Efficacy of enzyme replacement therapy in α-mannosidosis mice: a preclinical animal study

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α-Mannosidosis is a lysosomal storage disorder which manifests itself in the excessive storage of mannose-containing oligosaccharides in the lysosomes of multiple peripheral tissues and in the brain. Here we report on the correction of storage in a mouse model of α-mannosidosis after intravenous administration of lysosomal acid α-mannosidase (LAMAN) from bovine kidney, and human and mouse recombinant LAMAN. The bovine and the human enzyme were barely phosphorylated, whereas the bulk of the mouse LAMAN contained mannose 6-phosphate recognition markers. The clearance decreased from bovine to human to mouse LAMAN with plasma half-times of 4, 8 and 12 min, respectively. The apparent half-life of the internalized enzyme was dependent on the enzyme source as well as tissue type and varied between 3 and 16 h. The corrective effect on the storage of neutral oligosaccharides was time-, tissue- and dose-dependent, and the effects were observed to be transient. After a single dose of LAMAN the maximum corrective effect was observed between 2 and 6 days after injection. In general the corrective effect of the human LAMAN was higher than that of the mouse LAMAN and lowest for the bovine LAMAN. Injection of 250 mU human LAMAN/g body weight followed by a subsequent injection 3.5 days later was sufficient to clear liver, kidney and heart from neutral oligosaccharides. Surprisingly a decrease in mannose containing oligosaccharides was also observed in the brain, with storage levels reported at <30% than that found in controls. These data clearly underline the efficacy of enzyme replacement therapy for the correction of storage in α-mannosidosis and suggest that this treatment can substantially decrease storage in the brain.

INTRODUCTION

α-Mannosidosis is a lysosomal storage disorder that is caused by the deficiency of lysosomal α-mannosidase (LAMAN, EC 3.2.1.24) and is known to occur in man (1), cattle (2) and cat (3). The deficiency of LAMAN causes the intralysosomal accumulation of oligosaccharides carrying α1,2-, α1,3- and α1,6-mannosyl residues at their non-reducing termini. These oligosaccharides mainly originate from the intralysosomal degradation of glycoproteins with N-linked oligosaccharides. It should be noted that the stored oligosaccharides originate from the catabolism of dolichol-linked oligosaccharides and from misfolded glycoproteins redirected to the cytosol for degradation by the proteasome (4,5). These oligosaccharides are trimmed in the cytosol and imported into lysosomes by an ATP-dependent mechanism (5). The lysosomal storage is observed in a wide range of cell types and tissues, including neurons in all regions of the brain. The extent of storage, however, has been show to be cell-type and tissue-specific differences. The clinical phenotype of α-mannosidosis is heterogenous, ranging from severe infantile forms to mild juvenile forms with moderate mental retardation, dysostosis multiplex, coarsening of the face, impaired hearing, recurrent infections and mild hepatosplenomegaly (6). Multiple

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mutations are found in human α-mannosidosis, but a genotype–phenotype correlation is not apparent (7). Enzyme replacement and bone marrow transplantation (BMT) are the major therapeutic options in lysosomal storage disorders (8,9). Enzyme replacement therapy (ERT) is an effective means to improve the clinical manifestations in type I Gaucher disease (10) and has meanwhile been approved for several lysosomal storage disorders including some involving the brain (8). Reports on ERT in α-mannosidosis are so far lacking. The few attempts of BMT in human α-mannosidosis have had variable outcomes, but have indicated that successful engraftment can increase language, social and motor skills (11–13). A remarkable success of BMT that included metabolic correction in the brain has been observed in feline α-mannosidosis (14).

To obtain a more accessible model for the study of therapeutic modalities in α-mannosidosis we have generated a mouse model for α-mannosidosis by disrupting the gene for LAMAN. The morphological and biochemical alterations in mouse α-mannosidosis closely resemble those reported in human α-mannosidosis and the phenotype corresponds to a mild form of the human disease (15). Here we report on the evaluation of ERT in mouse α-mannosidosis with LAMAN purified from bovine kidney and from secretions of mammalian cells producing the recombinant mouse or human enzyme.

