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ARTICLE

Human acid α-glucosidase from rabbit milk has therapeutic effect in mice with glycogen storage disease type II

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Pompe's disease or glycogen storage disease type II (GSDII) belongs to the family of inherited lysosomal storage diseases. The underlying deficiency of acid α-glucosidase leads in different degrees of severity to glycogen storage in heart, skeletal and smooth muscle. There is currently no treatment for this fatal disease, but the applicability of enzyme replacement therapy is under investigation. For this purpose, recombinant human acid α-glucosidase has been produced on an industrial scale in the milk of transgenic rabbits. In this paper we demonstrate the therapeutic effect of this enzyme in our knockout mouse model of GSDII. Full correction of acid α-glucosidase deficiency was obtained in all tissues except brain after a single dose of i.v. enzyme administration. Weekly enzyme infusions over a period of 6 months resulted in degradation of lysosomal glycogen in heart, skeletal and smooth muscle. The tissue morphology improved substantially despite the advanced state of disease at the start of treatment. The results have led to the start of a Phase II clinical trial of enzyme replacement therapy in patients.

INTRODUCTION

The clinical spectrum of glycogen storage disease type II (GSDII) comprises infants, children and adults. All patients characteristically have acid α-glucosidase deficiency and suffer from progressive skeletal muscle weakness and wasting (1,2). Affected infants lack residual enzyme activity and show cardiomegaly as a conspicuous additional symptom. They die of cardiorespiratory failure within the first 2 years of life. The residual enzyme activity of affected children and adults slows the progression of the disease. Respiratory insufficiency with concomitant complications is the most serious and life-threatening problem in these patients.

There is currently no treatment for this fatal disease, but a series of *in vitro* and *in vivo* studies has demonstrated the feasibility of receptor-mediated enzyme replacement therapy (3–6). Subsequently, cell culture and transgenic animal technology were explored to produce the required recombinant human acid α-glucosidase (rhGAA) on a large scale (7–10). Recent studies have indicated that the enzymes produced in the different systems have the proper characteristics for therapeutic application (10,11). Based on favorable results with production of rhGAA in the milk of transgenic mice, we have chosen transgenic rabbits to realize the industrial production of rhGAA. The efficacy of this product has been demonstrated in mice with GSDII. This paper describes the biochemical and morphological effects after 6 months of weekly i.v. enzyme infusions.

RESULTS

To effect production of human acid α-glucosidase in the milk of transgenic rabbits, we have used a transgene construct that resulted in high expression of human acid α-glucosidase in the milk of transgenic mice (10). The construct consists of the human acid α-glucosidase gene cloned behind the bovine αS1-casein promoter. Transgenic rabbits of the selected strain produce up to 8 g rhGAA/l milk. The purified rhGAA has an apparent molecular mass of 110 kDa (Fig. 1a) and thereby is comparable to the acid α-glucosidase precursor secreted in human urine (12).

Short-term pilot experiments

The therapeutic effectiveness of the purified rhGAA was assessed in acid α-glucosidase-deficient knockout (KO) mice

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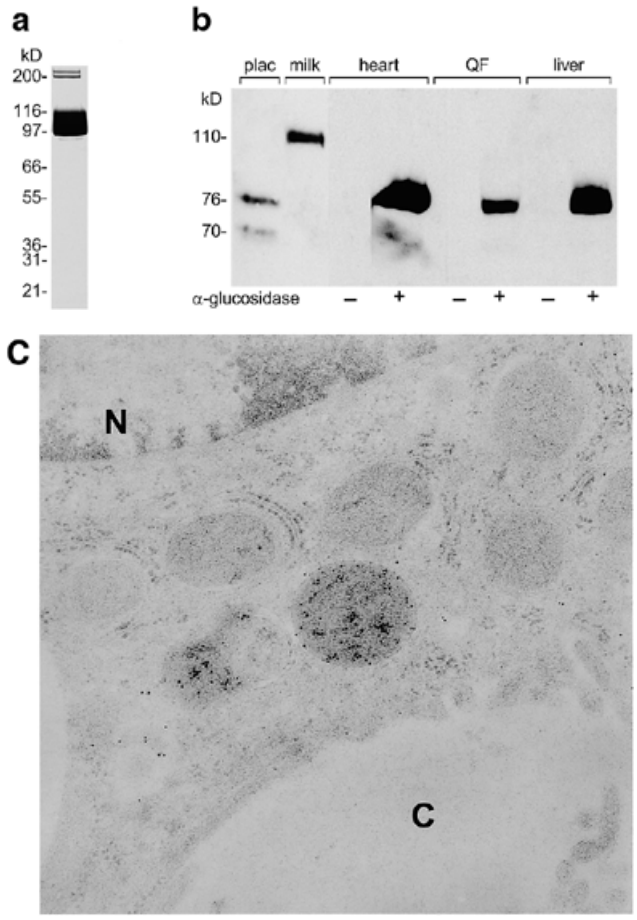

