Enzyme replacement therapy in a murine model of Morquio A syndrome

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Mucopolysaccharidosis IVA (MPS IVA) is an autosomal recessive disorder caused by a deficiency of N-acetylgalactosamine-6-sulfate sulfatase (GALNS), leading to accumulation of keratan sulfate (KS) and chondroitin-6-sulfate. The pharmacokinetics and biodistributions were determined for two recombinant human GALNSs produced in CHO cell lines: native GALNS and sulfatase-modifier-factor 1 (SUMF1) modified GALNS. Preclinical studies of enzyme replacement therapy (ERT) by using two GALNS enzymes were performed on MPS IVA mice. The half-lives in blood circulation of two phosphorylated GALNS enzymes were similar (native, 2.4 min; SUMF1, 3.3 min). After intravenous doses of 250 units/g body weight were administered, each enzyme was primarily recovered in liver and spleen, with detectable activity in other tissues including bone and bone marrow. At 4 h post-injection, enzyme activity was retained in the liver, spleen, bone and bone marrow at levels that were 20–850% of enzyme activity in the wild-type mice. After intravenous doses of 250 units/g of native GALNS, and 250, 600 or 1000 units/g of SUMF1-GALNS were administered weekly for 12 weeks, MPS IVA mice showed marked reduction of storage in visceral organs, sinus lining cells in bone marrow, heart valves, ligaments and connective tissues. A dose-dependent clearance of storage material was observed in brain. The blood KS level assayed by tandem mass spectrometry was reduced nearly to normal level. These preclinical studies demonstrate the clearance of tissue and blood KS by administered GALNS, providing the in vivo rationale for the design of ERT trials in MPS IVA.

INTRODUCTION

Mucopolysaccharidosis IVA (MPS IVA; Morquio A syndrome: MIM# 253000) is an autosomal recessive lysosomal storage disorder caused by the deficiency of the lysosomal enzyme, N-acetylgalactosamine-6-sulfate sulfatase (GALNS; EC 3.1.6.4). Classically affected patients, who have little GALNS activity, progressively accumulate glycosaminoglycans (GAGs), keratan sulfate (KS) and chondroitin-6-sulfate (C6S), primarily in the lysosomes of multiple organs, especially ligaments, connective tissues, bone and cartilage (1–3). MPS IVA varies clinically from a severe to attenuated form, characterized by systemic skeletal dysplasia with short stature, hypoplasia of the odontoid process, pectus carinatum, kyphoscoliosis, genu valgum, laxity of joints, hearing loss, corneal clouding, heart valvular disease and mild hepatosplenomegaly. Unlike other types of MPS, intelligence is normal (4–6).

The initial signs and symptoms in most MPS IVA patients have been identified by the age of 3 years. MPS IVA patients are usually evaluated during the second or third year of life for unusual skeletal features. At between 5 and 10 years, the patients often undergo major surgical operations in neck, hip, knee and leg regions. Most patients will be wheelchair-bound as teenagers (6). Patients with a severe form often do not survive beyond the second or third decade of life primarily related to cervical instability and pulmonary compromise.

Current treatment for Morquio A syndrome is supportive and limited mainly to symptomatic management of the joint pain and surgical operations for the orthopedic complications.

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Morquio A syndrome is a particularly attractive candidate for enzyme replacement therapy (ERT) because affected patients lack CNS involvement. Tremendous progress in research toward development of ERT has been made in the last three decades. Over 3500 Gaucher patients have been treated with dramatic clinical results since the early 1990s (7,8). Other apparent successes in ERT are provided for Fabry disease (9,10), MPS I (5), MPS II (11,12) and MPS VI (13–15). Those ERTs have proved dramatic improvements in visceral organs in treated patients but little or no improvement in the bone or brain. In animal models of MPS I, MPS VI and MPS VII, reduction or elimination of lysosomal storage in many tissues has been achieved by intravenous injections of recombinant enzymes (16–22).

To date, no in vivo ERT has been performed on MPS IVA. Recently, mouse models of MPS IVA that lack any detectable GALNS activity were generated by targeted disruption of the mouse Galns gene in embryonic stem cells (2,3,23,24). The generation of the knockout mouse with partial gene deletion and the mouse tolerant to repeated injections of human enzymes has made the preclinical evaluation of ERT possible.