RESULTS
Characterization of the LAMAN preparations
LAMAN purified from three different species was used for enzyme replacement in α-mannosidosis mice (Fig. 1). The

LAMAN from bovine kidney occurs as a mixture of polypeptides (11–48 kDa) generated by limited proteolysis from a common precursor (16). As expected for a lysosomal hydrolase purified from tissue and thereby exposed to endosomal/lysosomal phosphatase activity, the enzyme exhibited a low content of Man6P-recognition marker. When incubated with a Man6P-receptor affinity matrix, 6.4% of the LAMAN bound to the matrix in a Man6P-dependent manner. The recombinant mouse and human LAMAN were isolated from the secretions of Man6P-receptor deficient mouse fibroblasts and of Chinese hamster ovary (CHO) cells, respectively. The enzymes were largely isolated in their precursor forms, but contained a variable fraction (5–35%) of proteolytically processed forms of about 55 and 70 kDa (Fig. 1). The mouse LAMAN had a higher content of Man6P-recognition marker which mediated binding of 73.6% of the activity to the affinity matrix. Of the human LAMAN only 4.2% bound in a Man6P-dependent manner to the affinity matrix and was expressed as percentage of that in the mock-injected α-mannosidosis mice. Mass spectrometry and sensitivity to jack bean α-mannosidase showed that the oligosaccharides M2–M9 contain 2–9 mannose residues and a single N-acetylgalactosamine residue at the reducing terminus.

Figure 1. Polypeptide pattern of the bovine, mouse and human LAMAN. An amount of 10 μg of LAMAN purified from bovine kidney or the secretions of cells overexpressing the mouse or human LAMAN were separated by SDS–PAGE and stained with Coomassie Blue. The 130 kDa polypeptides (P) seen in the mouse and human LAMAN correspond to the precursors and the polypeptides ranging from 11 to 70 kDa to proteolytically processed forms of LAMAN. The 46–48 kDa polypeptides in the bovine LAMAN represent partially processed intermediates.

Figure 2. Thin-layer chromatography (TLC) of neutral oligosaccharides in liver, spleen, kidney and heart. The fraction of neutral oligosaccharides from equal amounts of tissues was separated by TLC. Lane 1 contains the sample from the mock-injected control mice (+/+(+)), lane 2 from the mock-injected α-mannosidosis mice (−/−), lanes 3–5 the samples from α-mannosidosis mice injected with 100 μl mouse LAMAN/g body weight and killed after 2, 4 and 10 h, respectively (Table 1). Oligosaccharides were detected with orcinol/sulphuric acid and quantified by densitometry. For calculation of the storage the amount of neutral oligosaccharides in α-mannosidosis mice was corrected for that in the mock-injected control mice. The storage in the LAMAN injected α-mannosidosis mice was expressed as percentage of that in the mock-injected α-mannosidosis mice. Mass spectrometry and sensitivity to jack bean α-mannosidase showed that the oligosaccharides M2–M9 contain 2–9 mannose residues and a single N-acetylgalactosamine residue at the reducing terminus.
Corrective effect of a single intravenous injection of the mouse LAMAN

In order to study the short- and the long-term effect of a single dose of LAMAN on the storage of neutral oligosaccharides, α-mannosidosis mice at the age of 9 weeks received 100 mU mouse LAMAN/g body weight intravenously. In order to control for the amount of injected enzyme and its clearance from circulation, blood was taken 5, 30 and 60 min after injection. Then 5 min after injection the LAMAN activity varied by < ± 6% indicating that the mice had received comparable amounts of enzyme. The enzyme was cleared from circulation with a half-life of <20 min. The mice were killed 2, 4 and 10 h after injection, and organ extracts were prepared for determination of the LAMAN activity and neutral oligosaccharides. The maximum activity of LAMAN in the organs was observed 2–4 h after injection (Table 1). In liver the activity was 6–7 times higher than in control mice, and in spleen and heart the maximum values exceeded those of controls. In kidney, at maximum one-fourth of the activity in the control mice was reached. The small activity seen in brain is likely to be due to enzyme located in the vascular system. Between 4 and 10 h after injection the enzyme activity decreased rapidly in liver, spleen and kidney with an apparent half-life of about 3 h. Western blot demonstrated that the internalized precursor of LAMAN was rapidly processed to mature forms (data not shown).

The hallmark of α-mannosidosis is the storage of neutral oligosaccharides in a wide variety of tissues (Fig. 2, lanes 1 and 2). These oligosaccharides contain 2–9 mannose residues and an N-acetylglucosamine residue at their reducing terminus and therefore result from the action of an endoglucoamidasase. The amount of neutral oligosaccharides in liver and spleen decreased progressively with time to 15% and to 7% of that observed in the mock-injected animals, whereas in the kidney and heart the storage was reduced only to about 49% and 74% (Fig. 2).

