Therapy for glycogen storage disease type II

Acid α-glucosidase production in milk
and enzyme replacement therapy in a mouse model

Enzym therapie voor glycogeen stapelingsziekte type II

Studies in een muismodel met menselijk α-glucosidase
geproduceerd in melk van transgene dieren

Agnes Geertruida Antoinette Bijvoet

Therapy for glycogen storage disease type II

Acid α-glucosidase production in milk
and enzyme replacement therapy in a mouse model

Enzym therapie voor glycogeen stapelingsziekte type II

Studies in een muismodel met menselijk α-glucosidase
geproduceerd in melk van transgene dieren

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus
Prof. Dr. P.W.C. Akkermans, M.A.
en volgens besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op

woensdag 16 juni 1999 om 11.45 uur

Door

Agnes Geertruida Antoinette Bijvoet

Geboren te Haarlem

Promotiecommissie

Promotor: Prof. Dr. H. Galjaard

Overige leden: Prof. Dr. M.F. Niermeijer
                   Prof. Dr. F.G. Grosveld
                   Prof. Dr. P.D. Verdouw

Copromotor: Dr. A.J.J. Reuser

Scope of the thesis

Glycogen storage disease type II is an autosomal recessive lysosomal storage disorder, which is caused by the deficiency of acid α-glucosidase. Skeletal muscle weakness is the common clinical feature of early and late-onset forms of this disease. Cardiomegaly and -insufficiency earmark the severe infantile phenotype. The aims of the experimental work described in this thesis were to investigate the pathogenesis of glycogen storage disease type II and to test the feasibility of enzyme replacement therapy using recombinant human acid α-glucosidase produced in the milk of transgenic mammals. A knockout mouse model of the disease was made and used as an experimental tool in these studies.

CONTENTS

A.G.A. Bijvoet, E.H.M. Van de Kamp, M.A. Kroos, J.H. Ding, B.Z. Yang, P. Visser,
C.E. Bakker, M.P. Verbeet, B. Oostra, A.J.J. Reuser, and A.T. Van der Ploeg,

Chapter 1

General introduction

Lysosomes and lysosomal storage diseases

Lysosomes

Almost all eukaryotic cells contain lysosomes. Cellular and extra-cellular (macro) molecules are degraded in these acidic cell-organelles and the products can be reutilized for metabolic functions and cell renewal (De Duve *et al.* 1955; Hers 1963; Hers and Van Hoof 1973). Other functions of "the lysosomal system" are more specialized. For instance, microorganisms are destroyed in lysosomes of polymorphonuclear granulocytes and macrophages, and aged erythrocytes are degraded in the lysosomal system of macrophages. Lysosomes in premature erythrocytes participate in cell maturation by the degradation of organelles. Low density lipoproteins particles deliver cholesterol to the lysosomal system of hepatocytes. The lysosomal system of osteoclasts is specialized in bone-resorption allowing remodeling of bone during growth, and thyreoglobuline is converted to thyroxine and tri-iodothyronine in lysosomes of the thryoid gland (Hers and Van Hoof 1973; De Duve 1985; Glaumann and Ballard 1987).

The (glyco-) proteins, (glyco-) lipids, nucleic acids and complex sugars that are degraded in the lysosomal system enter the lysosomes via endocytosis or autophagy. The degradation is performed by over 40 different lysosomal enzymes that act, often in concert, on a specific chemical bond (Gieselmann 1995; Walkley 1998). The monomeric degradation products are
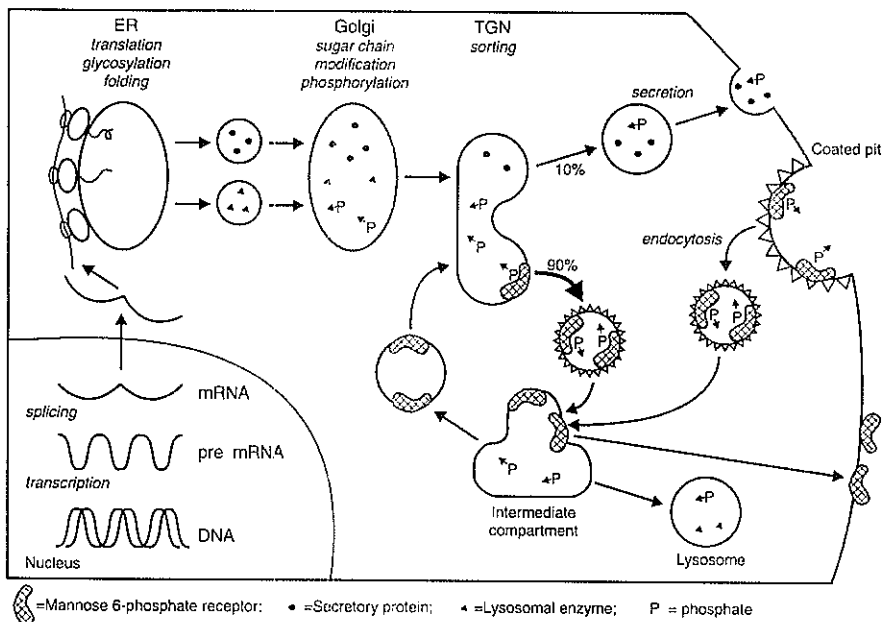
Figure 1. Schematic representation of synthesis and transport of soluble lysosomal proteins. (Adapted and modified from Van der Ploeg 1989).
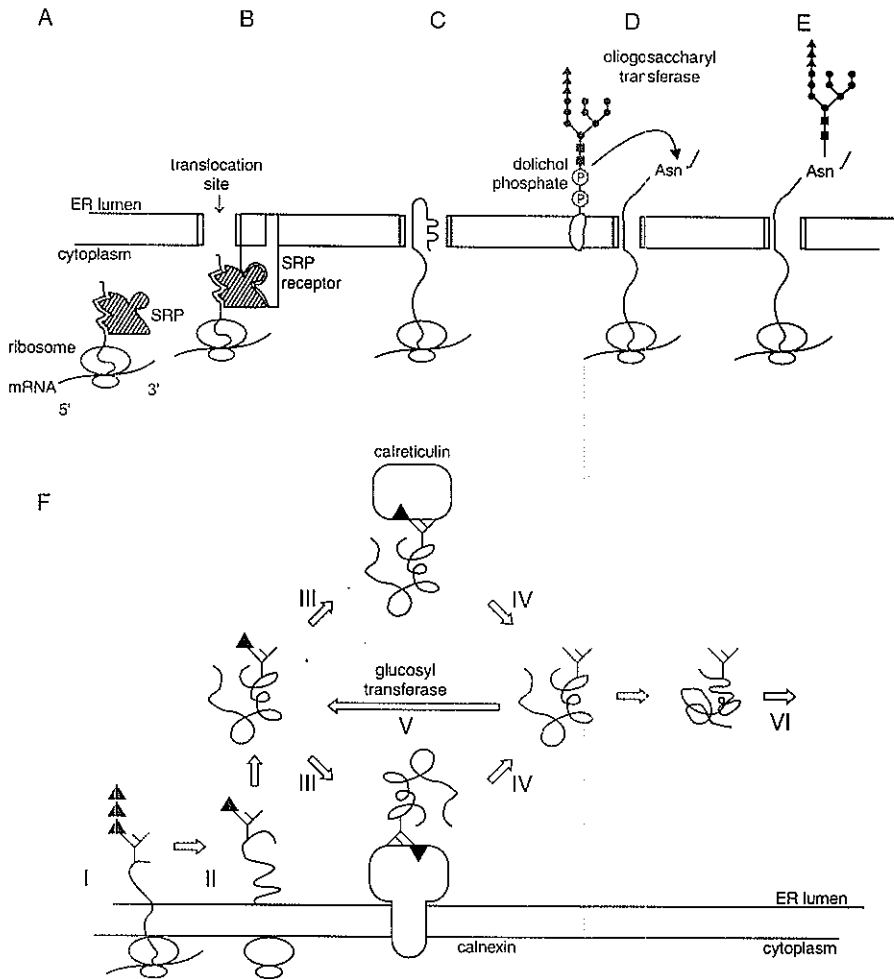
Figure 2. *Translocation, glycosylation and folding of lysosomal proteins in the endoplasmic reticulum (ER). A. The signal peptide is recognized by the signal recognition particle (SRP). B. The SRP-signalsequence-ribosome complex docks to the ER membrane. C. The protein is co-translationally imported in the ER lumen. D. A pre-formed oligosaccharide is transferred en block from a lipid carrier (dolicholphosphate) to an Asparagine (Asn) residue in the nascent polypeptide (E). F. Oligosaccharides are trimmed by glucosidases I and II (I and II). Monoglucosylated glycoproteins bind calreticulin or calnexin and are subjected to folding (III). Glucosidase II removes the remaining glucose residue, preventing further interaction with calnexin and calreticulin (IV) if they are not yet properly folded, they are reglucosylated by UDP-glucosyltransferase and re-enter the folding cycle (V). When the glycoproteins are correctly folded, they leave the cycle (VI).* ▲*, glucose;* ●*, mannose;* ■*, N-acetylglucosamine; P, phosphate;* ⌒⌒ *, signal sequence. (Adapted and modified from Trombetta and Helenius 1998).*

exported from the lysosome via passive diffusion or active transport and can be re-utilized by the cell. Most lysosomal enzymes are synthesized as precursor and are extensively modified during transport to the lysosomes (Fig. 1) (reviewed in Von Figura and Hasilik 1986; Kornfeld and Mellman 1989; Braulke 1996). A signal sequence at the N-terminus of lysosomal proteins is recognized, immediately after it has been formed, by a signal recognition particle (SRP). The complex of SRP, signal sequence and ribosomes with the messenger is transported to the endoplasmic reticulum (ER), where translation is completed, while the nascent enzyme enters the ER lumen (Fig. 2) (Andrews and Johnson 1996; Rapoport *et al.* 1996). The signal peptide of most soluble lysosomal proteins is cleaved off. The polypeptide is N-glycosylated by the attachment of a pre-formed oligosaccharide to asparagines in the consensus sequence Asn-X-Ser/Thr (X can be any amino acid, other than Pro), followed by trimming of the oligosaccharide (three glucose and one mannose residues are removed) (Kornfeld and Kornfeld 1985; Silberstein and Gilmore 1996).

Translocation and correct folding is assisted by chaperones that associate transiently to the newly synthesized protein (Rajagopalan *et al.* 1994; Hammond and Helenius 1995; Trombetta and Helenius 1998). In addition, chaperones prevent premature processing and export from the ER to the Golgi network. The chaperones calnexin and calreticulin are involved in glycoprotein folding and specifically bind to mono-glucosylated core glycans, which are transient intermediates in the oligosaccharide trimming process. The chaperone-protein complex may promote the folding process of the glycoprotein.

A model is proposed that the binding and release of the glycoprotein to calnexin or calreticulin is driven by cycles of deglucosylation and reglucosylation (Fig. 2F). Deglucosylation by glucosidase II is thought to inhibit substrate binding, while the addition of glucose to the glycoprotein by UDP-glucose: glycoprotein glycosyltransferase (UDP-GT) is believed to promote binding. UDP-GT is supposed to function as folding sensor that does not recognize correctly folded protein and in this way interrupts the on-and-off cycle (Trombetta and Helenius 1998). Besides calnexin and calreticulin other enzymes assist proteins to obtain the correct folding, for instance by the deformation and reformation of disulfide bonds (Bulleid 1993; Gilbert 1997).

Further modification of the carbohydrate chains takes place in the Golgi complex, resulting in high mannose, complex or hybrid type of oligosaccharide chains. Most lysosomal enzymes contain one or more high-mannose chains that are phosphorylated as the next necessary modification for transport to the lysosomes. N-acetylglucosamine (GlcNAc)-1-phosphate is transported to a mannose residue followed by the removal of the GlcNAc (Fig. 3) (Hasilik *et al.* 1981; Reitman and Kornfeld 1981). The resulting mannose 6-phosphate (M6P) groups are recognized by one of the two mannose 6-phosphate receptors, which are involved in lysosomal enzyme sorting (Kornfeld 1992; Pohlmann *et al.* 1995; Kasper *et al.* 1996).

There are two types of M6P receptors, a cation-independent receptor of 300 kD, in addition binding insulin like growth factor II (CI-M6P receptor/IGFII-R), and a cation-dependent receptor of 46 kD (CD-M6P receptor). Although both are involved in the sorting of lysosomal enzymes, there is a difference in affinity for the various enzymes, indicating that they may complement each other (Kasper *et al.* 1996). The receptor-ligand complexes are transported via

clathrin coated vesicles that bud off from the trans side of the Golgi complex and fuse to early lysosomes (endosomes) (Fig. 1) (Leborgne and Hoflack 1997; Schmid 1997). Due to the lower pH in this compartment, the complexes dissociate. The receptor cycles back to the Golgi complex or to the plasma membrane (Von Figura and Hasilik 1986) and the lysosomal enzymes continue their way to the lysosomes. About 10 % of the newly synthesized lysosomal enzymes is not correctly transported to the lysosome but secreted (Von Figura and Hasilik 1986). A second transport mechanism, used by membrane bound proteins and by a minority of the soluble proteins, is M6P receptor independent (Peters and Von Figura 1994; Sandoval and Bakke 1994). Some lysosomal proteins use both M6P receptor dependent as well as independent pathways.