Figure 1. SDS–PAGE analysis of purified rhGAA, conversion to mature enzyme in recipient mouse tissues and lysosomal localization. (**a**) Purified rhGAA (32 μg), stained with Coomassie brilliant blue, appears on a non-reduced 4–12% gradient gel as a single molecular species of 110 kDa, known as the acid α-glucosidase precursor (the 220 kDa doublet results from aggregation). (**b**) Conversion of the 110 kDa precursor to the mature 76 kDa enzyme in heart, skeletal muscle (quadriceps femoralis) and liver of a GSDII KO mouse. The mice received four injections. The tissues were analyzed for the presence of rhGAA by western blotting after immunoprecipitation with rabbit antibodies directed against purified human placental acid α-glucosidase. The enzyme on the blot was visualized with polyclonal murine antibodies against human acid α-glucosidase using chemiluminescence. Human placental acid α-glucosidase and rhGAA from rabbit milk are used as markers. (**c**) The intralysosomal localization of acid α-glucosidase in hepatocytes of treated KO mice is demonstrated using immunoelectron microscopy in 60 nm Lowicryl sections, using the same rabbit antibodies as described in (b). N, nucleus; C, bile canaliculus.

in a series of different experiments. A single enzyme dose (17 mg/kg) given to three FVB KO mice resulted, 2 days after administration, in normalization of acid α-glucosidase activity in all tissues except brain. In another initial test, one C57Bl/6 (B1 in Table 1) and two FVB KO mice (F1 and F2 in Table 1) received four rhGAA injections (40–68 mg/kg) within a 6 day interval. An acid α-glucosidase activity level of 20–60 times wild-type (WT) was reached in the liver of treated mice. The activities in heart and skeletal muscle were increased to three times the level in WT mice. The 76 kDa mature form of acid α-glucosidase was recovered from the target tissues and not the administered 110 kDa precursor (Fig. 1b). This provides evidence for uptake of the precursor in endosomes and lysosomes, in which organelles the 76 kDa mature enzyme is formed as a natural result of post-translational modification. A further demonstration of uptake in lysosomes is given in Figure 1c, showing acid α-glucosidase in the lysosome of a hepatocyte by immunoelectron microscopy. Uptake of the enzyme by liver and heart was accompanied by reduction in the glycogen content, but no such effect was seen in skeletal muscle (Table 1).

Long-term treatment

Encouraged by these results we decided to subject 6-month-old KO mice to long-term treatment (14–25 weeks). The protocol included 16 (12 FVB and four C57Bl/6) mice, half of which received rhGAA and the others placebo, once every week. The first injection dose was 68 mg/kg; the following doses were 17 mg/kg. One day after the 13th injection blood samples were taken to assess potential immune responses against the administered rhGAA. None of the C57Bl/6 mice had developed an antibody titer, but a titer was measured in serum of all treated FVB mice (~20% of the injected enzyme was immunoprecipitable per calculated total serum volume).

Three enzyme-treated and three placebo-treated [phosphate-buffered saline (PBS)] FVB mice (F4–F6 and F7–F9, respectively, in Table 2) were killed at this stage to evaluate the therapeutic effectiveness of enzyme administration. The acid α-glucosidase activity in liver of all treated mice was far above normal (>120 times) and the liver glycogen content had normalized (Table 2). Only one of the three mice, however, had increased enzyme activity in heart and skeletal muscle and this increase was not accompanied by a lowering of the glycogen concentration in these tissues.

It was reasoned that the effectiveness of enzyme replacement therapy could possibly be improved if we could succeed in counteracting the immunological response. To this end, we raised the enzyme dose 4-fold for both FVB and C57Bl/6 mice (although the latter did not have an antibody titer). After four additional high dose injections, the FVB mice (F10–F12 in Table 2) were analyzed and showed 60 times the WT acid α-glucosidase activity in liver, four times the WT activity in heart and five times the WT activity in skeletal muscle compared with untreated littermates (F13–F15 in Table 2). The lysosomal glycogen store of the liver was fully depleted. The glycogen content of the heart was reduced by 39% (on average 288 μg/mg in treated versus 470 μg/mg in untreated knockout mice) and an average reduction of 10% was observed in skeletal muscles (125 μg/mg treated versus 140 μg/mg untreated).

Two C57Bl/6 KO mice (B3 and B4) continued to receive weekly injections of 68 mg/kg until week 25, despite the detection of low antibody titers after the 20th injection (~5% of injected enzyme was immunoprecipitable per calculated total serum volume). Two days after the last injection, the acid α-glucosidase activity in all tissues of these mice was above normal (Fig. 2a and b), except for kidney (36% of WT) and brain (2% of WT). Importantly, the glycogen content of all tissues, except brain, had either reversed to normal or was significantly reduced when compared with the values measured in placebo-treated mice (B5 and B6) (Fig. 2c).