To determine how long the corrective effect of a single dose of LAMAN persists, mice were examined 4, 8 and 16 days after the injection of 100 mU mouse LAMAN/g body weight. At all time points the LAMAN activity in the organs (spleen, kidney, heart and brain) was in the range of non-treated or mock-injected α-mannosidosis mice, except for liver, where 4 days after injection the LAMAN activity (15.1 mU/g wet weight) was still about 5-fold higher than that in the mock-injected mice. The storage of neutral oligosaccharides in liver, spleen and kidney 4 days after injection was in the range seen 10 h after injection (compare Figs 2 and 3). In heart, storage had decreased from 74% after 10 h to 42% after 4 days. This indicates that the corrective effect seen after 10 h persists for about 4 days in spite of the fact that little or no LAMAN activity is detectable 10 h after injection in organs such as kidney or heart. After 4 days the storage of oligosaccharides clearly began to increase again. The increase observed between day 4 and day 16 after injection corresponded to 20–40% of the storage seen in the mock-injected α-mannosidosis mice (Fig. 3).

Comparison of the clearance and the corrective effect of the bovine, mouse and human LAMAN

To compare the corrective effect of the LAMAN preparations from the three discussed sources mice were injected with a dose of LAMAN that was expected to yield a partial correction. We therefore injected 50 mU LAMAN/g body weight. The bovine and the human LAMAN were rapidly cleared from circulation with half-lives of 4 and 8 min, respectively (Fig. 4). The clearance of the highly phosphorylated mouse LAMAN was slower and at least biphasic. About 85% of the enzymes were cleared with an apparent half-time of 12 min, while the apparent clearance of the remaining fraction was about 47 min (Fig. 4).

The mice were killed 2 days after injection and extracts from liver, spleen, kidney and heart were examined for neutral oligosaccharides (Fig. 5). With the exception of liver, the corrective effect was highest for the human LAMAN and lowest for the bovine LAMAN. The corrective effect of the mouse enzyme was intermediate. Only in liver was the corrective effect of the bovine enzyme (4% of the storage remaining) more pronounced than that of the human enzyme (14% of the storage remaining). In liver the corrective effect of mouse LAMAN was weakest (23% of the storage remaining).

### Table 1. LAMAN activity in tissue extracts of control and α-mannosidosis mice before and after injection of 100 mU mouse LAMAN/g body weight

<table>
<thead>
<tr>
<th>Genotype</th>
<th>LAMAN injected (100 mU/g body weight)</th>
<th>LAMAN (mU/g wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Spleen</td>
</tr>
<tr>
<td>(+/+) (n)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>(−/−) (n)</td>
<td>–</td>
<td>136.7 ± 29.1 (19)</td>
</tr>
<tr>
<td>(−/−) [a]</td>
<td>2 h[a]</td>
<td>2.9 ± 2.5 (21)</td>
</tr>
<tr>
<td>(−/−) [b]</td>
<td>4 h[b]</td>
<td>883</td>
</tr>
<tr>
<td>(−/−) [c]</td>
<td>10 h[c]</td>
<td>197</td>
</tr>
</tbody>
</table>

[a] All values were corrected for the mean α-mannosidase activity in serum at t₀ (2350 mU/ml serum). The correction factors were 0.98, 1.07 and 0.96 for 2.4 and 10 h, respectively.

[a] refers to control mice, [−−] refers to α-mannosidosis mice and n refers to the number of animals investigated.
Corrective effect of human LAMAN at high dose

The comparison of the bovine, mouse and human LAMAN indicated that the poorly phosphorylated human LAMAN had a relatively higher corrective potential in kidney and heart, two organs which are more resistant to metabolic correction than liver and spleen. To evaluate the corrective potential of the human enzyme we injected a single dose of 250 mU LAMAN and analyzed the mice 1–12 days after injection. In liver, storage was fully corrected 1 and 3 days after injection. After 6 and 12 days neutral oligosaccharides started to accumulate again, but reached only about 30% of the storage level before treatment (Fig. 6). Light microscopical examination of the liver revealed the almost complete disappearance of storage vacuoles, which in untreated α-mannosidosis mice are prominent in sinus endothelial cells, Kupffer cells and hepatocytes and reappear 12 days after injection (Fig. 7). In spleen and kidney, the storage of neutral oligosaccharides decreased to 12% and 18%, respectively. It is noteworthy that in spleen and kidney the maximum of correction was observed only after 3 and 6 days, respectively. In both organs neutral oligosaccharides started to reaccumulate 3 and 6 days after the injection (Fig. 5). The neutral oligosaccharides in the brain of α-mannosidosis mice were not affected by the treatment (data not shown).