GALNS is encoded by a member of the sulfatase gene family of which 17 different human sulfatase genes have been cloned (25). The amino acid residue Cys79 in exon 2 of human GALNS was identified in the catalytic site domain which is homologous with the active site domains of all known sulfatases (26,27). To catalyze the hydrolysis of their natural substrates, the conserved cysteine corresponding to Cys 79 in exon 2 of human GALNS must be modified into a formylglycine (FGly) post-translationally in all sulfatases (26). This modification is generated by the protein product of sulfatase modifier factor 1 (SUMF1) gene which encodes the formylglycine-generating enzyme (FGE) (28,29). The FGly is essential for the activity of the sulfatases because it contains an aldehyde hydrate that attacks the sulfate ester, which leads to the formation of the enzyme–substrate complex and to the cleavage of the sulfate residue (27). Coexpressing GALNS with SUMF1 in COS cells has been shown to increase the enzyme activity of GALNS (30,31).

To produce sufficient amounts of purified GALNS enzyme, high level expression of GALNS in Chinese hamster ovary (CHO) cells has been established as a source of selectively secreted human recombinant enzyme.

In this study, we report the pharmacokinetics, biodistribution and clinical response of ERT in comparison with two GALNS enzymes by using MPS IVA mouse models. These mouse-model studies provide the ‘proof of concept’ and critical preclinical information for the design of clinical trials of GALNS enzyme replacement in patients with Morquio A syndrome.

RESULTS

Characterization of human recombinant GALNS

Native (without SUMF1 coexpression) and SUMF1-GALNSs were stably overexpressed by different CHO cell lines. After purification, SUMF1-GALNSs had higher specific activities (270 715 ± 25 063 units/mg, n = 3) toward the artificial fluorogenic substrate compared with native-GALNS (213 855 ± 97 816 units/mg, n = 6). The SDS/PAGE analysis of the two enzyme preparations showed a similar M₆ (57, 39 and 19 kDa) of native and SUMF1-GALNSs (data not shown). These two GALNSs differed in the degree of uptake by MPS IVA human fibroblasts and MPS IVA mouse chondrocytes (Fig. 1), showing native GALNS has 41–52% of higher uptake.

The theoretical isoelectric point of GALNS was 6.25. The pI of SUMF1-GALNS should be the same as of GALNS since the sequence of GALNS was not disrupted by the co-transfection of SUMF1. The optimal pH for GALNS activity for both enzymes, also determined using 0.02 M sodium acetate buffer, was between pH 4.8 and pH 5.3. At a higher pH or lower pH, the activity decreased markedly (data not shown).

Pharmacokinetics and biodistribution

After a single intravenous dose of 250 units/g, both native and SUMF1-GALNSs were cleared from the circulation of the MPS IVA knockout mice in two phases—a rapid phase with a plasma t½ of 2.4 min for native GALNS and 3.3 min for SUMF1-GALNS and a second, longer phase with a plasma t½ of 20.6 min for native GALNS and 21 min for SUMF1-GALNS (Fig. 2). These findings suggested that these two enzymes had nearly the same clearance and remained detectable only for 30 min in blood circulation.

The biodistribution of each enzyme was determined after a single injection of 250 units/g (Fig. 3). At 4 h post-injection, the enzyme activities in liver were 25 ± 5.9 units/mg for native GALNS and 9.1 ± 0.2 units/mg for SUMF1-GALNS. The enzyme activities in spleen were 2.9 ± 1.2 units/mg for native GALNS and 2.1 ± 0.3 units/mg for SUMF1-GALNS. The enzyme activities in bone and bone marrow were 1.3 ± 0.9 units/mg and 2.6 ± 1.6 units/mg, respectively, for native GALNS and 2.0 ± 0.2 units/mg and 5.6 ± 0.3 units/mg.
respectively, for SUMF1-GALNS. At this dose, neither of the enzymes was detected or very little in the heart, lungs, kidneys or brain.