Secreted lysosomal enzymes carrying the M6P marker can bind to the CI-M6P receptor that is exposed on the plasma membrane, and are internalized via receptor mediated endocytosis (Kornfeld 1986). This way, they are transported from the extracellular environment to the lysosomes. Most lysosomal proteins undergo proteolytic processing before or after arrival in the lysosomal compartment (Neufeld 1991). This maturation is in some cases required to catalytically activate the enzyme.
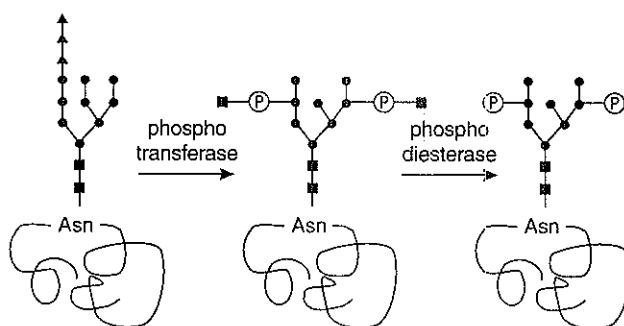
Figure 3. Phosphorylation of high mannose oligosaccharide chains. N-acetylglucosamine (GlcNAc)1-phosphate is linked to specific mannose residues by the enzyme N-acetylglucosamine phosphotransferase (phosphotransferase). The GlcNAc moiety is removed in mid Golgi by the enzyme N-actelyglucosamine-1-phosphodiesterase (phosphodiesterase). Asn, Asparagine residue; ●, mannose; ▓, N-acetylglucosamine; P, phosphate.

Lysosomal storage diseases

The deficiency of a lysosomal enzyme gives rise to intralysosomal accumulation of specific substrates. The deficiency of a so-called activator protein, assisting a lysosomal enzyme in its catabolic function, or the deficiency of a component of a larger lysosomal enzyme complex also can lead to a lysosomal storage disease. Inappropriate lysosomal enzyme targeting and defective export of degradation products from lysosomes are other causes of lysosomal diseases (Scriver *et al.* 1995). The nature of the storage products and the ultra structural appearance of the lysosomes are characteristic for the different storage diseases. Glycosaminoglycans are stored in the mucopolysaccharidoses (MPS) and sphingolipids in the lipidoses. Glycogen is stored in

glycogen storage disease type II and cystine and sialic acid when the respective transporter proteins are deficient.

Although lysosomal proteins are household proteins and ubiquitously expressed, the different lysosomal storage diseases show significant variations in tissue involvement and related clinical symptoms. Some clinical symptoms seem to relate directly to the storage process. For instance, hepatomegaly in the mucopolysaccharidoses is simply caused by expansion of the lysosomal system through osmotic swelling caused by the accumulation of glycosaminoglycans in hepatocytes. The skeletal deformities in this group of diseases are due to a similar storage process in chondrocytes losing their ability to maintain the cartilage matrix. In Gaucher disease the hepatomegaly is explained by an increase in cell number, probably caused by the stimulation of cell proliferation by the storage of glucosylcerebroside in macrophages. In glycogen storage disease type II there is a loss of muscle function that is easily understood by the observed loss of myofibrils and subsequently muscle cell structure, but details of the pathogenic process remain to be elucidated. It could be that the massively enlarged lysosomes burst through mechanical stress and that the fibers are destroyed through the release of proteolytic enzyme. Alternatively, it could be that the cellular decay is a secondary result of disturbed cellular metabolism (reviewed in Gieselmann 1995; Walkley 1998). Thus, the clinical symptoms are largely determined by the tissue specificity and nature of the substrate, despite the fact that the enzyme deficiencies are generalized. In general, however, symptoms often include skeletal malformations, mental retardation, and organomegaly, eye abnormalities, vascular problems, and motor and sensory disturbances. The lysosomal storage and the clinical course are progressive.

Lysosomal storage diseases are inherited as autosomal recessive traits, except Hunter and Fabry disease, which are X-linked. Although the individual lysosomal storage diseases are rare, the overall incidence of this family of diseases is about 1 in 5,000-10,000 (Galjaard and Reuser 1984; Reuser *et al.* 1994). Gaucher, Niemann-Pick en Tay-Sachs disease have a high frequency in Askenazi Jews (Neufeld 1991), Aspartylglucosaminuria and Salla disease in Finns (Neufeld 1991; Reuser *et al.* 1994), and adult forms of galactosialidosis and GM1-gangliosidosis in Japanese (d'Azzo *et al.* 1995; Suzuki *et al.* 1995).

Lysosomal storage diseases are clinically heterogeneous, and patients are usually classified on the basis of severity of symptoms and age of onset, in severe infantile, milder juvenile or adult subtypes. Sometimes, the classification is based on organ involvement such as neuronopathic and non-neuronopathic forms in Gaucher disease. Often however, there is overlap between subtypes and it is more appropriate to speak of a clinical spectrum. In general, the milder phenotype correlates with a higher level of residual enzyme activity.

Most genes and cDNAs involved in lysosomal storage diseases have been cloned. A genotype-phenotype correlation has been noted in most diseases in the sense that mutations resulting in the complete absence of active enzyme correlate with the most severe phenotype. However, for milder mutations resulting in residual enzyme activity, patients may show variable phenotypes despite an identical genotype (Gieselmann 1995). As has been proposed by Conzelmann and Sandhoff (1983/84), there is a "critical threshold" of enzyme activity above which the enzyme is capable to keep up with substrate influx and below which lysosomal

accumulation occurs. Therefore, the substrate influx, determined by the genetic background and non-genetic factors, can influence the clinical course (Scriver *et al.* 1995).

The diagnosis of a lysosomal storage disease requires interpretation and recognition of the clinical picture. Specific tests on urinary metabolites or blood may help to establish the diagnosis (like in oligosaccharidoses and mucopolysaccharidoses). Storage products can be measured in the tissues and lysosomal enzyme defects can be established in cells (leukocytes, fibroblasts and tissue biopsy samples) by assay of lysosomal enzyme activities using natural and/or artificial substrates. Sometimes, histology of biopsy specimens (liver, muscle, skin, etc.) may show evidence for storage. Prenatal diagnosis is possible by measuring lysosomal enzyme activities on chorionic villi or cultured amniotic fluid cells. Carrier detection based on enzyme activity levels is usually unreliable because of overlapping values of carriers and normal controls. Today's knowledge of frequently occurring mutations in the genes for many of the lysosomal enzymes and the possibilities of automated sequencing have brought carrier detection within reach and improved the accuracy of prenatal diagnosis. The mutations in families with affected relatives are relatively easy to define, but the exclusion of mutations in a non-related partner remains a problem (Neufeld 1991; Gieselmann 1995, 1998; Kleijer *et al.* 1995; Scott *et al.* 1996; Zhou *et al.* 1996).

At present, there is hardly any effective treatment for patients with a lysosomal storage disease. In principle, there are two strategies: the first is to deplete the storage product, and the second is to prevent storage by substrate deprivation. The subject is discussed in the section "Therapeutic interventions in lysosomal storage diseases".

Animal models of lysosomal storage diseases

The low incidence of lysosomal storage diseases, the clinical heterogeneity within each disease, and the restricted possibilities of tissue sampling impede studies on pathological processes and therapeutic interventions in lysosomal storage diseases. Most of these problems are not encountered when studying representative animal models. Genetic background variation can be eliminated and a variety of methods can be used. Moreover, it is possible to take samples in different stages of the disease and to do well-controlled experiments with sufficient numbers of animals. Natural animal models exist for some lysosomal storage diseases; other models have been made by drug administration and by genetic modification. The majority of the natural occurring models are large domestic animals, like cattle, sheep, goat, cat, and dog (reviewed in Jolly and Walkley 1997).

Only a few natural occurring mouse models are known: the twitcher mouse, deficient in galactosylceramidase and equivalent to human Krabbe disease, β-glucuronidase deficiency mice (Sly syndrome), mice with a mild form of sialidosis, mice with Niemann-Pick C disease and mice with Sanfilippo A disease (Potier *et al.* 1979; Duchen *et al.* 1980; Kobayashi *et al.* 1980; Loftus *et al.* 1997; Bhaumik *et al.* 1999). There is also a natural model for cathepsin L deficiency (*furless*), which has no known human equivalent (Roth *et al.* 1997; Nakagawa *et al.* 1998). The advantages of mouse models are evident: mice are small, relatively inexpensive to

house, they have a short reproduction cycle, and produce large litters. The developmental pattern is well known and in general similar to that of humans.

In addition to the natural mouse models, new techniques have enabled the generation of knockout mouse models for specific human diseases. These can be made via homologous recombination in embryonic stem cells and the injection of these cells in blastocysts. For the lysosomal diseases, this development started in 1992 with a mouse model for Gaucher disease (Tybulewicz *et al.* 1992). At present, knockout mouse models for many lysosomal storage disorders exist (Table 1). In addition, mouse models were created with deficiencies of acid phosphatase, cathepsin D, cathepsin L and both the CI-M6P receptor and CD-M6P receptor (Köster *et al.* 1993; Ludwig *et al.* 1993; Saftig *et al.* 1995, 1997; Wang *et al.* 1996; Nakagawa *et al.* 1998). The related diseases are not (yet) known in humans, possibly because of very early mortality or alternatively very mild phenotypic expression. Furthermore, mice can be generated with diseases that are very unlikely to occur in nature, for instance when two different genes are disrupted simultaneously (so called double knockout) (Sango *et al.* 1996).

The clinical, pathological and biochemical changes in the affected knockout mice are not always the same as in the human condition, despite the same lysosomal enzyme deficiency (Table 1). For some diseases the human phenotype is closely mimicked, but other mouse models present with an essentially normal phenotype or with totally unexpected manifestations. This could have various causes both at the level of developmental and biological pathways. (Erickson 1989; Wynshaw-Boris 1996). For instance, there are neurodevelopmental differences between mice and man. With respect to bone formation and remodeling, rodents only remodel bone at the surfaces of their smaller bones and not from the inside like larger mammals (Marks 1977). Differences in biochemical pathways are the reason why Tay-Sachs mice and Sandhoff mice have very different phenotypes, while in humans the phenotypes are almost identical (Yamanaka *et al.* 1994; Cohen-Tannoudji *et al.* 1995; Sango *et al.* 1995; Phaneuf *et al.* 1996). The enzyme hexosaminidase A (heterodimer of α and β-subunits) is deficient in both Tay-Sachs and Sandhoff disease. In mice, the hexosaminidase A substrate, GM2 ganglioside, is converted by sialidase into GA2 that in turn serves as a substrate for hexosaminidase B (homodimer of β-subunits) preventing in part the lysosomal accumulation of GM2 ganglioside in mice with a single hexosaminidase A deficiency (Sango *et al.* 1995). In humans, sialidase has no affinity for GM2 ganglioside, so that hexosaminidase A deficiency leads to glycolipid storage with severe symptoms.

In general, affected mice have a longer relative life span than humans with the comparable severe form of a lysosomal storage disease and differences are seen in age of onset and severity of symptoms. Evidently, this is in part caused by the different pace of the physiological processes in humans and mice. The embryonic development till birth takes only three weeks in mice compared to nine months in humans, and mice mature in six to eight weeks. Their average life span is 40 times shorter than that of humans. Taken the relative age, mice with severe infantile forms of lysosomal storage disease are not expected to live for more than a week, but usually they live much longer. In absolute terms of time, mice may not live long enough to develop the full clinical phenotype as seen in early-onset human diseases.