The human LAMAN activities recovered 24 h after injection were much higher than expected from the experiments with mouse LAMAN. In liver, the human LAMAN activity was still six times higher than in control liver. In spleen and kidney it accounted for 10–15% of that in control. To follow the uptake and stability of human enzyme, the LAMAN activity was determined in tissue extracts prepared 4, 16 and 24 h after injection of 250 mU human LAMAN/g body weight. Less than 20% of the injected LAMAN was recovered after 4 h in the tissues examined (liver 18%, kidney 0.4%, spleen 0.12% and heart 0.04%). When compared with the uptake of mouse LAMAN and taking into account that a 2.5-fold higher amount of the human LAMAN was injected, the activity recovered in liver, kidney and heart 4 h after injection was comparable for both enzyme preparations (compare Tables 1 and 2). Uptake by spleen appeared to be 2–3 times less efficient for the human enzyme. The biggest difference between the human and mouse enzyme was the higher stability of the human LAMAN internalized by liver, kidney and spleen. While the activity of the human LAMAN had decreased in these organs after 24 h to 17–33% of the 4 h value (Table 2), that of the mouse enzyme had decreased to 20–26% of the 4 h value already after 10 h (Table 1).

The corrective effect of the mouse (Fig. 3) and human LAMAN was only transient. Neutral oligosaccharides started to reaccumulate 3–6 days after injection. Increasing the amount of injected enzyme would be a mean to delay the reaccumulation. The corrective effect of a given dose of LAMAN is expected to be higher when administered as two half doses separated by an appropriate interval than as a single dose. Rather than increasing the amount of LAMAN to 500 mU/g body weight we administered two times 250 mU human LAMAN/g body weight each at 7 and 3.5 days prior to the analysis. This resulted in a full correction of the storage in kidney and heart (Fig. 8). Light microscopical examination demonstrated the absence of storage vacuoles in liver (data not shown).
not shown) and in tubular epithelia of kidney (Fig. 9). The residual storage in spleen was <20% of that in the mock-injected α-mannosidosis mice. Most notably, in brain the level of neutral oligosaccharides as quantified by thin layer chromatography (TLC) was only half of that in the brain of the mock-injected α-mannosidosis mice (Fig. 8).

To quantify the neutral oligosaccharides on a molar basis the neutral oligosaccharides extracted from the brain of the mock- and LAMAN-injected α-mannosidosis mice were reacted with 2-anthranilamide, which introduces a fluorescent tag at the reducing termini of the oligosaccharides. The fluorescently labelled oligosaccharides were separated by HPLC and quantified by comparison with an internal standard.

**Figure 6.** Neutral oligosaccharides in tissue extracts of α-mannosidosis mice after injection of a single dose of 250 mU of human α-mannosidase/g body weight. The mice were killed 1, 3, 6 and 12 days after injection. The neutral oligosaccharides in the tissue extracts of liver, spleen, kidney and heart were quantified by TLC and densitometry as in Figures 1 and 2.

**Table 2.** LAMAN activity in tissue extracts of α-mannosidosis mice 4–24 h after injection of 250 mU of human α-mannosidase/g body weight

<table>
<thead>
<tr>
<th>Hours after injection</th>
<th>LAMAN (mU/g wet weight)*</th>
<th>Liver</th>
<th>Spleen</th>
<th>Kidney</th>
<th>Heart</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>2774</td>
<td>157</td>
<td>81</td>
<td>21</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>2639</td>
<td>65</td>
<td>45</td>
<td>8</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>1549</td>
<td>31</td>
<td>26</td>
<td>10</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>796</td>
<td>24</td>
<td>16</td>
<td>6</td>
<td>3.0</td>
<td></td>
</tr>
</tbody>
</table>

All values were corrected for the mean LAMAN activity in serum at t₀ (5012 mU/ml serum). The correction factors were varied between 0.903 and 1.222.

*For LAMAN activity in tissue extracts of the control mice and non-injected mice see Table 1.

mock- and LAMAN-injected α-mannosidosis mice were reacted with 2-anthranilamide, which introduces a fluorescent tag at the reducing termini of the oligosaccharides. The fluorescently labelled oligosaccharides were separated by HPLC and quantified by comparison with an internal standard.
sidosis mice were injected with PBS (A) or 250 mU human LAMAN/g body weight (B) 7 and 3.5 days prior to killing. The outer stripe of the outer medulla is shown. Numerous clear vacuoles are seen in the thick ascending limb (TAL) of Henle’s loop of the mock-injected mouse (A, arrows) in contrast to the LAMAN-treated mouse (B). The proximal straight tubules (PST) are free of pathological vacuoles in either animals. Bars represent 20 μm.