At 24 h post-injection, the enzyme activities in liver were 2.0 ± 0.1 units/mg for native GALNS and 0.9 ± 0.6 units/mg for SUMF1-GALNS. The enzyme activities in spleen were 1.7 ± 0.4 units/mg for native GALNS and 0.7 ± 0.1 units/mg for SUMF1-GALNS. The enzyme activities in bone and bone marrow were 1.1 ± 0.4 units/mg and 0.8 ± 0.2 units/mg, respectively, for native GALNS and 1.4 ± 0.2 units/mg and 1.6 ± 0.5 units/mg, respectively, for SUMF1-GALNS (Fig. 3).

On the basis of these above findings in both enzymes and the higher yield, SUMF1-GALNS was selected for further studies of dose escalation.

To determine the effect that an increased dose of SUMF1-GALNS would have on the enzyme’s biodistribution, 1000 units/g was administered, and the GALNS activity in various tissues was assessed at 4 and 24 h post-injection (Table 1). The enzyme activities elevated 4–20 times in all tissues compared with those with 250 units/g injection. At 4 h post-injection, it was noted 11-fold or 6-fold increase of GALNS activity in bone and bone marrow, respectively, in comparison with 250 units/g injection. The substantial activity was detectable at both 4 h and 24 h postinjections in brain, heart, kidneys and lungs as well.

In SUMF1-GALNS, the total activities recovered at 4 h post-injection of 250 units/g, from the liver and spleen were 17.8 ± 3.6 and 1.18 ± 0.7%, respectively, of the administered dose while the total activities recovered at 24 h post-injection,

from the liver and spleen were 7.5 ± 2.6 and 0.44 ± 0.3%, respectively.

**Comparison of native and SUMF1-GALNS in clearance of storage in the MPS IVA mice**

First, we determined the effectiveness in clearance of storage material of a single dose of 250 units/g of both native and SUMF1-GALNSs. Twenty-four hours after a single dose of 250 units/g of either of enzyme, the accumulated storage material in Kupffer cells of liver, spleen and sinus lining cells in bone marrow was substantially cleared (data not shown). Clearance in other tissues including heart valves, brain, ligaments and connective tissues were not observed with a single dose.

To detect quantitative differences in the effectiveness of clearance of lysosomal storage, we used a protocol in which SUMF1-GALNS enzyme was given in 12 weekly treatments with different doses. The effect that repeated GALNS doses had on the depletion of storage in tissue was determined 1 week after 12 weekly intravenous administrations of 250 units/g of native GALNS and 250, 600 or 1000 units/g body weight of SUMF1-GALNS. All doses of SUMF1-GALNS of 250 units/g of native GALNS completely cleared the accumulated GAGs in the liver (Fig. 4A–D), spleen and sinus lining cells in bone marrow (Fig. 4E–H).

Ligaments and connective tissues surrounding the articular cartilage as well as osteoblasts and osteocytes in treated mice had less storage with a 600 units/g dose of SUMF1-GALNS (data not shown). Growth plate region did not show much improvement in column structure compared with untreated MPS IVA mice and the storage material was not completely cleared by even a higher dose (Fig. 4I–L).

Although MPS IVA patients have no CNS involvement clinically, untreated MPS IVA mice had storage material in hippocampus, cortex and meningeal. The parietal neocortical neurons had less storage than untreated in all treatment groups. Hippocampal neurons and meningeal cells had less storage in mice treated with a higher dose (data not shown). There was substantial reduction among the treated groups in the extent of clearance of storage in heart valves (Fig. 4M–P). It is noteworthy that murine antibodies to the heterologous human enzyme were not detected during these multiple-dose long-term studies by using MPS IVA tolerant mice (data not shown here) as described previously (3).

**Concentration of serum KS in the MPS IVA mouse**

Serum samples from untreated, 250 units/g of native or SUMF1-GALNS treated or wild-type control mice were analyzed for KS (Fig. 5). The baseline serum KS in wild-type controls was relatively constant, showing an average of 0.11 ± 0.03 μg/ml. The baseline serum KS in MPS IVA tolerant mice before treatment was elevated at an average level of 0.83 ± 0.069 μg/ml. There was a significant difference between treated and untreated groups (P < 0.0001). Serum KS concentrations in untreated mice did not change with age.