Table 1: Mouse models for lysosomal storage diseases

Disease	Enzyme/protein defect [secondary effect]	Phenotype[a]	Refs[b]
(Sphingo)lipidoses			
GM1-gangliosialidosis / Landing	β-galactosidase	+	(1,2)
GM2-gangliosialidosis / Sandhoff	β-chain [hexosaminidase A and B]	++	(3,4)
GM2-gangliosialidosis / Tay-Sachs	α-chain [hexosaminidase A and S]	0	(4-6)
GM2-gangliosialidosis / AMB variant	GM2 activator (sap3) (for α chain)	+	(7)
GM2-gangliosialidosis (double knockout)	α-chain and β-chain [hexosaminidase A, B and S]	x	(8)
Galactocerebrosidosis / Krabbe	β-galactocerebrosidase	++	(9)*
Glucocerebrosidosis / Gaucher	glucocerebrosidase	++	(10,11)
Sphingomyelin lipidosis / Niemann-Pick A, B	sphingomyelinase	++	(12,13)
Cholesteryl ester storage disease / Wolman	acid lipase	+	(14)
Metachromatic leukodystrophy	arylsulfatase A	0	(15,16)
Fabry	α-galactosidase A	+	(17)
Complex lipidosis	sphingolipid activator (prosaposin)	++	(18)
Oligosaccharidoses			
Aspartylglucosaminuria	aspartylglucosaminidase	++	(19,20)
Sialidosis / Mucolipidosis I	α-N-acetylneuraminidase	+	(21-23)*
Galactosialidosis	protective protein/cathepsin A [β-galactosidsase and α-neuraminidase]	++	(24)
Schindler disease	α-N-acetylgalactosaminidase	0	(25)
Mucopolysaccharidoses			
MPS I / Hurler/ Scheie	α-L-iduronidase	++	(26)
MPS II / Hunter	iduronate-sulfate sulfatase	?	(27)
MPS IIIA / Sanfilippo A	heparansulfate sulfatase	++	(28)*
MPS IIIB / Sanfilippo B	N-acetyl-α-D-glucosaminidase	++	(29)
MPS VI / Maroteaux-Lamy	arylsulfatase B	++	(30)
MPS VII / Sly	β-glucuronidase	++	(31-33)*
Glycogenosis			
Glycogenosis type II / Pompe	acid α-glucosidase	++	(34-36)
Other lysosomal diseases			
Cholesterol lipidosis / Niemann-Pick C	deficient cholesterol recycling	++	(37)*
Pycnodysostosis	cathepsin K	++	(38)
Lysosomal diseases unknown in human			
	acid phosphatase (LAP)	x	(39)
	cathepsin B	x/0	(40)
	cathepsin D	x	(41)
	cathepsin L	x	(42*,43)
	tartrate resistant acid phosphatase(TRAP)	x	(44)
	TRAP/LAP double KO	x	(39)
M6P receptors			
	CI-M6P(IGFII) receptor	x	(45)
	CD-M6P receptor	x	(46,47)
	CI-M6P/CD-M6P double KO	x	(48)

*" mouse phenotype compared with (early-onset) human phenotype: ++, close duplicate; +, minor deviations; 0, no obvious phenotype; x, no human equivalent known; ?, not (yet) known. *, natural mouse model.*

(1) Hahn et al. 1997; (2) Matsuda et al. 1997; (3) Sango et al. 1995; (4) Phaneuf et al. 1996 (5) Yamanaka et al. 1994; (6) Cohen-Tannoudji et al. 1995; (7) Liu et al. 1997; (8) Sango et al. 1996; (9) Duchen et al. 1980; (10) Tybulewicz et al. 1992; (11) Liu et al. 1998b; (12) Horinouchi et al. 1995; (13) Otterbach and Stoffel 1995; (14) Du et al. 1998; (15) Hess et al. 1996; (16) Gieselmann et al. 1998; (17) Ohshima et al. 1997; (18) Fujita et al. 1996; (19) Kaartinen et al. 1996; (20) Jalanko et al. 1998; (21) Potier et al. 1979; (22) Carrillo et al. 1997; (23) Rottier et al. 1998; (24) Zhou et al. 1995; (25) Wang et al. 1993; (26) Clarke et al. 1997; (27) Muenzer 1999; (28) Bhaumik et al. 1999; (29) Li et al. 1998; (30) Evers et al. 1996; (31) Birkenmeier et al. 1989; (32) Sands and Birkenmeier 1993; (33) Gwynn et al. 1998; (34) Bijvoet et al. 1998; (35) Raben et al. 1998a; (36) Raben et al. 1998b; (37) Loftus et al. 1997; (38) Saftig et al. 1998; (39) Saftig et al. 1997; (40) Deussing et al. 1998; (41) Saftig et al. 1995; (42) Roth et al. 1997; (43) Nakagawa et al. 1998; (44) Hayman et al. 1996; (45) Wang et al. 1994; (46) Köster et al. 1993; (47) Ludwig et al. 1993; (48) Pohlmann et al. 1995.

The phenotype of the mouse model of Gaucher disease obtained either by disruption of the glucocerebrosidase gene or insertion of a point mutation is quite exceptional in that mice die within 48 hours after birth (Tybulewicz *et al.* 1992; Liu *et al.* 1998b). The extent of lysosomal glycolipid storage is not sufficient to explain the short life span, the mice apparently succumb through a compromised epidermal permeability barrier caused by defective glucosylceramide metabolism (Tybulewicz *et al.* 1992; Willemsen *et al.* 1995; Liu *et al.* 1998b). A complicating factor in the generation of Gaucher disease knockout mice is the overlap of the 3' end of the glucocerebrosidase gene with *metaxin*, a gene responsible for embryonic lethality when inactivated (Winfield *et al.* 1997). This example emphasizes that modification of the intended gene may interfere with expression of other overlapping or neighboring genes, whereby the desired genotype-phenotype correlation is lost. Furthermore, the different genetic background of mice may lead to a different variation in phenotype. This has been demonstrated for various mouse models (Baribault *et al.* 1994; Sibilia and Wagner 1995; Threadgill *et al.* 1995; Rozmahel *et al.* 1996; Casal and Wolfe 1998).

Despite these considerations, mouse models of lysosomal storage diseases are of great value for understanding the pathogenesis, and for the development and testing of new therapeutic strategies.

Therapeutic interventions in lysosomal storage diseases

For most lysosomal storage diseases, there is at present no cure or adequate therapeutic intervention. Enzyme replacement therapy was considered feasible from the moment that lysosomal enzyme deficiencies were found to cause lysosomal storage diseases (De Duve 1964). However, the outcome of various attempts in different lysosomal storage diseases were disappointing due to the limited knowledge of the complexity of the lysosomal system and the limited supplies of enzyme (Tager *et al.* 1974; Desnick 1980).

Gradually, important information has been gathered on the regulation and activation of genes encoding lysosomal enzymes, lysosomal enzyme processing and the role of carbohydrate chains, phosphorylation and receptor mediated enzyme targeting (for details see above). The nature of the storage product and the organ specific site of storage in the different diseases require different therapeutic approaches for each disease. In principle, lysosomal storage diseases can be treated by prevention of storage or depletion of storage material, but in practice the possibilities for intervention are very limited.

Treatment of cystinosis with the drug cysteamine is a successful example of removing the storage product. The drug reacts with the stored cystine and forms transportable cysteine and cysteamine-cystine disulfide. This treatment improves renal function, especially in children, who are treated early in the disease process (Markello *et al.* 1993).

Other therapeutic approaches to remove the storage products are based on the capacity of cells to secrete and internalize enzymes. Fig. 4 illustrates exchange of lysosomal enzymes between adjacent cells via cell-to-cell contact and receptor mediated uptake. Carbohydrate
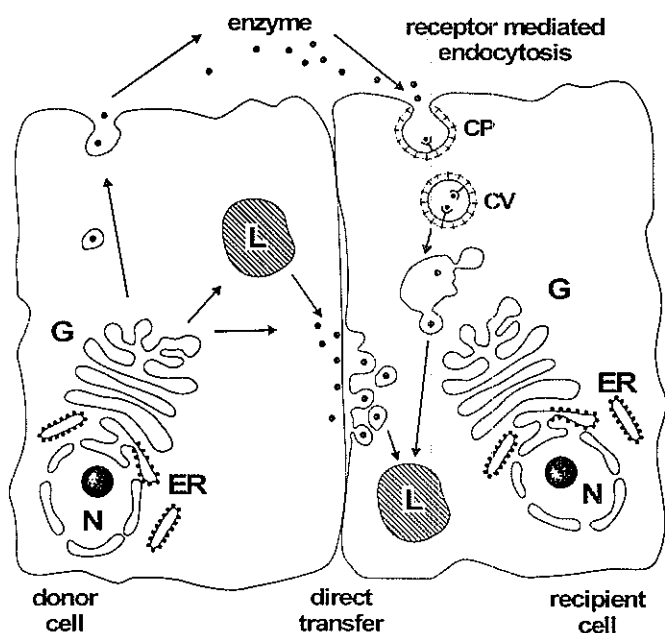
Figure 4. Schematic representation of the two known mechanisms of intercellular transport of lysosomal enzymes. Cells can exchange lysosomal enzymes via secretion and receptor mediated endocytosis. The enzyme-receptor complex is internalized in coated pits (cp), which pinch off from the plasma membrane to become coated vesicles (cv). These vesicles subsequently lose their coat and deliver the acquired enzyme to the lysosome (L). Alternatively, exchange can take place via direct cell to cell contact. The mechanism of this latter process is insufficiently understood. ER, endoplasmic reticulum; G, Golgi complex; N, nucleus; Y, receptor; ●, enzyme. (Adapted and modified from Bou-Gharios et al. 1993).

chains of lysosomal enzymes with mannose and mannose 6-phosphate groups in particular play a role in receptor mediated endocytosis and delivery of enzyme to lysosomes. Enzyme replacement is in principle possible through direct infusion of enzyme (enzyme replacement therapy), through transplantation or implantation of cells that secrete the enzyme (like bone marrow transplantation) or through introduction of the normal gene in somatic cells (gene therapy). The various approaches will be discussed below.

Enzyme replacement therapy

Most therapeutic attempts are based on replacement of the missing or inactive enzyme. The target tissues for enzyme replacement therapy vary by disease. Brain pathology is a serious problem, because therapeutic enzymes are not able to cross the blood-brain barrier. Twenty-five years of fundamental research have gradually led to the first successful application of enzyme replacement therapy. *In vitro* degradation of storage products was demonstrated after uptake of exogenous enzyme by cultured cells of patients with a lysosomal storage disease (Neufeld *et al.* 1975, 1980). For most enzymes the uptake can be mediated by the M6P receptor (Fig. 4), because of the presence of a M6P marker on one or more carbohydrate chains of the enzyme. M6P receptors are present on the cell surface of many cell types, like fibroblasts, myoblasts and hepatocytes. Other cell surface receptors that recognize carbohydrate chains and that can be used for enzyme targeting are mannose/N-acetylglucosamine receptors exposed on monocytes and mature macrophages, sialic acid receptors on glial cells, asialoglycoprotein receptor on hepatocytes and fucose receptors on fibroblasts and macrophages (Bou-Gharios *et al.* 1993).

Early clinical attempts at enzyme replacement therapy have failed for various reasons. For example, insufficient quantities and impure enzyme preparations were used and no advantage was taken of receptor mediated endocytosis. Furthermore, patients were not carefully selected and often had irreversible clinical symptoms (especially bone malformations and neurologic symptoms) at the start of treatment (Tager *et al.* 1974; Desnick 1980).

Currently, the only successful enzyme replacement therapy in human lysosomal storage diseases is that for the non-neuronopathic variant of Gaucher disease (type 1). About 2500 patients worldwide are presently under treatment. Some have been treated for over 10 years (Grabowski *et al.* 1998; Mistry and Abrahamov 1998). The enzyme preparation is purified from human placenta or produced in genetically engineered CHO-cells. In both cases, the enzyme is modified in the carbohydrate side chain so to fit the mannose receptor of lipid storing Kuppfer cells of the liver and tissue macrophages. Long term enzyme replacement results in regression of organomegaly and improvements in height, weight, sexual maturity, hematological parameters and liver function. Bone problems and pulmonary manifestations diminish, although skeletal responses develop relative slow. There is still debate about the proper enzyme dosage and administration frequency. About 15 % of the Gaucher patients receiving enzyme replacement therapy develop antibodies to the administered glucocerebrosidase, but 90 % of them became antibody negative (tolerized) after 28 months of continued therapy (Barton *et al.* 1990, 1991; Brady and Barton 1994; Grabowski *et al.* 1995, 1998; Rosenthal *et al.* 1995; Beutler 1998; Cohen *et al.* 1998; Elstein *et al.* 1998; Mistry and Abrahamov 1998).

Recently, preliminary information was released on the outcome of a phase II trial of enzyme replacement therapy in human MPS I. All patients involved in the trial showed a rapid reduction in liver size and in urinary GAG excretion. Substantial improvement in endurance/fatigue tests was observed in the majority of patients and also joint mobility improved. In addition, airway function improved in 9 of 10 patients. In 4 patients antibodies to iduronidase were observed, but the titers declined by 26 weeks. All patients continue on therapy (Kakkis *et al.* 1999).

In addition, two phase II trials of enzyme replacement were started in humans with Fabry disease. Intravenous administration of α-galactosidase A decreased the accumulated globotriao-sylceramide in plasma, liver, heart and kidneys (Desnick *et al.* 1999) and in liver and urine (Schiffmann *et al.* 1999). Some patients reported significant pain reduction and increased energy level (Schiffmann *et al.* 1999).

Based on the work described in this thesis, a phase II trial in patients with glycogen storage disease type II has recently begun with recombinant acid α-glucosidase produced in the milk of transgenic rabbits.

Animal models: Animal models have facilitated and stimulated studies on the efficacy of the various therapeutic approaches. Studies on MPS VII mice revealed positive effects in visceral organs. Reduced storage in brain and normal hearing and behavior were achieved when enzyme administration was started directly after birth at a time that the blood-brain barrier is still immature (Sands *et al.* 1994, 1997b; O'Connor *et al.* 1998; Vogler *et al.* 1998). Effects on brain pathology were not seen when therapy was started at 6 weeks after birth (Sands *et al.* 1997a). Even when enzyme replacement therapy was restricted to the first six weeks after birth, the effects were still seen after a year. Growth was improved, bone lengths were normal, and in three of five treated mice neuronal storage was reduced (Vogler *et al.* 1996).