Comparison of tissue sections of treated and untreated animals provided equally convincing evidence for the effectiveness of enzyme replacement therapy. This is illustrated in Figure 3 (skeletal muscle), Figure 4 (heart, smooth muscle and

Table 1. Acid α-glucosidase activity and glycogen content in tissues of KO mice after short-term treatment

	F1, α-glu	F2, α-glu	F3, PBS	B1, α-glu	B2, PBS	WT
Acid α-glucosidase activity (nmol/mg/h)						
Liver	1132	944	2.0	3375	2.0	50
Heart	24	10	0.3	60	0.2	16
Biceps	125	46	0.9	49	1.2	8.3
Liver	1132	944	2.0	3375	2.0	50
Glycogen content (μg/mg protein)						
Liver	70	13	147	23	406	12
Heart	1082	1259	1748	1971	3233	1
Muscle	86	116	87	90	86	1

epithelial cells of ducts of salivary gland) and Figure 5 (electron microscopy of heart muscle). Clearance of glycogen-containing vacuoles was observed in almost all muscle fibers of the gastrocnemius (Fig. 3a and b compared with c and d), the quadriceps femoralis (data not shown) and the longitudinal and circular skeletal muscle layers around the esophagus (Fig. 3f and h). Most muscle fibers had regained normal morphology. The presence of long arrays of central nuclei in many fibers suggests that dividing and differentiating satellite cells participate in the repair process (Fig. 3c, d and g). The pectoralis major showed partial correction with intra- and interfiber segmental variation (Fig. 3g compared with e). The correction of cardiomyocytes was impressive in some areas of the heart (Figs 4a and d and 5), but cells staining with periodic acid–Schiff (PAS) were still present in other areas. Smooth muscle cells of arteries and veins, prominent sites of glycogen storage in GSDII, had also lost most of their lysosomal glycogen, but were not in all instances fully corrected (Fig. 4b and e). Smooth muscle of the bladder showed little response as judged by the intensity and distribution of PAS staining, but degradation of lysosomal glycogen became evident using electron microscopy (data not shown). In contrast, smooth muscle of the stomach and digestive tract responded very well to enzyme administration with a dramatic reduction in PAS staining (Fig. 4c, f, g and j). A summary of the corrective effect of enzyme infusion is given in Table 3.

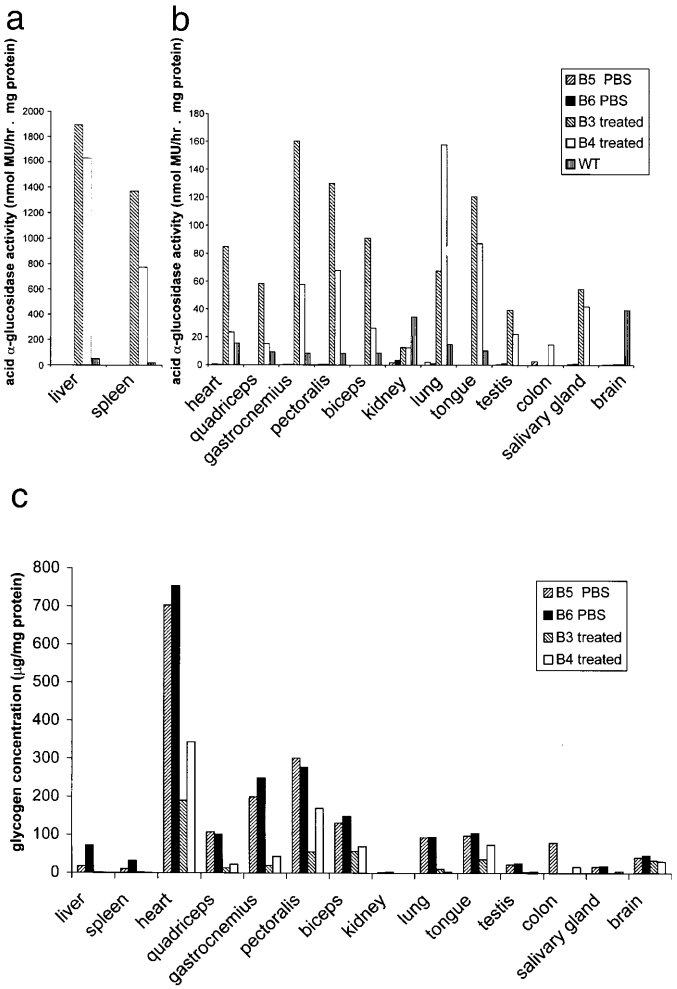

Figure 2. Acid α-glucosidase activity and glycogen content of mouse tissues after 25 weeks treatment with rhGAA. (**a** and **b**) The acid α-glucosidase activity was determined with the artificial substrate 4-methylumbelliferyl-α-D-glucopyranoside (MU) in liver and spleen (a) and other tissues (b). (**c**) The glycogen content was measured as described in Materials and Methods. Wild-type levels of testis, colon and salivary gland were not determined. Wild-type levels of other tissues were <5 μg/mg protein (data not shown).