Serum KS levels were decreased during the 12 weeks of treatment in all treatment groups from the baseline in untreated control mice (0.83 ± 0.085 μg/ml). The serum KS
decreased at 3 weeks after treatment and then decreased further over time. After 12 weekly infusions with 250 U/g of either of native or SUMF1-GALNS, the average level of serum KS concentration was 0.15 ± 0.045 or 0.14 ± 0.049 mg/ml, respectively. Thus, the final serum KS in native or SUMF1-GALNS treated mice was similar to that in normal control group. The groups treated with a higher dose (600 or 1000 units/g) showed no further reduction (data not shown).

**DISCUSSION**

This study had two purposes, namely (i) to compare biochemical properties of the SUMF1-GALNS enzyme with those of native GALNS enzyme; and (ii) to compare the response of MPS IVA mice to therapy with native or SUMF1-GALNS enzyme. Co-expression of SUMF1 together with GALNS cDNA resulted in an increase of specific activity. Most biochemical properties—molecular weight, optimal pH and blood clearance were not affected by coexpression of SUMF1. In vitro studies of the effect of inhibitors on endocytosis of SUMF1-GALNS enzyme by MPS IVA fibroblasts or chondrocytes showed clearly that the SUMF1-GALNS could be taken up by fibroblasts by Man6-P-mediated mechanisms, which target the mannose-6-receptor. Surprisingly, SUMF1-GALNS showed somewhat reduced Man6-P-dependent uptake compared with native GALNS, suggesting less phosphorylation. The basis for this difference in uptake by fibroblasts is unclear. The pathological improvement on MPS IVA mice did not show any difference between two enzymes at the same dose. However, to increase the production level, it appears to be beneficial to co-express SUMF1 in the GALNS over-expressing cell line to increase the yield of recombinant enzyme.

Cosma et al. (31) and Dierks et al. (32) identified FGE and its gene, SUMF1, which is responsible for multiple sulfatase deficiency. FGE is the post-translational modifying enzyme essential for the activation of multiple forms of sulfatases including GALNS. Normally, a sufficient amount of FGE required to modify sulfatases is constitutively expressed in the cell. However, when a specific recombinant sulfatase is excessively expressed, the relative amount of FGE is anticipated to decrease, making it necessary to coexpress FGE. The utility of FGE for overexpression of functional sulfatases was tested in previous studies. In vitro coexpression of SUMF1 with many sulfatase cDNAs in COS cells results in a synergistic increase in enzymatic activity, indicating that SUMF1 is both an essential and a limiting factor for most

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**Table 1.** Enzyme activity in tissue after 1000 units/g injection (n = 3)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>4 h post-injection</th>
<th>24 h post-injection</th>
<th>MPS IVA (untreated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>42.6 ± 10.4</td>
<td>9.7 ± 2.4</td>
<td>0.21 ± 0.49</td>
</tr>
<tr>
<td>Spleen</td>
<td>44.5 ± 25.6</td>
<td>3.2 ± 1.9</td>
<td>0.17 ± 0.23</td>
</tr>
<tr>
<td>Bone</td>
<td>22.2 ± 7.7</td>
<td>6.7 ± 2.6</td>
<td>0.025 ± 0.029</td>
</tr>
<tr>
<td>BM</td>
<td>35.4 ± 8.4</td>
<td>15.1 ± 3.4</td>
<td>0.021 ± 0.031</td>
</tr>
<tr>
<td>Brain</td>
<td>2.8 ± 0.6</td>
<td>0.2 ± 0.08</td>
<td>0.089 ± 0.069</td>
</tr>
<tr>
<td>Heart</td>
<td>2.4 ± 1.3</td>
<td>0.34 ± 0.04</td>
<td>0.009 ± 0.015</td>
</tr>
<tr>
<td>Kidney</td>
<td>7.2 ± 5.2</td>
<td>0.81 ± 0.42</td>
<td>0.011 ± 0.013</td>
</tr>
<tr>
<td>Lung</td>
<td>8.3 ± 2</td>
<td>0.73 ± 0.28</td>
<td>0.037 ± 0.052</td>
</tr>
</tbody>
</table>