DISCUSSION

Intravenous administration of the missing enzyme has been shown in several animal models of lysosomal storage disorders to effectively reduce the lysosomal storage in multiple visceral tissues (8). In the non-neuronopathic type I Gaucher disease which primarily affects cells of the monocyte/macrophage lineage, ERT with a modified form of the missing glucocerebrosidase that targets mannose receptors in monocytes/macrophage has become a powerful therapeutic option (10). A major problem for ERT of most of the lysosomal storage disorders is the involvement of the central nervous system. The blood–brain barrier apparently prevents the crossing of lysosomal enzymes from the blood to the interstitial space surrounding neuronal and glial cells of the central nervous system (9). In spite of the inaccessibility of neural cells to intravenously administered lysosomal enzyme, ERT may have some indirect beneficial effects on brain function in lysosomal storage disorders. The lysosomal damage may result from the clearance of storage material from brain endothelia and from the meninges which may improve the microcirculation and the flow of the cerebrospinal fluid (17). The assessment of the efficacy of ERT depends on the availability of a suitable animal model and of sufficient amounts of the missing enzyme. The generation of a mouse model for α-mannosidosis and the production of the recombinant LAMAN have made it possible to study the efficacy of ERT in this lysosomal storage disorder.

The major receptor systems responsible for the cellular uptake of circulating lysosomal enzymes are the Mr 300 kDa mannose 6-phosphate/insulin-like growth factor II receptors (MPR 300), which are expressed almost ubiquitously (18), and the mannose receptor, which is specific to cells of the monocyte/macrophage lineage (19). The asialoglycoprotein receptor present on hepatocytes and recognizing terminal galactose/N-acetylgalactosamine residues (20) can also contribute to the clearance, but in general is of lesser importance. This is explained by the predominance of high-mannose type oligosaccharides in lysosomal enzymes. The phosphorylation of mannose residues is an early event in the processing of N-linked oligosaccharides of lysosomal enzyme precursors and impairs their subsequent processing to sialylated complex type oligosaccharides. Lysosomal enzymes isolated from the secretions of cells are therefore enriched in precursor forms containing mannose 6-phosphate residues, whereas lysosomal enzymes isolated from tissues are enriched in forms that have been subjected to limited proteolysis and trimming of their oligosaccharides by phosphatases and glycosidases in lysosomes.

In the present study we have used LAMAN purified from bovine kidney. This enzyme preparation consisted of mature polypeptides with a low mannose 6-phosphate content. The recombinant human LAMAN isolated from the secretion of CHO cells consisted mainly of the precursor form, but was also poorly phosphorylated. The low mannose 6-phosphate content of this preparation is likely to be due to dephosphorylation during isolation. Occasionally, human LAMAN preparations were obtained, of which up to 40% bound to the MRP 300 column (C. Andersson and D.P. Roces, unpublished data). The recombinant mouse LAMAN which was purified from the secretions of mouse embryonic fibroblasts lacking mannose 6-phosphate receptors, had a high content of mannose 6-phosphate and was represented by the precursor

Figure 8. Light microscopy of kidney in the α-mannosidosis mice. α-Mannosidosis mice were injected with PBS (A) or 250 mU human LAMAN/g body weight (B) 7 and 3.5 days prior to killing. The outer stripe of the outer medulla is shown. Numerous clear vacuoles are seen in the thick ascending limb (TAL) of Henle’s loop of the mock-injected mouse (A, arrows) in contrast to the LAMAN-treated mouse (B). The proximal straight tubules (PST) are free of pathological vacuoles in either animals. Bars represent 20 μm.

Figure 9. Neutral oligosaccharides in tissue extracts of the α-mannosidosis mice twice injected with 250 mU human LAMAN/g body weight. Control (lane 1) and α-mannosidosis mice (lanes 2 and 3) were injected with PBS (lanes 1 and 2) or 250 mU human LAMAN/g body weight (lane 3) 7 and 3.5 days prior to analysis. Neutral oligosaccharides were prepared and quantified as in Figures 1 and 2. The migration of oligosaccharides composed of 2–9 mannose residues and a single N-acetylgalcosamine residue (M2–M9) is indicated. Arrows mark orcinol-positive material insensitive to LAMAN. The results in a duplicate set of mice (data not shown) varied by <15%.
2-anthranilamide and separated by HPLC. The position of the GlcNAc4Man3-Gal3 standard and of oligosaccharides composed of 2–9 mannose residues and a single N-acetylglucosamine residue (M2–M9) are indicated.