Table 2. Acid α-glucosidase activity and glycogen content in tissues of KO mice after long-term treatment

	13 injections						17 injections						WT
	F4, α-glu	F5, α-glu	F6, α-glu	F7, PBS	F8, PBS	F9, PBS	F10, α-glu	F11, α-glu	F12, α-glu	F13, PBS	F14, PBS	F15, PBS	
Acid α-glucosidase activity (nmol/mg/h)													
Liver	832	700	609	1.7	1.8	2.2	3018	3225	1871	4.0	3.2	2.3	50
Heart	6	0.9	1.9	0.6	0.5	0.6	51	61	64	0.6	0.3	0.5	16
Pectoralis	25	0.7	1.0	1.1	1.1	0.6	76	48	22	0.9	0.5	0.8	8.3
Quadriceps	15	1.0	0.9	0.8	0.7	0.5	24	52	13	0.6	0.3	0.5	
Gastrocnemius	40	1.0	0.8	0.5	0.6	0.5	33	75	24	0.5	0.4	0.4	
Glycogen content (μg/mg protein)													
Liver	3	2	3	15	35	22	4	0.3	–0.2	9	22	19	12
Heart	97	89	73	80	101	153	322	344	198	366	510	541	1
Pectoralis	68	66	109	46	62	90	121	127	111	96	71	172	1
Quadriceps	36	50	33	28	62	61	126	131	127	162	136	187	
Gastrocnemius	62	82	53	51	76	55	135	134	126	126	115	184	

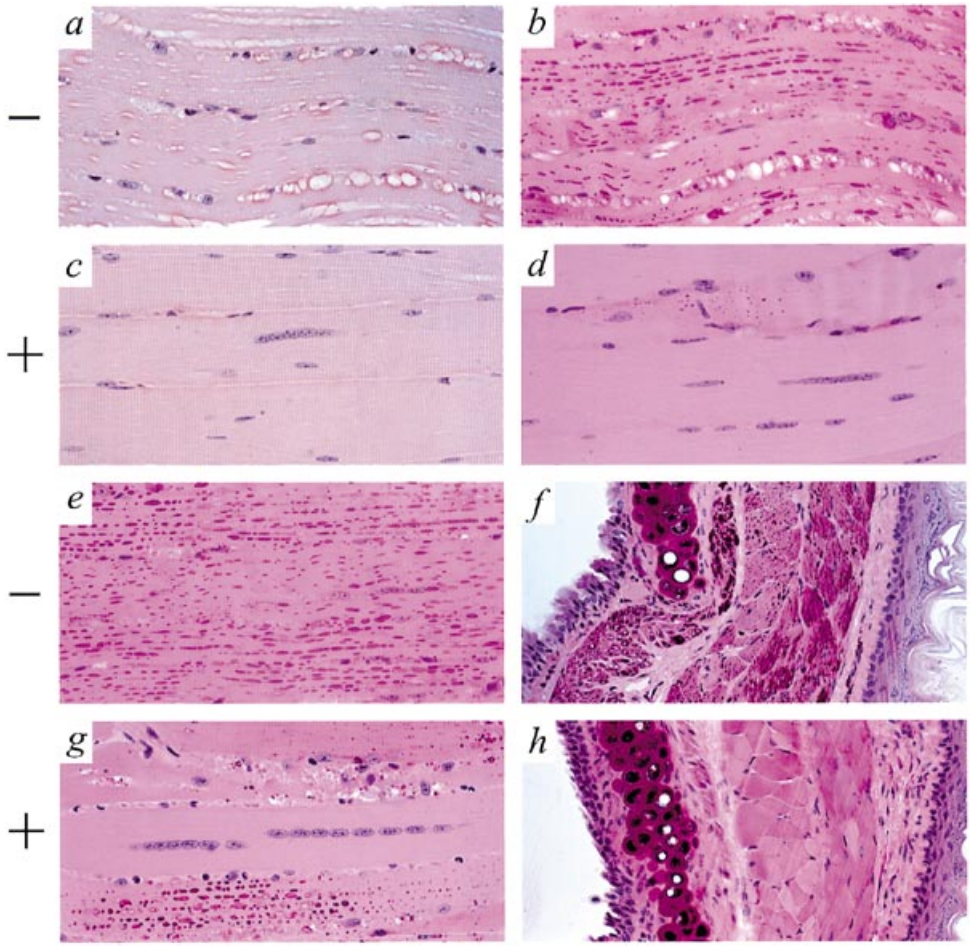

Figure 3. Correction of glycogen storage and improvement in skeletal muscle morphology of KO mice after prolonged treatment with rhGAA. (**a**–**d**) Gastrocnemius; (**e** and **g**) pectoralis; (**f** and **h**) esophagus and trachea. Sections (a) and (c) were stained with hematoxylin azofloxin, all others were stained with PAS to demonstrate glycogen storage. Sections (a), (b), (e) and (f) were from placebo-treated mice (–) and sections (c), (d), (g) and (h) from rhGAA-treated mice (+).