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**Figure 3.** Biodistribution and biostability of native and SUMF1-GALNSs in the liver, spleen, bone marrow and bone (clearance from the tissues). We infused 250 units/g body weight of the indicated enzymes into three MPS IVA mice for each treatment. After 4 and 24 h, the mice treated with 250 units/g were killed, and tissue samples were processed for biochemistry. Levels of enzyme observed in bone, bone marrow, liver and spleen are shown.
Figure 4. Reversal of storage after ERT. Light microscopy of liver, bone marrow, cartilage cell layer and brain from untreated (A,E,I,M), native GALNS-treated (B,F,J,N), SUMF1-GALNS-treated mice at 250 units/g (C,G,K,O), 600 (or 1000) units/g (D,H,L,P) of SUMF1-GALNS-treated mice. The liver Kupffer cells (A–D) had essentially complete clearance of storage by all the treatment groups. (A) An untreated MPS IVA mouse has extensive storage in sinus-lining cells and Kupffer cells. (B) After treatment with native GALNS, there is seen essentially complete removal of the lysosomal storage. (C) After treatment with SUMF1-GALNS (250 units/g) and (D) SUMF1-GALNS (600 units/g), there is similar normalization of the liver histology with essentially complete reduction in lysosomal storage. The sinus lining cells in bone marrow (E–H) had essentially complete clearance of storage by all the treatment groups. (E) The vacuolated osteoblasts line the cortical bone (arrow). The sinus lining cells in bone marrow (arrow) also contain a small amount of storage. The cells in the periosteum in an untreated MPS IVA mouse contain storage material (arrow). (F) Native GALNS. (G) SUMF1-GALNS (250 units/g). (H) SUMF1-GALNS (600 units/g). Osteoblasts, osteocytes and cells in the periosteum had less storage in all of the treated mice but the response was best in the three MPS IVA mice treated with a higher dose of enzyme. The cartilage cells had little response in all treated mice (I–L). (I) An untreated MPS IVA mouse shows storage material in chondrocytes and the thin disorganized cartilage layer. Vacuolated chondrocytes with lysosomal distension were obvious. (J) Native GALNS. (K) SUMF1-GALNS (250 units/g). (L) SUMF1-GALNS (600 units/g). The heart valves (M–P) showed clearance of storage by any of enzyme. Especially, mice treated with 600 (or 1000) units/g of SUMF1-GALNS had clearance to a greater extent than the clearance seen in the mice treated with 250 units of native- or SUMF1-GALNS. (M) The heart valves in an untreated MPS IVA mouse have abundant cytoplasmic vacuolization in the valve (arrow). (N) A reduction of storage is seen in the valve in mice treated with native GALNS (arrow) and (O) SUMF1-GALNS (250 units/g). (P) After treatment with SUMF1-GALNS (600 units/g), there was a more reduction in the amount of storage in the heart valve.
Sulfatases (31,32). Enhanced production by co-expressed SUMF1 was obtained in vivo on the arylsulfatase deficient mouse (33). FGE coexpression did not affect arylsulfatase mRNA or protein levels, suggesting that activation occurs at the post-translational level and that active and inactive arylsulfatase enzymes are colocalized in the cells (33). It was observed that part of the recombinant overexpressed sulfatases produced for ERT without co-expressed FGE is inactive due to insufficient post-translational modification.

The two recombinant GALNS enzymes had similar pharmacokinetics. The result of the current clearance study of MPS IVA shows a rapid clearance (half-life time) compatible with those of other phosphorylated lysosomal enzymes: α-iduronidase, 0.9 min (34); α-galactosidase A, 2–5 min (35); glycosylasparaginase, 4 min (36); β-glucuronidase, 5.2 min (37,38); arylsulfatase B, 13.7 min (20); and with non-phosphorylated, recombinant human α-N-acetylglucosaminidase (2 min) (39). Biphasic clearance in circulation has also been described for other lysosomal disorders; MPS I, IIIB and VI, Fabry disease, and aspartylglucosaminuria.

The biodistribution of two enzymes (native GALNS and SUMF1-GALNS) was similar except for liver. Most

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**Figure 5.** Level of serum KS in the MPS IVA mouse. Untreated MPS IVA mice \((n = 3)\), native GALNS treated \((n = 3)\), SUMF1-GALNS treated (250 units/g) \((n = 3)\) and control wild-type \((n = 3)\).
enzymes infused were recovered in the liver. The remainder of each was detected mainly in the spleen, bone marrow and bone. The fact that tissue distribution to liver was greater with native GALNS may result from greater phosphorylation, as suggested by the finding of higher uptake by GALNS-deficient fibroblasts. A 4-fold-increased dose of the SUMF1-GALNS altered the biodistribution.