Figure 10. HPLC-separation of 2-anthranilamide derivatized neutral oligosaccharides from brain. Neutral oligosaccharides from brain of α-mannosidosis mice injected with PBS (upper scan) or 250 mU human LAMAN/g body weight (lower scan) 7 and 3.5 days prior to killing were mixed with 220 pmol GlcNAc-Man3Gal3 as an internal standard, derivatized with 2-anthranilamide and separated by HPLC. The position of the GlcNAcMan3Gal3 standard and of oligosaccharides composed of 2–9 mannose residues and a single N-acetylglucosamine residue (M2–M9) are indicated.

A single intravenous administration of LAMAN led to a rapid and pronounced decrease of the lysosomal storage of neutral oligosaccharides irrespective of the source of the enzyme. The corrective effect was most pronounced in liver. This is also the tissue where the increase of the LAMAN activity relative to that in the wild-type mice was highest. Histological examination revealed a correction of storage in both parenchymal and non-parenchymal liver cells. This was true for the highly phosphorylated mouse LAMAN (data not shown) and for the poorly phosphorylated human LAMAN (Fig. 7). This was unexpected as an earlier study with β-glucuronidase had shown that non-phosphorylated enzyme forms localized almost exclusively to non-parenchymal liver cells, whereas the phosphorylated forms localized to both parenchymal and non-parenchymal liver cells (21). The corrective effect of LAMAN was transient in all tissues. The neutral oligosaccharides started to reaccumulate 2–6 days after the injection.

A remarkable correction of storage was observed when the injection of 250 mU human LAMAN/g body weight, which reduces storage in spleen and kidney by 80–90%, was repeated after 3.5 days. Within 1 week the storage disappeared not only in liver, but also in kidney and heart. More importantly, the concentration of neutral oligosaccharides also decreased in brain to about one-fourth. The latter cannot be attributed to an uptake of LAMAN into neural cells. In mice the blood–brain barrier matures within the first 2 weeks of life, and lysosomal enzymes administered intravenously after this period do not cross the blood–brain barrier (23–31). In the α-mannosidosis mice, the integrity of the blood–brain barrier is preserved. Intravenously administered Evans blue dye did not cross the blood–brain barrier and immunoglobulin G was excluded from extravascular tissue (unpublished data). In the brain homogenates of treated mice we observed trace amounts of LAMAN (Tables 1 and 2). These activities are attributed to extraneural cells, such as endothelia, choroid plexus epithelium and incompletely removed meninges. If it is unlikely that the administered LAMAN gets access to oligosaccharides stored in neuronal and glial cells alternative mechanisms have to be considered. Clearance of oligosaccharides via the blood circulation or via an improved flow of the cerebrospinal fluid may contribute to the decrease of neutral oligosaccharide storage in brain. Careful histological and biochemical analyses of the brain of treated and non-treated α-mannosidosis mice will be required to clarify in which cell types and regions of the brain ERT decreases the storage of neutral oligosaccharides.

In summary, this study yielded encouraging data on the efficacy of ERT in a mouse model of α-mannosidosis. A long-term study based on repeated injection of LAMAN and complemented by ultrastructural analysis of multiple tissues including brain and the measurement of cognitive abilities and motor skills is needed to evaluate the full potential of ERT in mouse α-mannosidosis.

MATERIALS AND METHODS

Expression and purification of the recombinant human LAMAN in CHO cells

Human LAMAN cDNA was isolated from HepG2 cDNA library and subcloned into an expression vector carrying a dihydrofolate reductase gene and the LAMAN cDNA under
the control of the human CMV-promotor was expressed in CHO cells deficient in dihydrofolate reductase. The CHO cells were cultured in a two-compartment CELLine flask (Integra Biosciences Inc.) in serum free ExCell 302 medium (JRH Biosciences) supplemented with 20 mM methotrexate at 37°C in a humidified atmosphere containing 5% CO₂. The medium was diluted using a Pellicon Biomax polysulphone filter with a 100 kDa cut-off against 4 vol 0.02 M Tris–HCl, pH 7.6. Ion-exchange chromatography was performed on DEAE-Sepharose FF (Amersham Pharmacia Biotech AB) using a NaCl gradient in 0.02 M Tris–HCl, pH 7.6. Active fractions were concentrated using an Amicon Centricon Plus-80 centrifugation filter with a 30 kDa cut-off and subjected to gel filtration in a HiPrep 26/60 Sephacryl High Resolution column (Amersham Pharmacia Biotech AB) in 0.02 M Tris–HCl, pH 7.6, containing 0.15 M NaCl. After concentration the final preparation had a specific activity of 9–15 U/mg.