DISCUSSION

Enzyme replacement therapy for lysosomal storage diseases is not a novel concept. The first speculations about the potential effectiveness of this type of therapy were made soon after the discovery that most lysosomal storage diseases are caused by lysosomal enzyme deficiencies (13–16). Close to 10 years of investigations and unsuccessful clinical trials went by before the idea of enzyme replacement therapy was largely abandoned (17). In retrospect, the disappointing results from those early days can be ascribed to the use of low doses of enzymes with unfavorable characteristics regarding immunotolerance and cellular targeting.

At present, the development of enzyme replacement therapy is again being actively pursued, with the focus on receptor-mediated endocytosis and the use of human recombinant enzymes. These efforts have been stimulated by the successful clinical application of receptor-mediated enzyme replacement therapy for Gaucher disease and the promising outcome of exploratory studies for other lysosomal diseases, including GSDII (5,8,10,11,18–21).

Enzyme replacement therapy requires a continuous supply of enzyme, but the natural sources are limited by demands of safety, species specificity and efficacy. The enzyme has to be stable and reach the lysosomes in the target tissues. It has to remain catalytically active and, preferably, should not elicit immunogenic responses. These characteristics are obtained by adequate post-translational modifications, and production in mammalian cells is the best option. The production of rhGAA has been explored in the milk of transgenic mice and in CHO cells and both systems seem attractive (7–10).

We have now made the step to large-scale enzyme production in the milk of transgenic rabbits and obtained production levels that are sufficiently high for clinical application (up to 8 g rhGAA/l rabbit milk). The therapeutic efficacy of the product has been demonstrated in several ways. The target tissues are reached, since the acid α-glucosidase activity in muscle, heart and other organs is increased by i.v. enzyme administration. The enzyme is transported to lysosomes, as illustrated indirectly by maturation of the 110 kDa recombinant precursor to mature 76 kDa enzyme and directly by immunoelectron microscopy and lowering of the glycogen concentration in the tissues. The lysosomal pathology is diminished and the tissue morphology is significantly restored despite the advanced pathology at the start of treatment. This is an important observation, because patients with GSDII are usually diagnosed after onset of clinical symptoms (2).

Table 3. Improvement of tissue morphology after prolonged enzyme treatment, as judged by light (LM) and electron microscopy (EM)

	LM	EM
Skeletal muscle		
Quadriceps	+++	
Gastrocnemius	++++	
Pectoralis	+	+
Triceps	+	
Diaphragm	+	++
Tongue	+	
Esophagus	+++	
Heart and smooth muscle		
Heart	++	++
Vessels[a]	++/–	+++/+
Lung	++	
Stomach	++++	
Ileum	+++	
Jejunum	++++	
Duodenum	+++	
Colon	+++	
Rectum	++++	
Bladder	–	+
Other tissues		
Liver	+++	+++
Spleen	++++	
Kidney	++++	
Salivary gland	++++	
Epidydimus	+++	
Plexi of Meissner and Auerbach	+++	
Cartilage	–	
Adipose tissue	+++	
Brain	–	

The effect of enzyme treatment was assessed semi-quantitatively by comparing LM and EM tissue sections of enzyme-treated (n = 2) and placebo-treated (n = 2) mice.
–, no difference between enzyme-treated and placebo-treated; +, local effects; ++, obvious differences; +++, very clear differences; ++++, tissue morphology close to normal.
[a]The effect varied in different vessels.

Although neurological symptoms have not been documented in GSDII, the inability of the enzyme to cross the blood–brain barrier remains a point of concern. Mental and motor developmental milestones need to be monitored closely if enzyme replacement therapy is to expand the lifespan of affected infants.

Based on our results, speculation can be made as to the prospects of enzyme replacement therapy in humans using rhGAA from rabbit milk. It is reassuring that this recombinant human enzyme is well tolerated by acid α-glucosidase-deficient mice over a relatively long period. The anaphylactic-type reaction (see Materials and Methods), observed after the third consecutive enzyme administration, was manageable by giving the mice clemastinum as a premedication. A similar premedication is advised for patients with Gaucher disease who are on enzyme therapy and react to imiglucerase. The different results obtained in our studies with FVB and C57Bl/6 mice suggest that the formation of neutralizing antibodies to recombinant human α-glucosidase can be a complication. The risk of antibody formation to the human enzyme is higher in (KO) mice than in humans and lower for juvenile and adult patients, with residual enzyme activity, than for infants, without activity. The recently published immuno-surveillance studies in Gaucher disease (22) may serve as an indicator: 13% of the patients receiving enzyme replacement therapy were reported to have an antibody response. Only a very small number of them developed true neutralizing antibodies and 90% became tolerant over time (22).