Encouraging results were also obtained with enzyme replacement therapy in MPS I dogs (Shull *et al.* 1994; Kakkis *et al.* 1996) and MPS VI cats (Crawley *et al.* 1996, 1997; Byers *et al.* 1997). Lysosomal storage in various organs was reduced and even a corrective effect on bone deformities was obtained when enzyme replacement therapy was started before skeletal maturity. However, no or little effects were seen in brain. Higher enzyme doses and more frequent injections in MPS I dogs resulted in enzyme activity in brain and heart valves, although no histological improvements were visible (Kakkis *et al.* 1996).

For application of enzyme replacement therapy in a broader range of lysosomal storage diseases a solution is needed for transfer of enzyme to the brain. Reversible permeabilization of the blood-brain barrier has been considered (Dobrenis *et al.* 1992), but even then, neurons are relatively inaccessible and have a low endocytotic activity. A possible strategy for efficient delivery of enzyme to neurons is the use of the tetanus toxin binding sites abundantly present in neuronal membranes. It has been demonstrated that conjugation of the non-toxic fragment C of tetanus toxin to β-hexosaminidase A leads to increased efficacy of enzyme uptake by neuronal cells *in vitro* (Dobrenis *et al.* 1992). An alternative way to cross the blood-brain barrier might be by conjugation of enzyme to an antibody to the transferrin receptor which is present on the brain capillary endothelial cells and might function as a molecular shuttle (Friden *et al.* 1993).

The time and costs involved in the development of novel techniques to produce therapeutic enzyme at industrial scale and the cost of lifelong treatment are other important restrictions to the clinical try-out of enzyme replacement therapy.

Transplantation

Transplantation of cells secreting the missing enzyme would potentially ensure a continuous supply of enzyme. Liver, stem cells derived from bone marrow and muscle and neural progenitor cells could serve as enzyme source. In addition, the transplanted cells may replace the affected cells and transfer enzyme to deficient cells via cell-to-cell contact (Fig. 4).

Over the past 15 years, bone marrow transplantation has been used most often to treat patients with lysosomal storage diseases. This therapy is associated with major problems and risks. A suitable donor is not always available. Graft-versus-host disease causes a high mortality: about 10 % if an HLA (human leukocyte antigen) identical sibling marrow donor was available and about 20-25 % if mismatched tissue was used (Hoogerbrugge *et al.* 1995). The necessary "conditioning" of the patient is a real burden, while the necessary immunosuppression may lead to dangerous side effects. The effect of bone marrow transplantation on patients with lysosomal storage diseases largely depends on the type and stage of the disease. Encouraging results have been obtained in patients with MPS I, MPS II, MPS III, metachromatic leukodystrophy and non-neuronopathic forms of Gaucher disease. In general, visceral symptoms improve, but skeletal lesions remain relatively unaffected by bone marrow transplantation. The maximal effect obtained is usually an arrest of further progression of the bone-lesions. The effect on neurological symptoms varies. Long-term follow-up data suggest a stabilization of neurological symptoms in MPS I patients who have received a transplantation before the age of two years. Patients with Tay-Sachs, Sandhoff and Farber disease, with significant neurological symptoms, do not benefit from bone marrow transplantation (reviewed in Hoogerbrugge *et al.* 1995; Hoogerbrugge and Valerio 1998).

Animal models: Experiments on animal models give the opportunity to perform well-controlled studies on the factors that play a role in the therapeutic process. In general, the best effects are obtained when the transplantation is done early in life. The progressive neuronal storage and the developing neurologic symptoms were reduced in cats with α-mannosidosis, dogs with fucosidosis and MPS I, when bone marrow transplantation was performed before maturity (Shull *et al.* 1987, 1988; Breider *et al.* 1989; Taylor *et al.* 1992; Walkley *et al.* 1994). In contrast, bone marrow transplantation on a GM1 gangliosidosis dog did not result in positive effects on neuronal storage and symptoms (O'Brien *et al.* 1990). In addition, studies on mouse models for Niemann-Pick A and Krabbe disease resulted in high enzyme levels and decreased storage in brain, next to improvement of visceral organs, due to infiltration of donor cells (Yeager *et al.* 1984; Hoogerbrugge *et al.* 1988; Miranda *et al.* 1997).

Infiltration of brain by bone marrow derived macrophages is only reported in diseases in which the blood-brain barrier becomes permeable as part of the disease process or when bone marrow transplantation is performed very early in life. The latter is illustrated by some positive effects on brain of MPS VII mice, next to positive effects on life span, although the pre-

transplantation-conditioning of these mice resulted in radiation induced skeletal damage and behavioral abnormalities (Sands *et al.* 1993; Bastedo *et al.* 1994). Interestingly, radiation induced detrimental effects could be prevented in MPS VII mice that were given enzyme replacement therapy during the first 6 weeks of life and bone marrow transplantation in week 7. Moreover, these mice had a very positive response and were almost in all aspects indistinguishable from their normal littermates at one year of age (Sands *et al.* 1997a). It remains to be tested whether this combination of therapies is beneficial to patients.

Gene therapy

Clinical trials: Limitations and disadvantages of enzyme replacement therapy and bone marrow transplantation have stimulated the development of gene therapy as an alternative method to provide a continuous supply of enzyme. The principle of gene therapy is to introduce the normal gene coding for the missing enzyme into somatic cells with the hope that the corrected cells secrete and provide enzyme to other tissues. More than 3000 individuals are involved in over 200 clinical trials on gene therapy by now, most having cancer or AIDS (Marshall 1995; Russell 1997). Protocols for the application of gene therapy in Gaucher, Hunter and Hurler disease are under examination (Poenaru 1996). Until now, however, only a few patients have had benefit from gene therapy. Problems are the inefficient delivery of genes and the transient expression (reviewed in Marshall 1995; Verma and Somia 1997; Russell 1997).

Vectors and approaches: A variety of vectors, non-viral and viral, are being explored to introduce the missing gene. They are designed to deliver the gene to the right place and to regulate its expression. All vectors used so far have limitations (Kay *et al.* 1997; Verma and Somia 1997). Retroviral vectors can only transduce dividing cells, and integrate random in the host genome. In contrast, adenoviral based vectors can infect both dividing and non-dividing cells and do not integrate in the host genome, but have other disadvantages, such as immune responses elicited by the viral proteins and silencing of the transgene. New generations of adenoviral vectors, with almost all virus genes deleted, are investigated to overcome these problems (Schiedner *et al.* 1998). Adeno-associated viral vectors are also able to transduce non-dividing cells and are relatively safe, but can only contain foreign DNA of limited size.

There are two main approaches to introduce the gene: *in vivo* gene therapy in which genes are delivered directly to the target cells and *ex vivo* gene therapy in which cells are taken from the patient, genetically engineered and transplanted back into the patient. An example is gene therapy on bone marrow derived cells. This procedure has the advantage that bone marrow of the patient can be used. Therefore, the risks of graft-versus-host disease are eliminated, and the conditioning of the patient can be less stringent.

The choice of muscle as donor organ for systemic delivery of therapeutic proteins to muscle or other tissues is less obvious. However, myoblasts have favorable features: they divide profusely and fuse during muscle regeneration with existing muscle fibers inserting their nuclei with the transgene into the muscles of the recipients. Muscle fibers can secrete enzymes and the risk of immune reactions is limited (Partridge and Davies 1995; Law *et al.* 1998). Even the injection of non-genetically modified myoblasts in muscle of a patient can be interpreted as gene

therapy, because the "normal" gene will be inserted into the genetically defective myofibers by fusion of the normal myoblasts with the diseased myofibers.

This latter approach is used in clinical trials on patients with muscular dystrophies. The outcome of these trials varies. Mosaic myofibers could be detected after injection of normal myoblasts in muscles of patients, indicating that the missing gene and its products were incorporated into genetically defective cells through cell-fusion. In some trials functional improvements of the patients were reported (reviewed in Law *et al.* 1998). Among the possible reasons for negative results were mentioned contamination of myoblast cultures with fibroblasts, sub-optimal myoblast harvest and transport procedures, immunoresponses and irreversible damage to muscles and nerves inherent to the injection procedures (number of injection sites). The use of genetically modified myoblasts, overproducing the missing protein, might improve the procedure.

Animal models: Fibroblasts and stem cells of bone marrow, muscle and neuronal origin have been used to test the effect of *ex vivo* gene therapy in animal models with lysosomal storage diseases with positive effects, but often it concerns single experiments with short term follow up (Salvetti *et al.* 1995a). Transplantation of bone marrow cells overexpressing the missing enzyme appears to have a corrective effect on the visceral organs of MPS VII (Wolfe *et al.* 1992; Marechal *et al.* 1993) and galactosialidosis mice (Zhou *et al.* 1995; Hahn *et al.* 1998), however, no effect was obtained with regard to brain or bone pathology. Implantation of genetically modified fibroblasts secreting β-glucuronidase resulted in detectable enzyme levels in liver, spleen, lung and brain of young adult MPS VII mice, however, a reduction of lysosomal storage was observed only in liver and spleen (Moullier *et al.* 1993a, 1993b). A similar experiment related to MPS I resulted in increased enzyme activity in liver and spleen of nude mice (Salvetti *et al.* 1995b). Implantation of retroviral infected fibroblasts in cats with MPS VII had a very short-term effect due to silencing of the transgene (Yogalingam *et al.* 1999). These preliminary results do not allow firm conclusions about the clinical applicability of *ex vivo* gene therapy.

Implantation of myoblasts transduced with the gene for human glucocerebrosidase in normal mice resulted in differentiation and fusion with mature myofibers (Liu *et al.* 1998a). Moreover, the transgenic product was secreted and could be detected in macrophages of liver and bone marrow. Injection of retroviral infected myoblasts in MPS VII mice following muscle injury resulted in efficient participation of infected cells in muscle regeneration. Depending on the regulatory sequences used in the gene-construct, stable expression and reduction of storage in distant organs could be obtained (Naffakh *et al.* 1993, 1996). These results hold promises for further development of this type of therapy and future application to patients.

Several methods are tried to increase the therapeutic efficacy for the central nervous system. Direct injection of an adenoviral vector in the brain of aspartylglucosaminuria deficient mice resulted in local reduction of storage in neuronal tissue (Peltola *et al.* 1998). Implantation of modified immature neuronal progenitor cells overexpressing β-glucuronidase or β-hexosamini-dase, in newborn MPS VII and normal mice respectively, resulted in the presence of donor cells throughout the cerebrum (Snyder *et al.* 1995; Lacorazza *et al.* 1996). The donor cells continued to express the enzyme, and a reduction of storage in neurons and glial cells was obtained in the

MPS VII mouse model, but disappointingly few donor cells were found in the cerebellum and brain stem. Transplantation of fibroblasts overexpressing β-glucuronidase in the brain of MPS VII mice only resulted in expression of the enzyme near the side of grafting (Taylor and Wolfe 1997).

Recently, promising results were obtained by *in vivo* gene delivery. Intravenous injection of adenovirus or adeno-associated virus containing the human β-glucuronidase gene resulted in enzyme activity and reduced lysosomal storage in liver and spleen of MPS VII mice (Ohashi *et al.* 1997; Watson *et al.* 1998). Moreover, when the adeno-associated virus vector was used in newborn MPS VII mice, therapeutically levels of enzyme activity were achieved in several organs, including liver, heart and brain, resulting in reduction or prevention of lysosomal storage (Daly *et al.* 1999b). Intramuscular injection of the adeno-associated virus resulted in high, but localized expression of the transgene. The secretion of β-glucuronidase and the correction of more distant organs were inefficient, limiting the therapeutic efficacy of this approach for MPS VII (Watson *et al.* 1998; Daly *et al.* 1999a).

Conclusion: So far, the effects of gene therapy are limited. The present generation of vectors only mediates local, short-term expression and some (especially adenovirus based) vectors cause strong immuno-logical reactions. New generations of vectors, such as gutless adenovirus vectors and lentivirus (retroviral-related) vectors, will deliver genes more accurately and efficiently. These vectors will allow better long-term control of gene expression. Focus is on the development of safe and stable expression systems with high efficacy *in vivo*. In addition, new approaches will be developed not using viral vectors, but cationic lipid carriers and targeted gene repair. This latter approach is based on the ability of chimeric RNA-DNA oligonucleotides to localize the target site (a point mutation), form a stable complex, and use the cells' DNA-repair machinery to correct the mutation (Kmiec 1999). *In vivo* application of this technique resulted in single base pair alterations in the factor IX gene in hepatocytes (Kren *et al.* 1998).

Substrate deprivation

A completely different approach of therapy of lysosomal storage diseases is substrate deprivation, whereby the efforts are focussed on the lowering of substrate synthesis and lysosomal import, instead of on the increase of lysosomal enzyme activity. The patient must, of course, tolerate the partial depletion of the substrate and there has to be some residual enzyme activity to catabolize the remaining substrate. When patients have a severe infantile form of disease, these low enzyme levels can possibly be obtained by enzyme replacement therapy, bone marrow transplantation, or gene therapy.

