committee of the Albert Einstein College of Medicine.

Histology, electron microscopy (EM), and immunostaining

For morphological studies using light microscopy animals were deeply anesthetized with pentobarbital and perfused via an intracardiac catheter with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. For EM analysis, tissues were post-fixed in 4% paraformaldehyde and 2% glutaraldehyde in 0.1M phosphate buffer, pH 7.2. Paraffin and Epon embedding, for light and electron microscopic studies respectively, were carried out using routine methods. For immunocytochemical studies, 40 µm sections were cut on a vibratome. Monoclonal antibodies to LAMP1 (Uthayakumar and Granger, 1995) from the Developmental Studies Hybridoma Bank, The University of Iowa, GM2 ganglioside (a gift from Dr. Philip Livingston, Memorial Sloan Kettering), and F4/80 (Serotec) were applied at predetermined dilutions followed by indirect immunolabeling using appropriate bridging antibodies. Peroxidase-anti-peroxidase labeling was detected with diaminobenzidine (Sigma, St. Louis, MO) using routine histochemical procedures (Walkley, 1995).

Lysosomal enzyme assays

Tissue homogenates were prepared by two methods. In method 1, tissues were homogenized on ice in 10–20 volumes 0.1 M citrate buffer, pH 5.5, with 25 strokes of a glass-to-glass tissue homogenizer (Kontes #8855000–0022) mounted on an Eberbach Con-Torque tissue grinder. Homogenates were centrifuged for 15 min at 10,000 × *g* and supernatants recovered for enzyme assay and protein determination. Tissue homogenates were assayed in triplicate with fluorogenic 4-methyl umbelliferyl (4-MU) substrates at 37°C based on standard protocols (Galjaard, 1980). β-N-Acetyl-D-hexosaminide N-acetylhexosaminohydrolase (β-hexosaminidase) (E.C. 3.2.1.52) was assayed at pH 4.5 for 0.5 h with 5 mM 4-MU-2-acetamidodeoxy-D-glucopyranoside in 0.2 M citric acid–0.34 M dibasic sodium phosphate; α-D-mannoside mannohydrolase (α-mannosidase) (E.C. 3.2.1.24) was assayed at pH 3.75 for 1 h with 4 mM 4-MU-D-mannopyranoside in 0.1 M citric acid–0.2 M dibasic sodium phosphate buffer with 1.5 mM ZnCl₂; GM1 ganglioside β-galactosidase (β-galactosidase) (E.C. 3.2.1.23) was assayed at pH 4.4 for 1 h with 1 mM 4-MU-D-galactopyranoside in 0.1 M citric acid–0.2 M dibasic sodium phosphate buffer with 100 mM NaCl; and β-D-glucuronide glucuronohydrolase (β-glucuronidase) (E.C. 3.2.1.31) was assayed at pH 4.8 for 1 h with 10 mM 4-MU-D-glucuronide in 0.1 M acetate buffer. Results are expressed as nanomoles substrate cleaved per hour per mg protein assayed by the Lowry method (Lowry *et al.*, 1951).

In method 2, tissues were homogenized in 0.1% Triton X-100 (v/v), freeze-thawed 3 times and sonicated for 10 sec three times (Ystrom Systems USA, power setting 7), centrifuged 5 min at $500 \times g$ and the supernatant assayed on the same day. Assays were performed essentially as described previously (Hopwood and Elliott, 1982) in 50 mM sodium acetate buffer, pH 5, with 34 μ M tetrasaccharide substrate (glucosamine-N-sulfate-(1,4)-iduronic or glucuronic acid-(1,4)-glucosamine-N-sulfate-(1,4)- $\{1-^3\text{H}\}$ idonic, gluconic, or anhydroidonic acid) in a final volume of 12 μ l. Each assay contained 30 μ g protein measured by the Lowry method (Lowry *et al.*, 1951) compared to Dade Human Protein Standard (Baxter Healthcare Corp., USA) and was incubated at 37°C for 16 h. α -N-Acetylglucosaminidase was measured as described (Hopwood and Elliott, 1982) using 60 μ M disaccharide substrate (N-acetylglucosaminide-(1,4)- $\{1-^3\text{H}\}$ -idonic, -gluconic, or -anhydroidonic acid) in 50 mM sodium acetate pH 4.5 with 20–30 μ g protein for 7 h at 37°C. Acetyl-CoA glucosamine N-acetyltransferase and glucosamine-6-sulfatase were assayed using a monosaccharide and a disaccharide substrate, respectively, as previously published (Hopwood and Elliott, 1981; Freeman and Hopwood, 1989). Iduronate-2-sulfatase activity was assayed using a disaccharide substrate (Hopwood, 1979).

High-resolution and gradient gel electrophoresis of glycosaminoglycans isolated and treated with sulfamidase

Glycosaminoglycans (GAGs) were isolated from urine and tissues as described previously (Hopwood and Harrison, 1982; Byers *et al.*, 1998). The amount and types of GAGs present in urine were assayed using high resolution electrophoresis (Hopwood and Harrison, 1982) or gradient gel electrophoresis (Turnbull *et al.*, 1997; Byers *et al.*, 1998). GAG fractions were either subjected (or not) to digestion with recombinant human sulfamidase and run on 30–40% linear gradient polyacrylamide gels as described (Byers *et al.*, 1998). GAG samples (10–25 μ l) containing 0.5–5.7 μ g of uronic acid, and marker standards (5–10 μ l) were combined with 10% glycerol and trace amounts of phenol red and bromophenol blue. Electrophoresis was performed at 350V for 16 h or until the phenol red dye front was 1 cm from the bottom of the gel. Oligosaccharides within the resolving gel were stained with alcian blue/silver (Merrill *et al.*, 1981).

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Abbreviations

MPS, mucopolysaccharidosis; EM, electron microscopy; 4-MU, 4-methylumbelliferyl; PAS, periodic acid Schiff; GAG, glycosaminoglycan.

Note Added in Proof

While this manuscript was in press we identified a point mutation in the sulfamidase gene of affected mice and have shown that mutant cDNA is inactive in CHO cell transfectants (R.Bhattacharya, B.Gliddon, T.Beccari, G.Yogalingam, M.Bhaumik, J.J.Hopwood and P.Stanley, manuscript in preparation).

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