Expression and purification of the recombinant mouse LAMAN

The cDNA encoding the mouse LAMAN (27) was subcloned in the expression vector pMPSVEH (28). A polyhistidine tail (six residues) had been added to the C-terminal part of the enzyme. Mouse embryonic fibroblasts deficient in the small and large mannose 6-phosphate receptors (mpr–/− MEF) (29) were transfected and stably expressing clones were selected with 50 μg/ml hygromycin. For production of the recombinant mouse LAMAN the cells were cultured in medium supplemented with 10% FCS in a humidified atmosphere containing 5% CO₂. The secreted recombinant mouse LAMAN was purified from conditioned medium using a three-step procedure. In the first step the medium was dialyzed against 20 mM sodium phosphate buffer pH 7.8 containing 500 mM sodium chloride and then loaded onto a Probond column (Invitrogen). The retained enzyme was eluted with a gradient 0–0.35 M of imidazole (total volume 80 ml) in 20 mM sodium phosphate buffer pH 6.0 containing 500 mM sodium chloride. The LAMAN containing fractions were dialyzed against 10 mM sodium phosphate, pH 6.0 and loaded onto a DEAE–cellulose. The enzyme was eluted in a 0–0.25 M sodium chloride gradient (total volume 80 ml) in 10 mM sodium phosphate buffer. Finally mouse LAMAN was adsorbed to ConA-Sepharose (loading buffer 20 mM Tris–HCl, pH 7.4, containing 1 mM MgCl₂, 1 mM MnCl₂, 1 mM CaCl₂ and 0.5 M NaCl), and eluted with α-mannopyranoside (0.0–1.0 M) in the same buffer. The final preparation had a specific activity of 17–25 U/mg.

Purification of bovine LAMAN

Bovine LAMAN was purified from kidney as described (16). The final preparation had a specific activity of 10 U/mg.

Determination of phosphorylation of LAMAN

LAMAN was incubated overnight with a MPR 300 affinity matrix as described (30). LAMAN activity was determined in the unbound fraction and the fractions eluted with 5 mM glucose 6-phosphate and 5 mM mannose 6-phosphate.

Injection of the mice

LAMAN was injected into the tail vein of 8–14-week-old α-mannosidosis mice (final volume up to 5.3 μl/g body weight). Mock-injected mice received the same volume of 10 mM phosphate, pH 7.4 in 0.15 M NaCl (phosphate-buffered saline PBS). In a single experiment the mice originating from up to three litters did not differ in age. Then 5 min after injection, blood was taken from the retro-orbital plexus to control for the amount of injected enzyme. Serum was prepared and stored at −20°C.

Preparation of organ extracts

Mice were anaesthetized with 20 μl of a solution of 10 mg/ml Ketavet (Parke Davis) and 2 mg/ml Rompun (Bayer) in 0.15 M NaCl and perfused with PBS. Organs (liver, spleen, kidney, heart and brain) were collected and stored at −78°C. About 50–70 mg of each organ were homogenized at 4°C in 9 vol (per weight) of 10 mM Tris–HCl, 150 mM NaCl, 1 mM PMSF (in isopropanol), 1 mM iodoacetamide and 5 mM EDTA. Triton X-100 was added to a final concentration of 0.5% w/v (1% for liver). After incubation for 30 min on ice, the samples were sonicated and then centrifuged for 15 min at 13 000g. The supernatant was stored at −20°C.

Enzyme assays

For determination of the LAMAN activity in the organ extracts and in the serum, 10–50 μl of enzyme sample was incubated in 0.2 ml of 0.2 M sodium citrate pH 4.6, 0.08% NaN₃, 0.4% BSA, 0.15% NaCl, and 10 mM p-nitrophenyl-α-mannopyranoside as substrate for 0.5–5 h at 37°C. An amount of 1 ml of 0.4 M glycine/NaOH, pH 10.4 was added to stop the reaction. Absorbance was read at 405 nm (ε = 18 500 M⁻¹ cm⁻¹). All the determinations were done in duplicate and with the appropriate blanks.

Western blotting

For western blotting of human LAMAN 20–40 μg of protein was separated on a 10% SDS–PAGE. After SDS–PAGE electrophoresis, the proteins were transferred to PVDF membranes using a semi-dry blotting system. Transfer efficiency was checked with Ponceau staining. The membranes were subsequently blocked with 5% skimmed milk powder and incubated with a rabbit antiserum raised against the recombinant human LAMAN. After washing in PBS, 0.1% Tween 20, the blots were incubated with horseradish peroxidase (HRP) coupled secondary antibodies. Signals were visualized using the ECL-Detection System (Amersham, Freiburg, Germany).