In summary, we have accomplished the large-scale production of rhGAA in the milk of transgenic rabbits and have shown the therapeutic potential of this enzyme in KO mice. While these studies were in progress, a Phase I clinical trial was completed, assessing the safety, tolerance and pharmacokinetics of rhGAA in healthy volunteers. These combined results have set the stage for a Phase II clinical trial on the safety and potential efficacy of rhGAA in patients with GSDII.

MATERIALS AND METHODS

KO mice

The KO mice used in this study were obtained by targeted disruption of exon 13 of the *Gaa* gene and have a complete deficiency of acid α-glucosidase (23). The mice were backcrossed for two generations in either an fvb or C57Bl/6 background. They were genotyped by PCR analysis. The mice were housed in a controlled facility and fed regular chow *ad libitum*.

Transgenic rabbits and rhGAA production

Expression cassette c8agluEx1 was used to generate transgenic rabbits (10). The rabbits were genotyped by PCR analysis and Southern blotting and producer animals were selected on the basis of high rhGAA activity levels in the milk. Recombinant human acid α-glucosidase was purified from the milk in a five-step procedure. In short, milk was defatted by low speed centrifugation and caseins were removed by tangential flow filtration. Acid α-glucosidase was purified from the resulting whey fraction by chromatography on an anion exchanger (QFF), two hydrophobic interaction columns (Phe HP and Source 15Phe), and stored frozen below –50°C.

SDS–PAGE and western blotting

The purified enzyme was analyzed by SDS–PAGE on a 4–12% gradient gel (Novex, San Diego, CA). For analysis of acid α-glucosidase in mouse tissues, the enzyme was immunoprecipitated from tissue homogenates with rabbit antibodies raised against human placental acid α-glucosidase complexed to protein A–Sepharose 4B as described (9). The complex was washed, boiled in sample buffer and applied to 8% SDS–polyacrylamide gels. Acid α-glucosidase was blotted onto nitrocellulose filters and visualized with mouse anti-human placental acid α-glucosidase antibodies using the enhanced chemiluminescence (ECL) detection kit (Amersham, Roosendahl, The Netherlands).

Treatment protocol

rhGAA from rabbit milk was brought to final concentrations of 2.5 (low dose) and (high dose) 10 mg/ml in PBS, filter sterilized