More activity was detected in all tissues investigated including bone and bone marrow. At the higher dose, enzymatic activity was clearly detectable in the brain, consistent with the recent reports that enzyme reaches the brain when given in higher doses. Several recent studies have shown that longer treatment with higher doses of enzyme resulted in improved therapeutic responses in brain in mouse models of MPS VII, asparaglygcosaminuria and β-mannosidosis (36-38,40). These results indicated that therapeutic enzyme can be delivered across the blood–brain barrier in the adult mouse if administered at higher doses than are used in conventional ERT trials and if the larger dose of enzyme is administered over a sufficient period.

We observed clearance of storage materials in some specific cell types in bone and bone marrow (sinus lining cells, ligaments, connective tissues in articular cartilage, osteoblasts, osteocytes) by a long-term treatment. Limited pathological response in the growth plate region is likely due to difficulty of access to the cartilage cell layers since the region is avascular. Therefore, a specific bone-targeting system may be required to deliver the enzyme to bone (41-43).

The likely explanation for the attenuated phenotype in MPS IVA mice compared with that found in Morquio A patients lies in differences in the amount of synthesis and distribution of KS between murine and human tissue. Two different types of KS (KS I and KS II) are found in human. The synthesis of KS II in the mouse and rat is much reduced (44). The cartilage cells in rodents do not have KS II chains in their aggrecan, which is the major proteoglycan containing KS in other mammals (44). The absence of KS II in murine aggrecan probably explains why MPS IVA mice have almost no skeletal clinical phenotype. KS in mouse serum was 10-50 times less than that in human serum and was actually undetectable in wild-type and even MPS IVA mice by conventional ELISA method previously developed (45,46). However, the mouse model has definite pathological abnormalities in some tissues, including cartilage and connective tissues as well as 5-10 times elevation of serum KS level. This elevation of KS in MPS IVA mouse is contributed mainly by KS I which is contained in aggrecan, fibromodulin and osteoatherin derived from cartilage and bone. Serum KS is normalized after ERT and improvement of pathological findings is observed in ligaments and connective tissues and some types of cells in bone, indicating that the present ERT is effective by clearing KS storage.

Thus, this is also the first report of a sensitive TMS method to quantitate serum KS. Establishment of a sensitive method to detect KS should be useful for monitoring the clinical course and clinical response to treatment in individual MPS IVA patients. The TMS method to assay not only KS but other GAGs such as DS and HS (47) simultaneously should be a powerful tool for screening all types of MPS and monitoring response to therapy.

The availability of a simple method to overexpress and purify recombinant human GALNS (23,24), the generation of GALNS-deficient mice (2,3) and the development of a rapid and sensitive TMS for KS have permitted the evaluation of two GALNS enzymes in the mouse model of MPS IVA. The resulting findings provide the first preclinical evidence in vivo that GALNS administration depletes the accumulated GAGs in the lysosomes of affected tissues and reduces the corresponding elevation in serum KS.

The findings demonstrate that GALNS is delivered to the target sites of pathology in the murine model of Morquio A syndrome. These results provide important preclinical data for the design of GALNS enzyme replacement trials in patients with Morquio A syndrome.

MATERIALS AND METHODS

Murine model

Two types of MPS IVA mice were used for the experiments. One was MPS IVA knockout mouse (Galns \(^{-/-}\)) with partial deletion of exon 2 resulting in the frame shift (2) and the other one was MPS IVA mouse tolerant to human GALNS protein (Galns\(^{tm0b795\times mc765\times jot}\)) (3). Tolerant mice contained both a transgene (cDNA) expressing inactive human GALNS in intron 1 and an active site mutation (C76S) in adjacent exon 2. To study the long-term effectiveness and side effects of therapies in the absence of immune responses, we developed the MPS IVA mouse tolerant to multiple infusions of human enzyme. Both mice were produced by homologous recombination in 129/Sv embryonic stem (ES) cells, by a replacement-type vector containing both the neomycin-resistance gene, for positive selection, and the thymidine kinase gene, for negative selection.