Lowering the biosynthesis of GM2 ganglioside of Tay-Sachs disease mice, by treatment with N-Butyldeoxynojirimycin (a water-soluble compound, noncytotoxic over a broad range of concentrations), prevents the lysosomal storage and the associated neuropathology (Platt *et al.* 1997). These results are promising, and substrate deprivation may also be effective for the treatment of other sphingolipidoses, although the correction of neurological symptoms has yet to be proven and the long-term effects remain to be awaited. Moreover, an additional risk is illustrated in a genetic model of substrate deprivation. Mice knockout for both the gene involved

in GM2 ganglioside synthesis as well as the Hex B gene have no GM2 ganglioside storage and a prolonged life span, but develop later in life oligosaccharide storage resulting in new disease manifestations (Liu *et al.* 1999).

Animal models and therapy

In general, animal models and especially knockout mouse models are useful for the initial evaluation of novel therapeutic approaches, although differences between humans and mice have to be kept in mind. The different therapeutic approaches do not necessarily apply equally well to all lysosomal storage diseases and need to be optimized for each disease and its variants separately. In some cases, a combination of therapies may lead to the most optimal results. It is uncertain whether the damage to tissues, which may start prenatally, is reversible. This holds in particular for lesions of the brain and skeleton. Therapies will be more successful when applied in an early stage of the disease process.

Biotechnology for the production of lysosomal proteins

Enzyme replacement therapy requires a continuous supply of enzyme with pharmacological quality. The enzyme sources are limited by demands on safety, species specificity and efficacy. The enzyme preparation has to be stable and to reach the lysosomes in the target tissue. It has to remain catalytically active or should become active during transport to or in the lysosome. Preferably, the enzyme should not elicit immunogenic responses. These characteristics may be obtained by adequate post-translational modifications.

Human placenta and human urine can serve as natural sources of various lysosomal enzymes. However, the concentration of lysosomal enzymes in the urine is very low, and extraction from urine is bound to fail by logistic obstacles. Enzyme purified from human placentas (Ceredase®; Genzyme Cambridge, MA) is successfully applied for treatment of patients with the non-neuronopathic variant of Gaucher disease. For targeting purposes, the enzyme must be modified in the carbohydrate side chain in order to fit the mannose receptor on the cell surface of macrophages, the cell type predominantly involved in this disease (Barton et al. 1990, 1991). The total quantity of enzyme needed for the worldwide treatment of Gaucher patients, is difficult to produce solely by purification from human placentas (about 50,000 placentas are needed per patient per year) and the product costs are very high ($80,000-312,000 per patient of 50 kg per year) (Grabowski et al. 1995, 1998). In addition, there is a risk of contaminating human pathogens in blood products. The use of enzyme from non-mammalian sources, like yeast and fungi introduces the almost unavoidable risk of an immunologic response.

Biotechnological production of recombinant human proteins became an opportunity since the cloning of human cDNAs and genes involved in lysosomal storage diseases. Several systems are explored for the production of recombinant proteins (see Table 2). However, the choice is limited when it comes to the production of lysosomal proteins, which need to be glycosylated for intralysosomal stability, and preferably equipped with a targeting signal for efficient delivery

to tissues and lysosomes (reviewed in Houdebine 1994; Colman 1998). Bacteria are unable to perform complicated glycosylation and some essential post-translational modifications, while yeast and fungi secrete inappropriately glycosylated enzymes. Transgenic plants would be an attractive production system, because they can be cultured in large fields at low costs. The eukaryotic cell machinery mediating protein modification is considerably conserved, although phosphorylation of mannose residues can not be performed in plants. The production of human lysosomal glucocerebrosidase in transgenic tobacco plants is actually investigated in search for a low-cost alternative for the presently used enzyme preparations. Tobacco synthesized glucocerebrosidase has a similar apparent molecular mass as human placental derived glucocerebrosidase, is glycosylated, and, most significantly, is enzymatically active (Cramer *et al.* 1996). Delivery of glucocerebrosidase to macrophages is not dependent on M6P receptor mediated transport. The M6P marker, needed for transport of most other lysosomal enzymes to the lysosomes, may be added via *in vitro* phosphorylation (Boose *et al.* 1990). The idea is promising and detailed studies including *in vitro* and *in vivo* experiments have to provide insight in the real potential of this low cost production system.

Table 2: Production systems for recombinant human lysosomal enzymes

System	Advantages	Disadvantages
Bacteria	Low cost Large-scale production	No glycosylation Limited post-translational modifications
Yeast & fungi	Low cost Large scale production	Limited post-translational modifications
Transgenic plants	Very low cost Glycosylation feasible Negligible infection risk Large-scale production	No phosphorylation Extraction a problem
Insect cells / Baculovirus	Probably low cost Similar post-translational modification system	No phosphorylation
Mammalian cells	Proven Similar post-translational modification system	Expensive Low-scale production Risk of introducing pathogens
Transgenic animals	Potentially low cost Large-scale production Similar post-translational modification system	Large time to market Risk of introducing pathogens

[a]: *adapted and modified from Houdebine 1994; Colman 1998.*

Baculovirus infected insect cells

Several groups have investigated the production of lysosomal enzymes in baculovirus transfected insect-cells (Martin *et al.* 1988; Boose *et al.* 1990; Itoh *et al.* 1990; Hiraiwa *et al.* 1993; Coppala *et al.* 1994; Bonten *et al.* 1995; Bromme and McGrath 1996; Wu *et al.* 1996; Steed *et al.* 1998; Tilkorn *et al.* 1999). Insect-cells utilize apparently similar post-translational modification and transport systems as mammalian cells, and have been used successfully for relative large-scale production of recombinant proteins (Luckow and Summers 1988). Recombinant human lysosomal enzymes produced in these cells are glycosylated and catalytically active. However, differences in proteolytic and oligosaccharide processing were observed, and notably the M6P marker was lacking (Boose *et al.* 1990; Itoh *et al.* 1990; Hiraiwa *et al.* 1993; Wu *et al.* 1996). As a consequence of the latter, recombinant human acid α-glucosidase produced in insect cells is poorly internalized by cultured fibroblast from patients with GSDII (Wu *et al.* 1996). In contrast, recombinant human α-galactosidase A produced in insect cells is able to correct in part the enzyme deficiency in fibroblasts of patients with Fabry disease (Coppala *et al.* 1994). The uptake was postulated to be largely M6P receptor independent. For β-hexosaminidase B it was demonstrated that the M6P marker could be added by *in vitro* phosphorylation (Boose *et al.* 1990). The differences in post-translational processing in insect cells compared to mammalian cells limit the application of this system for the production of therapeutic lysosomal enzymes.

Mammalian cell culture

Several non-human mammalian cells have a post-translational modification system that is very similar to the human and they are actually used to produce human recombinant proteins. For instance, production in Chinese hamster ovary-K1 (CHO) cells has been explored for many lysosomal enzymes. In general, the recombinant proteins purified from the medium of genetically engineered CHO-cells are usually quite similar to the native proteins (Anson *et al.* 1992; Ioannou *et al.* 1992; Bielicki *et al.* 1993, 1995, 1998; Kakkis *et al.* 1994; Unger *et al.* 1994; Enomaa *et al.* 1995; Fuller *et al.* 1995; Van Hove *et al.* 1996; Weber *et al.* 1996; Litjens *et al.* 1997; Nagano *et al.* 1998). The enzymes contain the M6P recognition signal and are internalized by cultured fibroblasts of patients, resulting in correction of the storage phenotype of these cells. Thus, CHO cells seem to be useful for production of recombinant human lysosomal enzymes. In practice, large-scale recombinant human glucocerebrosidase production in genetically engineered CHO cells has been realized (Cerezyme®; Genzyme Cambridge, MA) and this enzyme has the same therapeutic effect in Gaucher type 1 patients as the natural glucocerebrosidase purified from human placenta (Grabowski *et al.* 1995). However, the change of production system has not lowered the production costs.

Transgenic animals

Studies on the production of a lysosomal enzyme in the milk of transgenic animals form a substantial part of this thesis. The system is attractive as potentially low-cost alternative to other production systems. The biosynthesis of lysosomal enzymes proceeds similar in the different mammalian species, and mammary gland cells are designed to secrete glycoproteins in high

concentration. The cells are densely packed in the tissue and are provided with the most optimal flow of nutrients via the circulation (reviewed in Houdebine 1994; Wall *et al.* 1997; Colman 1998; Janne *et al.* 1998). Hybrid gene constructs are used to acquire mammary gland specific expression. They consist of a 5' flanking untranslated promoter region of a milk specific protein coupled to the DNA sequences of the desired human protein. Production levels vary considerably and may depend on many factors (Rosen *et al.* 1996). Transgenes constructed with genomic sequences give better expression levels than cDNA constructs do.

Regulatory elements: Regulatory sequences of α_{S1}, α_{S2}, β and κ-casein, β-lactoglobulin, α-lactalbumin and whey acidic protein have been applied (Houdebine 1994; Wall *et al.* 1997; Colman 1998). The α_{S2} and κ-casein sequences tested so far lack regulatory sequences necessary for their effective expression in the mammary gland (Rijnkels *et al.* 1995; Branyi *et al.* 1996). Variation in the architecture of the different hybrid transgenes that were used hampers a systematic comparison of milk protein gene regulatory sequences for their ability to direct high level, tissue specific, expression of transgenes (Bawden *et al.* 1994; Houdebine 1994; Wall *et al.* 1997). Moreover, the critical evaluation of various transgenes and controlling elements is a time consuming enterprise because it needs to be done in the lactating mammary gland. To accelerate the process of transgene evaluation, Kerr *et al.* (1996) have developed an initial screening protocol based on jet-injection of naked DNA into lactating mammary glands.

Transgene expression is often integration site dependent and copy number independent. The knowledge about regulatory elements is increasing (Rosen *et al.* 1996; Drohan 1997; Malewski 1998). The role of a locus control region (LCR) at the 5' and 3' ends of the transgene construct and tissue specific enhancers in both the 5' and 3' flanking region as well as in introns have been recognized (Fig. 5) (Grosveld *et al.* 1987; Greaves *et al.* 1989; Chamberlain *et al.* 1991;
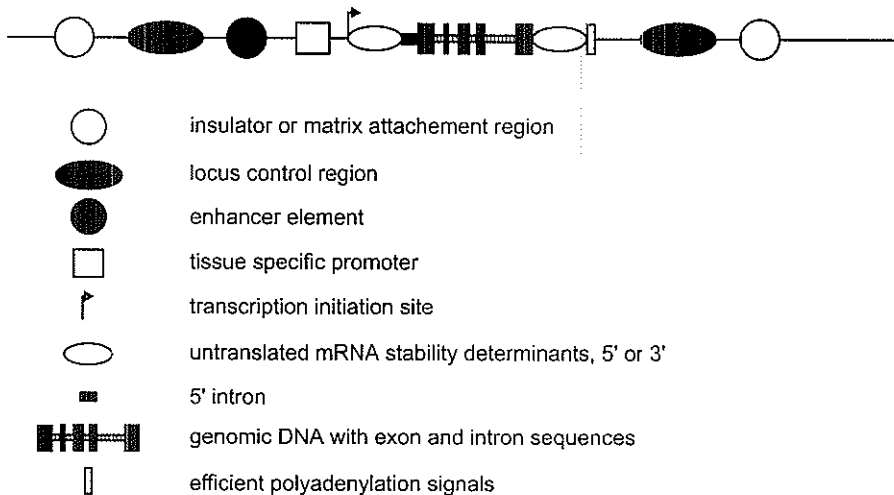

Figure 5. A schematic representation of a transgene showing the types and locations of the various elements needed for efficient transgene expression. (Adapted and modified from Houdebine 1994; Rosen et al. 1996).

McKnight *et al.* 1992; Houdebine 1994; Jones *et al.* 1995; Rosen *et al.* 1996; Drohan 1997). They are usually identified as regions that are hypersensitive to nucleases (like DNase I) and may be clustered binding sites for several transcription factors. In addition, some elements (insulators) were detected that insulate a nearby gene from the effects of neighboring genes, regulatory elements or regions of heterochromatin, that can overrule the control of the transgene (Dillon and Grosveld 1993). Insulators may also confine enhancers and LCRs to act within the domain (Chung *et al.* 1993; Wijgerde *et al.* 1995; Wall *et al.* 1997). The addition of insulators or matrix attachment regions to transgene constructs may overcome the integration site dependent and copy number independent expression.

More knowledge of regulatory elements will improve the design of transgenes and result in higher and more constant production of the recombinant protein in the milk. In addition, improved techniques for gene transfer may lead to higher efficacy of transgene integration, transgene stability and embryo survival (Houdebine 1994; Wall *et al.* 1997).