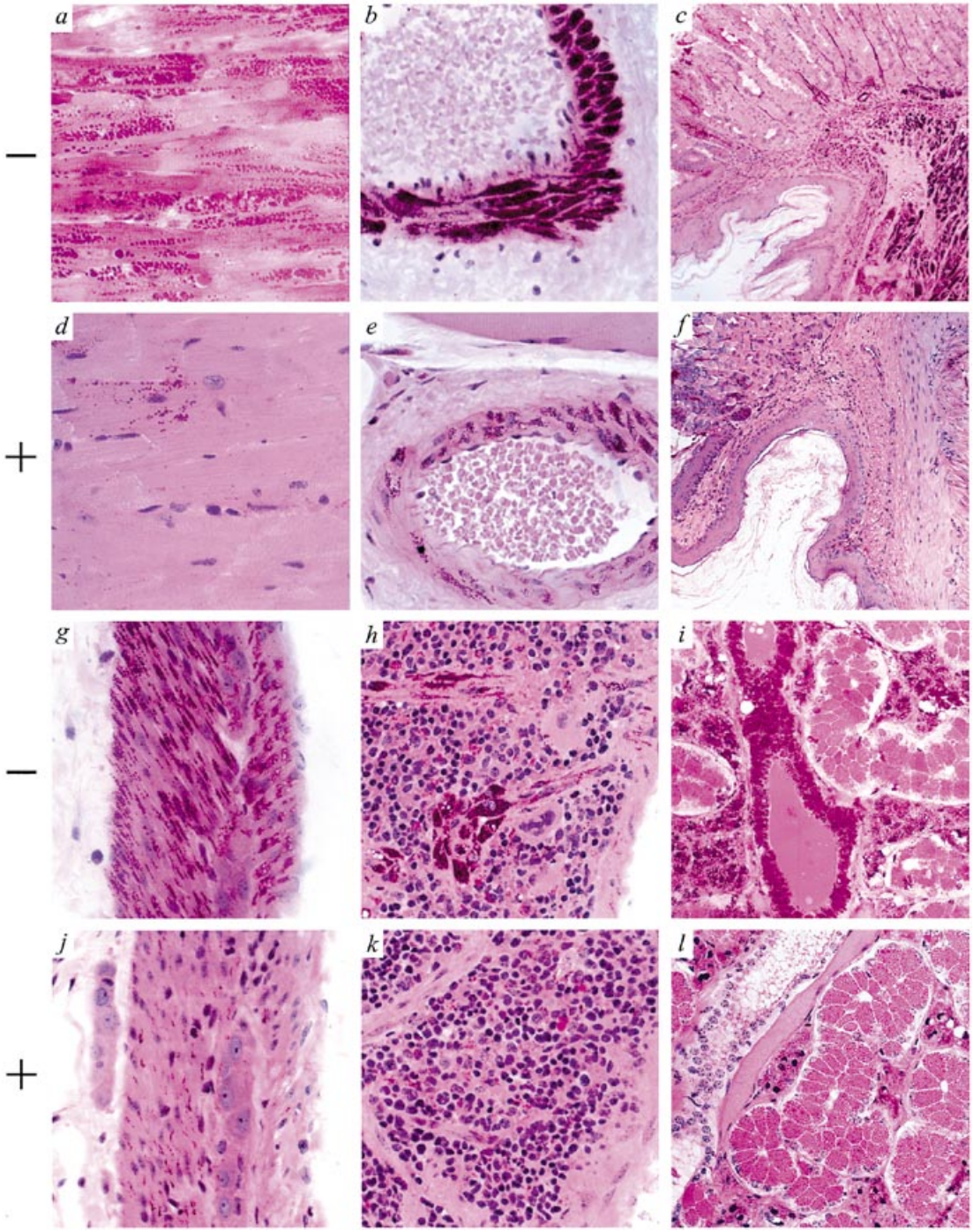

Figure 4. Correction of glycogen storage and improved morphology of heart, smooth muscle and salivary glands of KO mice after prolonged treatment with rhGAA. (**a** and **d**) Heart; (**b** and **e**) muscular artery; (**c** and **f**) stomach; (**g** and **j**) colon; (**h** and **k**) spleen; (**i** and **l**) salivary gland. The sections were stained with PAS. Sections (a)–(c) and (g)–(i) are from placebo-treated mice (–) and sections (d)–(f) and (j)–(l) from rhGAA-treated mice (+).

and injected in 200 µl volume into the tail vein. Single dose injections of enzyme were given to three 3-month-old FVB female KO mice. Three other KO females received PBS as placebo. Two groups of mice were used for short-term treatment: three 3-month-old KO females with an FVB background in the first group and two 8-month-old KO littermates with a C57Bl/6 background in the second group (one female and one male). Two mice from the first and one from the second group were treated with enzyme. The mice were injected four times with 40–68 mg/kg rhGAA within a 6 day interval. An i.p. injection of 0.05 mg/kg clemastinum (Tavegil; Sandoz Pharma, Basel, Switzerland) was given 30 min before the last enzyme infusion because an anaphylactic