Both affected mice were totally deficient in GALNS activity and progressively accumulated KS in both blood and the lysosomes of most tissues (liver, spleen, heart valves, brain, bone marrow, osteocytes, osteoblasts, peritoneum, ligaments, chondrocytes, connective tissues and kidneys). They had no clinical disease phenotype and survived a normal laboratory life span (>2 years). All experiments were conducted with the highest standards of humane animal care.

Production, purification and characterization of the recombinant human GALNS

Two human GALNSs were produced and purified, in order to compare their pharmacokinetics, biodistribution and pathological and biochemical effects on MPS IVA mice. Two GALNSs, designated ‘native GALNS’ and ‘SUMF1-GALNS,’ were produced by stable, amplified expression of the normal human GALNS cDNA in CHO-K1 cells, respectively. To produce SUMF1-GALNS, CHO cells overexpressing native GALNS were stably transfected with a human SUMF1 cDNA (31,32). Enzyme produced by this modified cell line provided a higher yield with a fully active form. The molecular weights of GALNS and GALNS-SUMF1 were estimated by SDS–PAGE.

The theoretical isoelectric point of GALNS was determined by using Expasy tool at the web site: http://www.expasy.ch/cgi-bin/pi_tool.
Both GALNS enzymes were purified from the medium using a two-column procedure (48). Briefly, batches of medium containing the enzyme were filtered through 0.2 μm capsule filters (Pall Gelman, East Hills, NY). Filtered medium was concentrated 15-fold and then dialyzed against 25 mM sodium acetate and 1 mM β-glycerophosphate buffer (pH 5.5). The concentrated and dialyzed medium containing GALNS enzyme was loaded onto a CM-sepharose CL-6B (Sigma-Aldrich, St Louis, MO) column. The enzyme was eluted with 200 ml of a linear gradient of 0–0.1 M NaCl, pH 5.5. The fractions with GALNS activity were pooled and concentrated. The concentrated fractions were applied to a 400 ml Sephacryl S-100 HR (Amersham Biosciences, Piscataway, NJ) gel filtration column equilibrated with 25 mM sodium acetate, 1 mM β-glycerophosphate and 100 mM NaCl at pH 5.5. The column was eluted with equilibration buffer. Fractions with high GALNS activity were pooled, concentrated and analyzed under denaturing conditions by 10% SDS–PAGE gel. Aliquots of the purified and concentrated GALNS were assayed for enzyme activity using 4-methylumbelliferyl-β-D-galactopyranoside-6-sulphate as a substrate (Moscorden Substrate, Rotterdam, The Netherlands). The enzyme assay was done as described (49).

One unit is defined as the enzyme catalyzing 1 nmol of 4-methylumbelliferyl-β-D-galactopyranoside-6-sulphate per hour. Total protein was determined by micro-Lowry protein assay (50) with bovine serum albumin used as a standard. The enzyme was stored in sterile vials at −80°C until used for infusions.

**In vitro uptake assay**

Human primary GALNS deficient fibroblasts were plated and were allowed to grow to confluence. Each GALNS enzyme was added to the media at a concentration 2000 units/ml in the presence or absence of 2 mM mannose 6-phosphate (M6P) (Sigma-Aldrich, St Louis, MO). After 32 h of incubation at 37°C, the media was removed, the cells were washed three times with 0.9% NaCl and resuspended in 0.1 ml of 1% sodium deoxycholate. GALNS activity and protein were measured.

The uptake efficiency of both GALNS enzymes was also studied by MPS IVA knockout Galns−/− chondrocytes. Protocol for chondrocyte cell culture was as follows. The xiphoid tissues were taken from mice under sterile conditions and washed in Hanks' balanced cell solution. The tissues were incubated in chondrocyte growth medium (CMG) with 0.4% pramoxine for 1 h at 37°C and then incubated with 0.05% bacterial collagenase for 18 h at 37°C. The resultant cells (chondrocytes) were filtered through 80 μm nylon membrane into a vessel with CMG and centrifuged. After washing four times with collagenase free medium, the chondrocytes were used for culture. The cells were used in a monolayer culture right after the explants were taken. To make sure whether the cells still produce type II collagen and have the chondrocytic phenotype, Col2A expression level was measured. After reaching ~90% confluence, the GALNS enzyme solution was added to a final concentration of 2000 units/ml. The inhibitory experiment by adding M6P was the same procedure described above for fibroblasts.