Recombinant proteins: In general, the production of heterologous recombinant proteins is explored in mice, because they are well-known laboratory animals and have a small reproduction time. Within 4 months after microinjection, there is enough protein to characterize the biochemical and functional properties. In addition, the stability of the transgene in subsequent generations can be determined. The choice of species for large-scale production will be influenced by the amount of protein needed for clinical development and application, and the length of time to milk production (littersize and milk volume). In the natural biological setting of the transgenic system, the desired protein can be produced at much higher concentration than in cell culture systems (Houdebine 1994; Colman 1996, 1998; Drohan 1997). However, the use of transgenic animals for enzyme production bears the potential risk of introducing pathogens in the product. Using specified pathogen free animals can minimize this risk. The largest disadvantage of transgenic animal technology is the length of time required to obtain a transgenic herd. In the future this problem can be partially overcome by applying the recently developed nuclear transfer technique instead of conventional microinjection and breeding (Wolf *et al.* 1998).

Besides milk, also urine of transgenic animals can be a source of lysosomal enzymes. The principle of recombinant protein production in urine was recently demonstrated for recombinant human growth hormone in transgenic mice (Kerr *et al.* 1998). The hybrid transgene construct typically contains the promoter of a protein highly expressed in bladder epithelial cells (uroplakin II) to direct expression and secretion of the foreign protein. The system may be applicable for the production of recombinant human lysosomal enzymes as well, because lysosomal enzymes are naturally present in urine and are glycosylated, phosphorylated and catalytically active. Protein production in urine has advantages over production in milk: both males and females produce the transgenic product from birth on and lifelong, and purification is probably easier to perform from urine than from milk. However, the protein concentration in urine will be lower than in milk and the collection of urine may be more complicated than of milk.

Isolation and purification

Isolation and purification are important procedures in all production systems. The enzyme has to be pure and free of toxins and pathogens. Purification of proteins from milk involves removal of lipids by low speed centrifugation, followed by precipitation, chromatography and viral filtration. For example, recombinant α_1-antitrypsin is purified from the milk of transgenic sheep to >99 % purity, via such kind of procedures (Colman 1996, 1998). The separation of highly homologous proteins remains difficult, but this problem is common to all production systems.

References

Andrews, D.W. and Johnson, A.E. (1996) The translocon: more than a hole in the ER membrane? *Trends Biochem Sci* 21: 365-369.

Anson, D.S., Taylor, J.A., Bielicki, J., Harper, G.S., Peters, C., Gibson, G.J. and Hopwood, J.J. (1992) Correction of human mucopolysaccharidosis type-VI fibroblasts with recombinant N-acetylgalactosamine-4-sulphatase. *Biochem J* 284: 789-794.

Baribault, H., Penner, J., Iozzo, R.V. and Wilson-Heiner, M. (1994) Colorectal hyperplasia and inflammation in keratin 8-deficient FVB/N mice. *Genes Dev* 8: 2964-2973.

Barton, N.W., Furbish, F.S., Murray, G.J., Garfield, M. and Brady, R.O. (1990) Therapeutic response to intravenous infusions of glucocerebrosidase in a patient with Gaucher disease. *Proc Natl Acad Sci USA* 87: 1913-1916.

Barton, N.W., Brady, R.O., Dambrosia, J.M., Di Bisceglie, A.M., Doppelt, S.H., Hill, S.C., Mankin, H.J., Murray, G.J., Parker, R.I., Argoff, C.E., Grewal, R.P., Yu, K.T. and collaborators (1991) Replacement therapy for inherited enzyme deficiency. Macrophage-targeted glucocerebrosidase for Gaucher's disease. *N Engl J Med* 324: 1464-1470.

Bastedo, L., Sands, M.S., Lambert, D.T., Pisa, M.A., Birkenmeier, E. and Chang, P.L. (1994) Behavioral consequences of bone marrow transplantation in the treatment of murine mucopolysaccharidosis type VII. *J Clin Invest* 94: 1180-1186.

Bawden, W.S., Passey, R.J. and Mackinlay, A.G. (1994) The genes encoding the major milk-specific proteins and their use in transgenic studies and protein engineering. *Biotechnol Genet Eng Rev* 12: 89-137.

Beutler, E. (1998). Enzyme replacement therapy for Gaucher's disease. *Baill Clin Haem* 10: 751-763.

Bhaumik, M., Hohnson, L., Dobrenis, K., Bhattacharyya, R., Wurzelmann, S., Finnamore, P., Muller, V.J., Rozaklis, T., Hopwood, J.J., Walkley, S.U. and Stanley, P. (1999) A mouse model of Sanfilippo Syndrome (Mucopolysaccharidosis IIIA). *5th Symposium on Mucopolysaccharide and related diseases*, Vienna, Austria: 57.

Bielicki, J., Hopwood, J.J., Wilson, P.J. and Anson, D.S. (1993) Recombinant human iduronate-2-sulphatase: correction of mucopolysaccharidosis-type II fibroblasts and characterization of the purified enzyme. *Biochem J* 289: 241-246.

Bielicki, J., Fuller, M., Guo, X.H., Morris, C.P., Hopwood, J.J. and Anson, D.S. (1995) Expression, purification and characterisation of recombinant human N-acetylgalactosamine-6-sulphatase. *Biochem J* 311: 333-339.

Bielicki, J., Hopwood, J.J., Melville, E.L. and Anson, D.S. (1998) Recombinant human sulphamidase: expression, amplification, purification and characterization. *Biochem J* 329: 145-150.

Bijvoet, A.G.A., Van de Kamp, E.H.M., Kroos, M.A., Ding, J.H., Yang, B.Z., Visser, P., Bakker, C.E., Verbeet, M.P., Oostra, B., Reuser, A.J.J. and Van der Ploeg, A.T. (1998) Generalized glycogen storage and cardiomegaly in a knockout mouse model of Pompe disease. *Hum Mol Genet* 7: 53-62.

Birkenmeier, E.H., Davisson, M.T., Beamer, W.G., Ganschow, R.E., Vogler, C.A., Gwynn, B., Lyford, K.A., Maltais, L.M. and Wawrzyniak, C.J. (1989) Murine mucopolysaccharidosis type VII. Characterization of a mouse with β-glucuronidase deficiency. *J Clin Invest* 83: 1258-1256.

Bonten, E.J., Galjart, N.J., Willemsen, R., Usmany, M., Vlak, J.M. and d'Azzo, A. (1995) Lysosomal protective protein/cathepsin A. Role of the "linker" domain in catalytic activation. *J Biol Chem* 270: 26441-26445.

Boose, J.A.; Tifft, C.J., Proia, R.L. and Myerowitz, R. (1990) Synthesis of a human lysosomal enzyme, β-hexosaminidase B, using the baculovirus expression system. *Protein Expr Purif* 1: 111-120.

Bou-Gharios, G., Abraham, D. and Olsen, I. (1993) Lysosomal storage diseases: mechanisms of enzyme replacement therapy. *Histochem J* 25: 593-605.

Brady, R.O. and Barton, N.W. (1994). Enzyme replacement therapy for Gaucher disease: critical investigations beyond demonstration of clinical efficacy. *Biochem Med Metab Biol* 52: 1-9.

Branyi, M., Aszodi, A., Devinoy, E., Fontaine, M.L., Houdebine, L.M. and Bosze, Z. (1996) Structure of the rabbit κ-casein encoding gene - expression of the cloned gene in the mammary gland of transgenic mice. *Gene* 174: 27-34.

Braulke, T. (1996) Origin of lysosomal proteins. *Subcell Biochem* 27: 15-49.

Breider, M.A., Shull, R.M. and Constantopoulos, G. (1989) Long-term effects of bone marrow transplantation in dogs with mucopolysaccharidosis I. *Am J Pathol* 134: 677-692.

Bromme, D. and McGrath, M.E. (1996) High level expression and crystallization of recombinant human cathepsin S. *Protein Sci* 5: 789-791.

Bulleid, N.J. (1993) Protein disulfide-isomerase: role in biosynthesis of secretory proteins. *Adv Protein Chem* 44: 125-150.

Byers, S., Nuttall, J.D., Crawley, A.C., Hopwood, J.J., Smith, K. and Fazzalari, N.L. (1997) Effect of enzyme replacement therapy on bone formation in a feline model of mucopolysaccharidosis type VI. *Bone* 21: 425-431.

Carrillo, M.B., Milner, C.M., Ball, S.T., Snoek, M. and Campbell, R.D. (1997) Cloning and characterization of a sialidase from the murine histocompatibility-2 complex: low levels of mRNA and a single amino acid mutation are responsible for reduced sialidase activity in mice carrying the Neu1a allele. *Glycobiology* 7: 975-986.

Casal, M.L. and Wolfe, J.H. (1998) Variant clinical course of mucopolysaccharidosis type VII in two groups of mice carrying the same mutation. *Lab Invest* 78: 1575-1581.

Chamberlain, J.W., Vasavada, H.A., Ganguly, S. and Weissman, M.S. (1991). Identification of cis sequences controlling efficient position-independent tissue-specific expression of human major histocompatibility complex class I genes in transgenic mice. *Mol Cell Biol* 11: 3564-3572.

Chung, J.H., Whiteley, M. and Felsenfeld, G. (1993) A 5' element of the chicken β-globin domain serves as an insulator in human erythroid cells and protects against position effect in Drosophila. *Cell* 74: 505-514.

Clarke, L.A., Russell, C.S., Pownall, S., Warrington, C.L., Borowski, A., Dimmick, J.E., Toone, J. and Jirik, F.R. (1997) Murine mucopolysaccharidosis type I: targeted disruption of the murine α-L-iduronidase gene. *Hum Mol Genet* 6: 503-511.

Cohen-Tannoudji, M., Marchand, P., Akli, S., Sheardown, S.A., Puech, J.P., Kress, C., Gressens, P., Nassogne, M.C., Beccari, T., Muggleton-Harris, A.L. and et al. (1995) Disruption of murine Hexa gene leads to enzymatic deficiency and to neuronal lysosomal storage, similar to that observed in Tay-Sachs disease. *Mamm Genome* 6: 844-849.

Cohen, I.J., Katz, K., Kornreich, L., Horev, G., Frish, A. and Zaizov, R. (1998). Low-dose high-frequency enzyme replacement therapy prevents fractures without complete suppression of painful bone crises in patients with severe juvenile onset type I Gaucher disease. *Blood Cells Mol Dis* 24: 296-302.

Colman, A. (1996) Production of proteins in the milk of transgenic livestock: problems, solutions, and successes. *Am J Clin Nutr* 63: 639S-645S.

Colman, A. (1998) Production of therapeutic proteins in the milk of transgenic livestock. *Biochem Soc Symp* 63: 141-147.

Coppala, G., Yan, Y., Hantzopoulos, P., Segura, E., Stroh, J.G. and Calhoun, D.H. (1994) Characterization of glycosylated and catalytically active recombinant human α-galactosidase A using a baculovirus vector. *Gene* 144: 197-203.

Cramer, C.L., Weissenborn, D.L., Oishi, K.K., Grabau, E.A., Bennett, S., Ponce, E., Grabowski, G.A. and Radin, D.N. (1996) Bioproduction of human enzymes in transgenic tobacco. *Ann N Y Acad Sci* 792: 62-71.

Crawley, A.C., Brooks, D.A., Muller, V.J., Petersen, B.A., Isaac, E.L., Bielicki, J., King, B.M., Boulter, C.D., Moore, A.J., Fazzalari, N.L., Anson, D.S., Byers, S. and Hopwood, J.J. (1996) Enzyme replacement therapy in a feline model of Maroteaux-Lamy syndrome. *J Clin Invest* 97: 1864-1873.

Crawley, A.C., Niedzielski, K.H., Isaac, E.L., Davey, R., Byers, S. and Hopwood, J.J. (1997) Enzyme replacement therapy from birth in a feline model of mucopolysaccharidosis type VI. *J Clin Invest* 99: 651-662.

Dai, Y., Roman, M., Naviaux, R.K. and Verna, I.M. (1992) Gene therapy via primary myoblasts: long term expression of factor IX protein following transplantation in vivo. *Proc Nat Acad Sci USA* **89**: 10892-10895.

Daly, T.M., Okuyama, T., Vogler, C., Haskins, M.E., Muzyczka, N. and Sands, M.S. (1999a) Neonatal intramuscular injection with recombinant adeno-associated virus results in prolonged β-glucuronidase expression in situ and correction of liver pathology in mucopolysaccharidosis type VII mice. *Hum Gene Ther* **10**: 85-94.

Daly, T.M., Vogler, C., Levy, B., Haskins, M.E. and Sands, M.S. (1999b) Neonatal gene transfer leads to widespread correction of pathology in a murine model of lysosomal storage disease. *Proc Natl Acad Sci USA* **96**: 2296-2300.

d'Azzo, A., Andria, G., Strisciuglio, P. and Galjaard, H. (1995) Galactosialidosis. *In: The metabolic and molecular bases of inherited disease*. Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. (Eds) New York, McGraw-Hill, Inc. **II**: 2825-2837.

De Duve, C., Pressman, B.C., Gianetto, R., Wattiaux, R. and Appelmans, F. (1955) Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem J* **60**: 604-617.

De Duve, C. (1964). From cytosis to lysosomes. *Fed Proc Am Soc Exp Biol* **23**: 1045-1049.

De Duve, C. (1985). *A guided tour of the living cell*. New York, The Rockefellar University Press, Scientific American Books Inc.

Desnick, R.J. (1980). *Enzyme therapy in genetic diseases:2*. New York, Alan R. Liss, Inc.