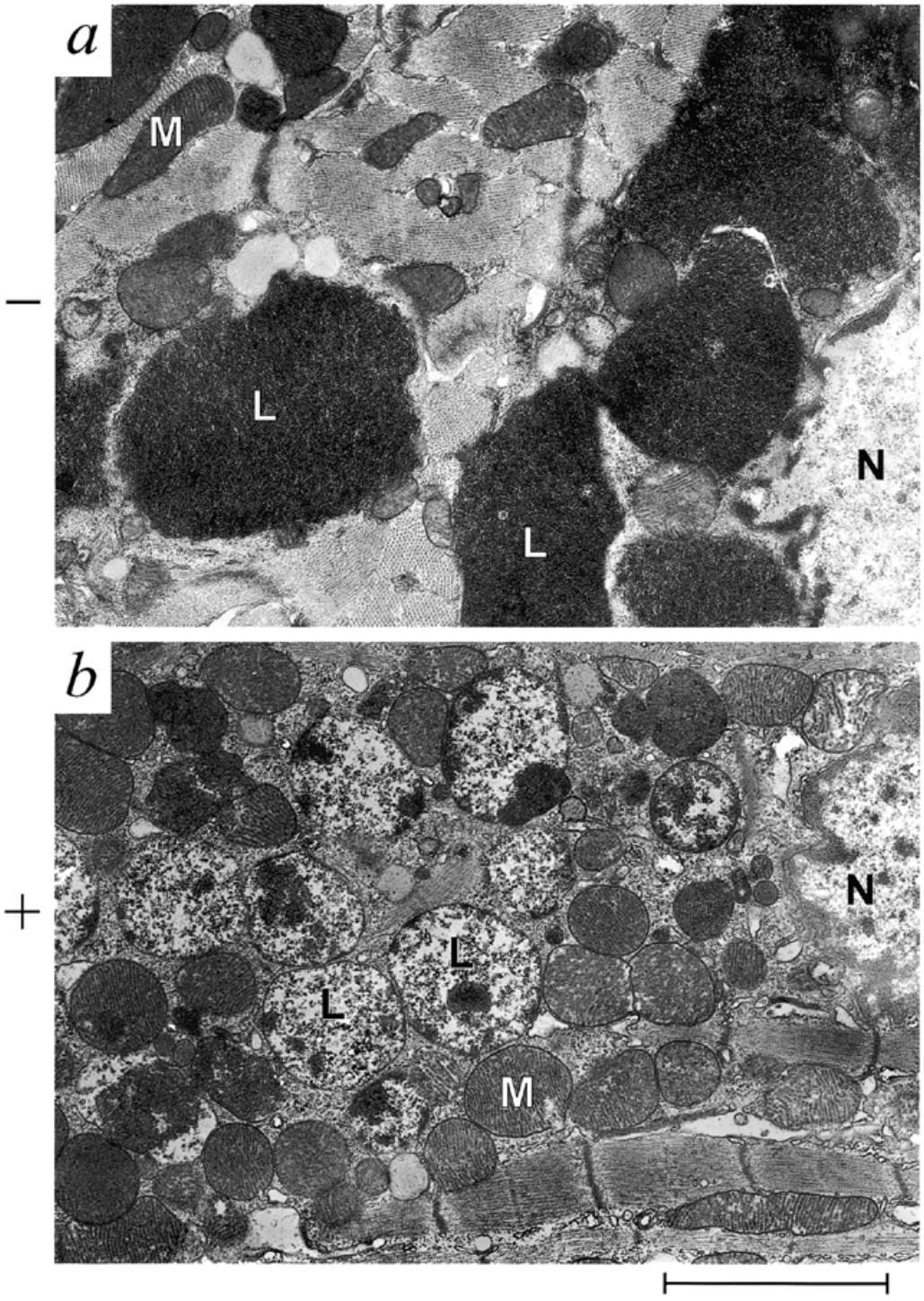

Figure 5. Electron microscopy showing degradation of lysosomal glycogen in heart muscle of KO mice after prolonged treatment with rhGAA. (**a**) Placebo-treated (–); (**b**) rhGAA-treated (+). N, nucleus; L, lysosome; M, mitochondria. Bar, 2 μm.

reaction occurred shortly after the third injection. Mice were fasted for 16 h before killing to deplete non-lysosomal glycogen. Tissues were collected after perfusion with PBS.

For prolonged treatment, 12 KO females with an FVB background and four KO males with a C57Bl/6 background, all 6 months of age, were included. Weekly injections were given. Eight mice (six females and two males) received enzyme, the other eight (six females and two males) PBS as placebo. The first injection was a high dose of 68 mg/kg; the following 13 injections were low dose (17 mg/kg); the last 11 were again high dose (68 mg/kg). Intraperitoneal injections with clemastinum were given from the third week on, 30 min before enzyme/placebo administration, to

prevent anaphylactic shock. Blood samples were taken in weeks 13, 16, 18 and 21. Six FVB mice were killed on day 2 after the 13th injection and six more after the 18th injection. The four C57Bl/6 mice were killed 2 days after the 25th injection. Mice were fasted for 16 h before killing. Tissues were collected after perfusion with PBS.

Biochemical assays

Tissues homogenized in PBS were assayed for acid α-glucosidase activity with 4-methylumbelliferyl-α-D-glucopyranoside as substrate, at pH 4.3 as described (23). The glycogen concentration in the tissue samples was measured after dialysis of the homogenates against PBS. Glycogen was degraded to glucose with a mixture of α-amlysase and α-amyloglucosidase. The amount of liberated glucose was determined by the glucose oxidase method (24).

Histology

For light microscopy, tissues were fixed with 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) and embedded in glycol methacrylate according to standard procedures. Sections of 4 μm were stained with PAS reagent and hematoxylin azofloxin. For electron microscopy, glutaraldehyde-fixed tissue specimens were post-fixed with 1% OsO_4 in 0.1 M cacodylate buffer containing 50 mM $K_3Fe(CN)_6$ according to De Bruijn (25) and embedded in epon. For immunoelectron microscopy, tissues were fixed with 1% acrolein and 0.4% glutaraldehyde in 0.1 M cacodylate buffer and embedded in Lowicryl. Ultrathin sections were immunostained by incubation with rabbit anti-human placental acid α-glucosidase antibodies, followed by an incubation with goat anti-rabbit IgG coupled to 10 nm colloidal gold.

Antibody titers

An estimate of the serum titers of antibodies against rhGAA was obtained as follows. Known aliquots of rhGAA were incubated overnight with serial dilutions of mouse sera and protein A–Sepharose beads. The serum titers were calculated from the percentage of precipitated acid α-glucosidase, essentially as described by De Jonge *et al*. (26).

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