Isolation of neutral oligosaccharides

Tissue samples (50–60 mg) were cut into small pieces and homogenized with 0.6 ml H₂O (HPLC grade) at 4°C. After freezing (−20°C) and thawing twice and ultrasonic treatment, proteins were precipitated by the addition of 4 vol methanol and extracted by the addition of 4 vol chloroform/H₂O (1:3) (31). The supernatant was desalted by incubation for 1 h at
4°C with mixed-bed ion-exchange resin (AG 501-X8, 20–50 mesh). The unbound material was lyophilized and resuspended in water (1 ml/mg tissue).

Separation of mannose oligosaccharides by TLC
Neutral oligosaccharides extracted from equal aliquots of tissue were loaded onto TLC plates (20 x 20 Silica gel F60, Merck). After drying the plates at room temperature for ~1 h, the oligosaccharides were separated by developing overnight with n-butanol/acetic acid/H2O (100:50:50). After drying (~1 h at room temperature and then 5 min at 110°C), the plates were developed for 4 h in n-propanol/nitromethane/H2O (100:80:60). For staining, the plates were sprayed with 0.2% orcinol in H2SO4 (20% in water), and heated at 110°C. The size of the oligosaccharides was determined by MALDI-TOF.

Digestion of oligosaccharides with α-glucosidase or jack bean α-mannosidase
To avoid the interference with glycogen derived oligosaccharides 20 μl of the oligosaccharide extracts from liver and heart were incubated overnight at 37°C with 40 U/ml α-glucosidase from Bacillus steathermophilus (Sigma) in 20 mM phosphate, pH 6.8. The incubation mixture was heated at 96°C to denature the proteins. After centrifugation for 10 min at 13 000g, the supernatant was desalted by incubation for 1 h at 4°C with an ion-exchange resin (AG 501-X8, 20–50 mesh), lyophilized and resuspended in 20 μl of water and then separated by TLC.

To verify the nature of the oligosaccharides, 20 μl of the oligosaccharide extracts were incubated overnight at 37°C with 30 U/ml α-mannosidase from jack bean (Sigma) in 0.1 M sodium acetate, pH 5.0, containing 2 mM ZnCl2. After incubation the oligosaccharides were prepared as described for samples digested with α-glucosidase and separated by TLC.

Quantitative analysis of neutral oligosaccharides in organs
The oligosaccharides (0.3 μl) were mixed with 220 pmol of a decasaccharide which served as an internal standard and had the composition GlcNAcMan3Gal1. The mixture was lyophilized and resuspended in 5 μl of a DMSO/acetic acid (7:3) containing 0.34 μl 2-anthranilamide (Aldrich) and 1 μl NaBH3CN (Fluka). After incubation for 2 h at 65°C, the samples were purified by paper chromatography (developed with ethyl acetate). The oligosaccharides, which remain at the starting point, were extracted by sonication of the paper in water. After lyophilization and dissolving in 300 μl acetonitrile/80 mM ammonium formate pH 4.4 (65:35), the oligosaccharides were loaded onto a Gluco-Sepharose column (Ludger) and eluted with the acetonitrile/ammonium formiate buffer at a flow rate of 0.4 ml/min. Fluorescence (excitation 350 nm, emission 450 nm) was recorded (Shimadzu, RF-10A XL) and the mass of the oligosaccharides determined by MALDI-TOF.

MALDI-TOF
Samples from HPLC fractions were lyophilized and dissolved in 2–3 μl water. Water extracts from TLC plates were derivatized with 1-phenyl-3-methyl-5-pyrazolone and dissolved in 2–3 μl water. 2,5-Dihydroxybenzoic acid (DHB, 5 mg/ml in water) was used as matrix. DHB (0.5 μl) and 1 μl of sample were spotted onto the Anchorchip target (Bruker Daltonik), and dried under room temperature. Mass spectrometric analysis was performed on a Reflex III MALDI-TOF (Bruker Daltonik) with a 337 nm UV laser.

Histological examinations
For electron microscopy, small sections of liver and kidney collected at killing time were immersed in 0.1 M phosphate, 6% glutaraldehyde pH 7.4. Tissue samples were post-fixed with 2% osmium tetroxide, dehydrated and embedded in Araldite. Semi-thin sections were stained with toluidine blue. Ultrathin sections were processed according to standard techniques. For histochemical investigations, the sections were immersed with Bouin’s solution diluted 1:4 in 10 mM phosphate pH 7.4, 0.15 M NaCl. Embedding was performed in low melting point paraffin (Wolff, Wetzlar, Germany). Serial sections (7 μm) were cut and mounted on glass slides covered with Biobond (British Biocell, London, UK). Central sections of each series were stained with haematoxylin and eosin for standard light microscopy.

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