Desnick, R.J., Ioannou, Y.A. and Eng, C.M. (1999) Fabry disease: preclinical and clinical studies of α-galactosidase A replacement. *5th Symposium on Mucopolysaccharide and related diseases*, Vienna, Austria: 109.

Deussing, J., Roth, W., Saftig, P., Peters, C., Ploegh, H.L. and Villadangos, J.A. (1998) Cathepsins B and D are dispensable for major histocompatibility complex class II-mediated antigen presentation. *Proc Natl Acad Sci USA* **95**: 4516-4521.

Dillon, N. and Grosveld, F. (1993). Transcriptional regulation of multigene loci: multi level control. *Trends Genet* **9**: 134-137.

Dobrenis, K., Joseph, A. and Rattazzi, M.C. (1992) Neuronal lysosomal enzyme replacement using fragment C tetanus toxin. *Proc Natl Acad Sci USA* **89**: 2297-2301.

Drohan, W.N. (1997) The past, present and future of transgenic bioreactors. *Thromb Haemost* **78**: 543-547.

Du, H., Duanmu, M., Witte, D. and Grabowski, G.A. (1998) Targeted disruption of the mouse lysosomal acid lipase gene: long-term survival with massive cholesteryl ester and triglyceride storage. *Hum Mol Genet* **7**: 1347-1354.

Duchen, L.W., Eicher, E.M., Jacobs, J.M., Scaravilli, F. and Teixeira, F. (1980) Hereditary leucodystrophy in the mouse: the new mutant twitcher. *Brain* **103**: 695-710.

Elstein, D., Abrahamov, A., Hadas-Halpern, I., Meyer, A. and Zimran, A. (1998). Low-dose low-frequency imiglucerase as a starting regimen of enzyme replacement therapy for patients with type I Gaucher disease. *Qjm* **91**: 483-488.

Enomaa, N., Danos, O., Peltonen, L. and Jalanko, A. (1995) Correction of deficient enzyme activity in a lysosomal storage disease, aspartylglucosaminuria, by enzyme replacement and retroviral gene transfer. *Hum Gene Ther* **6**: 723-731.

Erickson, R.P. (1989) Why isn't a mouse more like a man? *Trends Genet* **5**: 1-3.

Evers, M., Saftig, P., Schmidt, P., Hafner, A., McLoghlin, D.B., Schmahl, W., Hess, B., von Figura, K. and Peters, C. (1996) Targeted disruption of the arylsulfatase B gene results in mice resembling the phenotype of mucopolysaccharidosis VI. *Proc Natl Acad Sci USA* **93**: 8214-8219.

Friden, P.M., Walus, L.R., Watson, P., Doctrow, S.R., Kozarich, J.W., Bäckman, C., Bergman, H., Hoffer, B., Bloom, F. and Granholm, A.C. (1993) Blood-brain barrier penetration and in vivo activity of an NGF conjugate. *Science* **259**: 373-377.

Fujita, N., Suzuki, K., Vanier, M.T., Popko, B., Maeda, N., Klein, A., Henseler, M., Sandhoff, K. and Nakayasu, H. (1996) Targeted disruption of the mouse sphingolipid activator protein gene: a complex phenotype, including severe leukodystrophy and wide-spread storage of multiple sphingolipids. *Hum Mol Genet* **5**: 711-725.

Fuller, M., Van der Ploeg, A., Reuser, A.J.J., Anson, D.S. and Hopwood, J.J. (1995) Isolation and characterisation of a recombinant, precursor form of lysosomal acid α-glucosidase. *Eur J Biochem* **234**: 903-909.

Galjaard, H. and Reuser, A.J.J. (1984) Genetic aspects of lysosomal storage diseases. *In: Lysosomes in biology and pathology.* Dingle, J.T., Dean, R.T. and Sly, W., (Eds) Elsevier Science Publishers BV: 315-345.

Gieselmann, V. (1995) Lysosomal storage diseases. *Biochim Biophys Acta* **1270**: 103-136.

Gieselmann, V., Matzner, U., Hess, B., Lullmann-Rauch, R., Coenen, R., Hartmann, D., D'Hooge, R., DeDeyn, P. and Nagels, G. (1998) Metachromatic leukodystrophy: molecular genetics and an animal model. *J Inherit Metab Dis* **21**: 564-574.

Gilbert, H.F. (1997) Protein disulfide isomerase and assisted protein folding. *J Biol Chem* **272**: 29399-29402.

Glaumann, H. and Ballard, F.J. (1987). *Lysosomes: Their role in protein breakdown.* London, Academic Press.

Grabowski, G.A., N.W., B., Pastores, G., Dambrosia, J.M., Banerjee, T.K., McKee, M.A., Parker, C., Schiffman, R., Hill, S.C. and Brady, R.O. (1995) Enzyme therapy in type 1 Gaucher disease: Comparative efficacy of mannose-terminated glucocerebrosidase from natural and recombinant sources. *Ann Intern Med* **122**: 33-39.

Grabowski, G.A., Leslie, N. and Wenstrup, R. (1998). Enzyme therapy for Gaucher disease: the first five years. *Blood Rev* **12**: 115-133.

Greaves, D.R., Wilson, F.D., Lang, G. and Kioussis, D. (1989). Human CD2 3' flanking sequences confer high-level T cell-specific, position-independent gene expression in transgenic mice. *Cell* **56**: 979-986.

Grosveld, F., Van Assendelft, G.E., Greaves, D.R. and Kollias, G. (1987). Position-independent, high-level expression of the human beta-globin gene in transgenic mice. *Cell* **51**: 975-985.

Gwynn, B., Lueders, K., Sands, M.S. and Birkenmeier, E.H. (1998) Intracisternal A-particle element transposition into the murine β-glucuronidase gene correlates with loss of enzyme activity: a new model for β-glucuronidase deficiency in the C3H mouse. *Mol Cell Biol* **18**: 6474-6481.

Hahn, C.N., del Pilar Martin, M., Schröder, M., Vanier, M.T., Hara, Y., Suzuki, K., Suzuki, K. and d'Azzo, A. (1997) Generalized CNS disease and massive GM1-ganglioside accumulation in mice defective in lysosomal acid β-galactosidase. *Hum Mol Genet* **6**: 205-211.

Hahn, C.N., Martin, M.P., Zhou, X.Y., Mann, L.W. and d'Azzo, A. (1998) Correction of murine galactosialidosis by bone marrow-derived macrophages overexpressing human protective protein/cathepsin A under control of the colony-stimulating factor-1 receptor promoter. *Proc Natl Acad Sci USA* **95**: 14880-14885.

Hammond, C. and Helenius, A. (1995) Quality control in the secretory pathway. *Curr Opin Cell Biol* **7**: 523-529.

Hasilik, A., Waheed, A. and Von Figura, K. (1981) Enzymatic phosporylation of lysosomal enzymes in the presence of UDP-N-acetylglucosamine. Absence of the activity in I-cell fibroblasts. *Biochem Biophys Res Commun* **98**: 761-767.

Hayman, A.R., Jones, S.J., Boyde, A., Foster, D., Colledge, W.H., Carlton, M.B., Evans, M.J. and Cox, T.M. (1996) Mice lacking tartrate-resistant acid phosphatase (Acp 5) have disrupted endochondral ossification and mild osteopetrosis. *Development* **122**: 3151-3162.

Hers, H.G. (1963) α-glucosidase deficiency in generalized glycogen storage disease (Pompe's disease). *Biochem J* **86**: 11-16.

Hers, H.G. and Van Hoof, F. (1973) *Lysosomes and storage diseases.* New York, Academic Press.

Hess, B., Saftig, P., Hartmann, D., Coenen, R., Lullmann-Rauch, R., Goebel, H.H., Evers, M., von Figura, K., D'Hooge, R., Nagels, G., De Deyn, P., Peters, C. and Gieselmann, V. (1996) Phenotype of arylsulfatase A-deficient mice: relationship to human metachromatic leukodystrophy. *Proc Natl Acad Sci USA* **93**: 14821-14826.

Hiraiwa, M., O'Brien, J.S., Kishimoto, Y., Galdzicka, M., Fluharty, A.L., Ginns, E.I. and Martin, B.M. (1993) Isolation, characterization, and proteolysis of human prosaposin, the precursor of saposins (sphingolipid activator proteins). *Arch Biochem Biophys* **304**: 110-116.

Hoogerbrugge, P.M., Poorthuis, B.J., Romme, A.E., van de Kamp, J.J., Wagemaker, G. and van Bekkum, D.W. (1988) Effect of bone marrow transplantation on enzyme levels and clinical course in the neurologically affected twitcher mouse. *J Clin Invest* **81**: 1790-1794.

Hoogerbrugge, P.M., Brouwer, O.F., Bordigoni, P., Ringden, O., Kapaun, P., Ortega, J.J., O'Meara, A., Cornu, G., Souillet, G., Frappaz, D. and et al. (1995) Allogeneic bone marrow transplantation for lysosomal storage diseases. The European Group for Bone Marrow Transplantation. *Lancet* **345**: 1398-1402.

Hoogerbrugge, P.M. and Valerio, D. (1998) Bone marrow transplantatioin and gene therapy for lysosomal storage diseases. *Bone Marrow Transplant* **21**: S34-S36.

Horinouchi, K., Erlich, S., Perl, D.P., Ferlinz, K., Bisgaier, C.L., Sandhoff, K., Desnick, R.J., Stewart, C.L. and Schuhman, E.H. (1995) Acid sphingomyelinase deficient mice: a model of types A and B Niemann-Pick disease. *Nat Genet* **10**: 288-292.

Houdebine, L.M. (1994) Production of pharmaceutical proteins from transgenic animals. *J Biotechnol* **34**: 269-287.

Ioannou, Y.A., Bishop, D.F. and Desnick, R.J. (1992) Overexpression of human α-galactosidase A results in its intracellular aggregation, crystallization in lysosomes, and selective secretion. *J Cell Biol* **119**: 1137-1150.

Itoh, K., Oshima, A., Sakuraba, H. and Suzuki, Y. (1990) Expression, glycosylation, and intracellular distribution of human β-galactosidase in recombinant baculovirus-infected Spodoptera frugiperda cells. *Biochem Biophys Res Commun* **167**: 746-753.

Jalanko, A., Tenhunen, K., McKinney, C.E., LaMarca, M.E., Rapola, J., Autti, T., Joensuu, R., Manninen, T., Sipila, I., Ikonen, S., Riekkinen, P., Jr., Ginns, E.I. and Peltonen, L. (1998) Mice with an aspartylglucosaminuria mutation similar to humans replicate the pathophysiology in patients. *Hum Mol Genet* **7**: 265-272.

Janne, J., Alhonen, L., Hyttinen, J.M., Peura, T., Tolvanen, M. and Korhonen, V.P. (1998) Transgenic bioreactors. *Biotechnol Annu Rev* **4**: 55-74.

Jolly, R.D. and Walkley, S.U. (1997) Lysosomal diseases of animals: an essay in comparative pathology. *Vet Pathol* **34**: 527-548.

Jones, B.K., Monks, B.R., Liebhaber, S.A. and Cooke, N.E. (1995). The human growth hormone gene is regulated by a multicomponent locus control region. *Mol Cell Biol* **15**: 7010-7021.

Kaartinen, V., Mononen, I., Voncken, J.W., Noronkoski, T., Gonzalez-Gomez, I., Heisterkamp, N. and Groffen, J. (1996) A mouse model for the human lysosomal disease aspartylglucosaminuria. *Nat Med* **2**: 1375-1378.

Kakkis, E.D., Matynia, A., Jonas, A.J. and Neufeld, E.F. (1994) Overexpression of the human lysosomal enzyme α-L-iduronidase in Chinese hamster ovary cells. *Protein Expr Purif* **5**: 225-232.

Kakkis, E.D., McEntee, M.F., Schmidtchen, A., Neufeld, E.F., Ward, D.A., Gompf, R.E., Kania, S., Bedollia, C., Chien, S.L. and Shull, R.M. (1996) Long-term and high-dose trials of enzyme replacement therapy in the canine model of mucopolysaccharidosis I. *Biochem Mol Med* **58**: 156-167.

Kakkis, E.D., Muenzer, J., Tiller, G., Waber, L., Belmont, J., Passage, M., Izykowski, B., Phillips, J., Walot, I., Doroshow, R., Hoft, R., Yu, K.T., Okazaki, S., Lewis, D., Lachman, R. and Neufeld, E.F. (1999). Enzyme replacement therapy in mucopolysaccharidosis I: Results of a human clinical trial. *5th Symposium on Mucopolysaccharide and related diseases*, Vienna, Austria: A25.

Kasper, D., Dittmer, F., von Figura, K. and Pohlmann, R. (1996) Neither type of mannose 6-phosphate receptor is sufficient for targeting of lysosomal enzymes along intracellular routes. *J Cell Biol* **134**: 615-623.

Kay, M.A., Liu, D. and Hoogerbrugge, P.M. (1997) Gene therapy. *Proc Natl Acad Sci USA* **94**: 12744-12746.