The enzyme activity was expressed in units, and the percent inhibition was calculated as follows: (units without inhibitor − units with inhibitor)/ (units without inhibitor) × 100.

**Optimal pH**

The optimal pH of the enzymes was determined by using the substrate 4-methylumbelliferyl-β-D-galactopyranoside-6-sulphate in 0.1 M NaCl and 0.1 M sodium acetate at pH 3.0, 3.5, 4.0, 4.5, 5.0 and 5.5. GALNS enzyme activity was measured for each enzyme at different pH values for triplicate.

**Injections, specimen collection and enzyme activity**

To compare the pharmacokinetics of the two GALNS enzymes, 3-month-old homozygous mutant MPS IVA knockout mice were used for each group of experiment. The native or SUMF1-GALNS was diluted in PBS and was injected intravenously through the lateral tail vein. Mock-treated mice received PBS.

To determine the clearance of the enzyme from the blood circulation, a dose of 250 units/g of native or SUMF1-GALNS was injected into the tail vein of three 3-month-old mice and blood samples were collected by retro-orbital puncture at different intervals after the infusion. To establish the tissue distribution of the enzyme, three 3-month-old Galns−/− mice treated with 250 units/g of native or SUMF1-GALNS were sacrificed 4 and 24 h later and liver, spleen, kidney, heart, lung, brain, femur bone and bone marrow were obtained. Moreover, three 3-month-old Galns−/− mice treated with 1000 units/g of SUMF1-GALNS were sacrificed 4 and 24 h later. Mice were anesthetized by intraperitoneal injection of 50 μl of a solution of 100 mg ketamine/ml (Sigma-Aldrich, St Louis, MO) and were perfused with 50 ml of 0.9% saline, to remove heme, which interfered with the fluorometric GALNS enzymatic assay. Tissues were homogenized immediately in 1 ml of homogenization buffer consisting of 25 mM Tris–HCl, pH 7.2 and 1 mM phenylmethylsulfonyl fluoride. GALNS assays on dilutions of tissue extracts were incubated for 24 h, and the activity was expressed as units/mg protein, as determined by micro-Lowry assay.

**Treatment protocols**

Three 3-month-old tolerant mice in each group received for 12 weeks infusions (native GALNS: 250 units/g of body weight, SUMF1-GALNS: 250, 600 and 1000 units/g of body weight) through tail vein. Mice were sacrificed 1 week after the last injection, and the organs were removed for histopathology analysis with light or electron microscopy. Three untreated age-matched MPS IVA mice served as controls.

**Pathology: response to ERT**

For morphological evaluation, liver, spleen, kidney, brain, heart, femur and bone marrow from 6-month-old MPS IVA mice treated with 250 units/g of native GALNS (n = 3), 250, 600 and 1000 units/g of SUMF1-GALNS (each dose:
n = 3) or PBS buffer (n = 3) were collected at necropsy, immersion-fixed in 4% paraformaldehyde/2% glutaraldehyde in PBS, post-fixed in osmium tetroxide and embedded in Spurr’s resin. For evaluation of lysosomal storage by light microscopy, toluidine blue-stained 0.5-μm-thick sections were examined. Tissues from the treated and untreated mice were evaluated for reduction in storage without knowledge of their treatment.

KS assay

LC/MS/MS method was used for the analysis of the disaccharides produced from KS (47). API-4000 mass spectrometer equipped with a turbo ionspray was used (Applied Biosystems, Foster City, CA). KS in mouse serum were digested to the disaccharide by keratanase II (Seikagaku Corporation, Tokyo, Japan). Analysis of disaccharides was performed by liquid chromatography (LC) tandem mass spectrometry using multiple reactions monitoring in negative ion mode. Separation of LC was performed on a Hypercarb (2.0 mm i.d. × 150 mM, 5 μm) with a gradient elution of acetonitrile-0.1 M ammonium bicarbonate (pH 10). Flow rate of mobile phase was 0.2 ml/min. This method was applied to the determination of KS in serum of mouse samples.

Conflict of Interest statement

None declared.

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