Kerr, D.E. and Wall, R.J. (1996) Jet-gun transfection of the lactating ovine mammary gland in vivo with plasmid DNA containing mammary specific promoters. *J Anim Sci* **74 suppl**: 121.

Kerr, D.E., Liang, F., Bondioli, K.R., Zhao, H., Kreibich, G., Wall, R.J. and Sun, T.T. (1998) The bladder as a bioreactor: urothelium production and secretion of growth hormone into urine. *Nat Biotechnol* **16**: 75-79.

Kleijer, W.J., van der Kraan, M., Kroos, M.A., Groener, J.E., van Diggelen, O.P., Reuser, A.J.J. and van der Ploeg, A.T. (1995). Prenatal diagnosis of glycogen storage disease type II: enzyme assay or mutation analysis? *Pediatr Res* **38**: 103-106.

Kmiec, E.B. (1999) Targeted gene repair. *Gene Therapy* **6**: 1-3.

Kobayashi, T., Yamanaka, T., Jacobs, J.M., Teixeira, F. and Suzuki, K. (1980) The Twitcher mouse: an enzymatically authentic model of human globoid cell leukodystrophy (Krabbe disease). *Brain Res* **202**: 479-483.

Kornfeld, R. and Kornfeld, S. (1985) Assembly of asparagine-linked oligosaccharides. *Annu Rev Biochem* **54**: 631-664.

Kornfeld, S. (1986) Trafficking of lysosomal enzymes in normal and disease states. *J Clin Invest* **77**: 1-6.

Kornfeld, S. and Mellman, I. (1989) The biogenesis of lysosomes. *Annu Rev Cell Biol* **5**: 483-525.

Kornfeld, S. (1992) Structure and function of the mannose 6-phosphate/insulinlike growth factor II receptors. *Annu Rev Biochem* **61**: 307-330.

Köster, A., Saftig, P., Matzner, U., von Figura, K., Peters, C. and Pohlmann, R. (1993) Targeted disruption of the M(r) 46,000 mannose 6-phosphate receptor gene in mice results in misrouting of lysosomal proteins. *EMBO J* 12: 5219-5223.

Kren, B.T., Bandyopadhyay, P. and Steer, C.J. (1998) In vivo site-directed mutagenesis of the factor IX gene by chimeric RNA/DNA oligonucleotides. *Nat Med* 4: 285-290.

Lacorazza, H.D., Flax, J.D., Snyder, E.Y. and Jendoubi, M. (1996) Expression of human β-hexosaminidase α-subunit gene (the gene defect of Tay-Sachs disease) in mouse brains upon engraftment of transduced progenitor cells. *Nat Med* 2: 424-429.

Law, P., Goodwin, T., Fang, Q., Vastagh, G., Jordan, T., Jackson, T., Kenny, S., Duggirala, V., Larkin, C., Chase, N., Phillips, W., Williams, G., Neel, M., Krahn, T. and Holcomb, R. (1998) Myoblast transfer as a platform technology of gene therapy. *Gene Ther Mol Biol* 1: 345-363.

Leborgne, R. and Hoflack, B. (1997) Mannose 6-phosphate receptors regulate the formation of clathrin-coated vesicles in the TGN. *J Cell Biol* 137: 335-345.

Li, H.H., Yu, W.H., Zhao, H.Z., Rozengurt, N., Lyons, K.M., Anagnostaras, S.G. and Neufeld, E.F. (1998) A mouse model for Sanfilippo syndrome type B. *Am J Hum Genet* 64 **suppl:** A15.

Litjens, T., Bielicki, J., Anson, D.S., Friderici, K., Jones, M.Z. and Hopwood, J.J. (1997) Expression, purification and characterization of recombinant caprine N-acetylglucosamine-6-sulphatase. *Biochem J* 327: 89-94.

Liu, Y., Hoffmann, A., Grinberg, A., Westphal, H., McDonald, M.P., Miller, K.M., Crawley, J.N., Sandhoff, K., Suzuki, K. and Proia, R.L. (1997) Mouse model of GM2 activator deficiency manifests cerebellar pathology and motor impairment. *Proc Natl Acad Sci USA* 94: 8138-8143.

Liu, C., Bahnson, A.B., Dunigan, J.T., Watkins, S.C. and Barranger, J.A. (1998a) Long-term expression and secretion of human glucocerebrosidase by primary murine and human myoblasts and differentiated myotubes. *J Mol Med* 76: 773-781.

Liu, Y., Suzuki, K., Reed, J.D., Grinberg, A., Westphal, H., Hoffmann, A., Doring, T., Sandhoff, K. and Proia, R.L. (1998b) Mice with type 2 and 3 Gaucher disease point mutations generated by a single insertion mutagenesis procedure (SIMP). *Proc Natl Acad Sci USA* 95: 2503-2508.

Liu, Y., Wada, R., Kawai, H., Sango, K., Deng, C., Tai, T., McDonald, M.P., Araujo, K., Crawley, J.N., Bierfreund, U., Sandhoff, K., Suzuki, K. and Proia, R.L. (1999). A genetic model of substrate deprivation therapy for a glycosphingolipid storage disorder. *J Clin Invest* 103: 497-505.

Loftus, S.K., Morris, J.A., Carstea, E.D., Gu, J.Z., Cummings, C., Brown, A., Ellison, J., Ohno, K., Rosenfeld, M.A., Tagle, D.A., Pentchev, P.G. and Pavan, W.J. (1997) Murine model of Niemann-Pick C disease: mutation in a cholesterol homeostasis gene. *Science* 277: 232-235.

Luckow, V.A. and Summers, M.D. (1988) Trends in the development of baculovirus expression vectors. *Biotechnology* 6: 47-55.

Ludwig, T., Ovitt, C.E., Bauer, U., Hollinshead, M., Remmler, U., Lobel, P., Ruther, U. and Hoflack, B. (1993) Targeted disruption of the mouse cation-dependent mannose 6-phosphate receptor results in partial missorting of multiple lysosomal enzymes. *EMBO J* 12.

Malewski, R. (1998) Computer analysis of distribution of putative cis- and trans-regulatory elements in milk protein gene promoters. *BioSystems* 45: 29-44.

Marechal, V., Naffakh, N., Danos, O. and Heard, J.M. (1993) Disappearance of lysosomal storage in spleen and liver of mucopolysaccharidosis type VII mice after transplantation of genetically modified bone marrow cells. *Blood* 82: 1358.

Markello, T.C., Bernardini, I.M. and Gahl, W.A. (1993) Improved renal function in children with cystinosis treated with cysteamine. *New Engl J Med* 328: 1157-1162.

Marks, S.C. (1977). Pathogenesis of osteopetrosis in the microphthalmic mouse: reduced bone resorption. *Am J Anat* 149: 269-275.

Marshall, E. (1995). Gene therapy's growing pains. *Science* 269: 1050-1055.

Martin, B.M., Tsuji, S., LaMarca, M.E., Maysak, K., Eliason, W. and Ginns, E.I. (1988) Glycosylation and processing of high levels of active human glucocerebrosidase in invertebrate cells using a baculovirus expression vector. *DNA* 7: 99-106.

Matsuda, J., Suzuki, O., Oshima, A., Ogura, A., Noguchi, Y., Yamamoto, Y., Asano, T., Takimoto, K., Sukegawa, K., Suzuki, Y. and Naika, M. (1997) β-Galactosidase-deficient mouse as an animal model for GM1-gangliosidosis. *Glycoconj J* 14: 729-736.

McKnight, R.A., Shamay, A., Sankaran, L., Wall, R.J. and Hennighausen, L. (1992). Matrix-attachement regions can impart positions-independent regulation of a tissue-specific gene in transgenic mice. *Proc Natl Acad Sci USA* 89: 6043-6947.

Miranda, S.R., Erlich, S., Visser, J.W., Gatt, S., Dagan, A., Friedrich, V.L., Jr. and Schuchman, E.H. (1997) Bone marrow transplantation in acid sphingomyelinase-deficient mice: engraftment and cell migration into the brain as a function of radiation, age, and phenotype. *Blood* 90: 444-452.

Mistry, P.K. and Abrahamov, A. (1998). A practical approach to diagnosis and management of Gaucher's disease. *Baill Clin Haem* 10: 817-838.

Moullier, P., Bohl, D., Heard, J.M. and Danos, O. (1993a) Correction of lysosomal storage in the liver and spleen of MPS VII mice by implantation of genetically modified skin fibroblasts. *Nat Genet* 4: 154-159.

Moullier, P., Marechal, V., Danos, O. and Heard, J.M. (1993b) Continuous systemic secretion of a lysosomal enzyme by genetically modified mouse skin fibroblasts. *Transplantation* 56: 427-432.

Muenzer (1999) Targeted disruption of the mouse iduronate sulfatase gene. *5th Symposium on Mucopolysaccharide and related diseases*, Vienna, Austria: 61.

Naffakh, N., Pinset, C., Montarras, D., Pastoret, C., Danos, O. and Heard, J.M. (1993) Transplantation of adult-derived myoblasts in mice following gene transfer. *Neuromusc Disord* 3: 413-417.

Naffakh, N., Pinset, C., Montarras, D., Li, Z., Paulin, D., Danos, O. and Heard, J.M. (1996). Long-term secretion of therapeutic proteins from genetically modified skeletal muscles. *Hum Gene Ther* 7: 11-21.

Nagano, S., Yamada, T., Shinnoh, N., Furuya, H., Taniwaki, T. and Kira, J. (1998) Expression and processing of recombinant human galactosylceramidase. *Clin Chim Acta* 276: 53-61.

Nakagawa, T., Roth, W., Wong, P., Nelson, A., Farr, A., Deussing, J., Villadangos, J.A., Ploegh, H., Peters, C. and Rudensky, A.Y. (1998) Cathepsin L: critical role in li degradation and CD4 T cell selection in the thymus. *Science* 280: 450-453.

Neufeld, E.F., Lim, T.W. and Shapiro, L.J. (1975) Inherited disorders of lysosomal metabolism. *Annu Rev Biochem* 44: 357-376.

Neufeld, E.F. (1980) The uptake of enzymes into lysosomes. An overview. *In: Enzyme therapy in genetic diseases: 2*. Desnick, R.J. (Ed) New York, Alan R. Liss: 77-84.

Neufeld, E.F. (1991) Lysosomal storage diseases. *Annu Rev Biochem* 60: 257-280.

O'Brien, J.S., Storb, R., Raff, R.F., Harding, J., Appelbaum, F., Morimoto, S., Kishimoto, Y., Graham, T., Ahern-Rindell, A. and O'Brien, S.L. (1990) Bone marrow transplantation in canine GM1 gangliosidosis. *Clin Genet* 38: 274-280.

O'Connor, L.H., Erway, L.C., Vogler, C.A., Sly, W.S., Nicholes, A., Grubb, J., Holmberg, S.W., Levy, B. and Sands, M.S. (1998) Enzyme replacement therapy for murine mucopolysaccharidosis type VII leads to improvements in behavior and auditory function. *J Clin Invest* 101: 1394-1400.

Ohashi, T., Watabe, K., Uehara, K., Sly, W.S., Vogler, C. and Eto, Y. (1997) Adenovirus-mediated gene transfer and expression of human β-glucuronidase gene in the liver, spleen, and central nervous system in mucopolysaccharidosis type VII mice. *Proc Natl Acad Sci USA* 94: 1287-1292.

Ohshima, T., Murray, G.J., Swaim, W.D., Longenecker, G., Quirk, J.M., Cardarelli, C.O., Sugimoto, Y., Pastan, I., Gottesman, M.M., Brady, R.O. and Kulkarni, A.B. (1997) α-Galactosidase A deficient mice: a model of Fabry disease. *Proc Natl Acad Sci USA* 94: 2540-2544.

Otterbach, B. and Stoffel, W. (1995) Acid sphingomyelinase-deficient mice mimic the neurovisceral form of human lysosomal storage disease (Niemann-Pick disease). *Cell* 81: 1053-1061.

Partridge, T.A. and Davies, K.E. (1995) Myoblast-based gen therapies. *Brit Med Bull* 51: 123-137.

Peltola, M., Kyttala, A., Heinonen, O., Rapola, J., Paunio, T., Revah, F., Peltonen, L. and Jalanko, A. (1998) Adenovirus-mediated gene transfer results in decreased lysosomal storage in brain and total correction in liver of aspartylglucosaminuria (AGU) mouse. *Gene Ther* 5: 1314-1321.

Peters, C. and Von Figura, K. (1994) Biogenesis of lysosomal membranes. *FEBS Letters* 346: 108-114.

Phaneuf, D., Wakamatsu, N., Huang, J.Q., Borowski, A., Peterson, A.C., Fortunato, S.R., Ritter, G., Igdoura, S.A., Morales, C.R., Benoit, G., Akerman, B.R., Leclerc, D., Hanai, N., Marth, J.D., Trasler, J.M. and Gravel, R.A. (1996) Dramatically different phenotypes in mouse models of human Tay-Sachs and Sandhoff diseases. *Hum Mol Genet* 5: 1-14.

Platt, F.M., Neises, G.R., Reinkensmeier, G., Townsend, M.J., Perry, V.H., Proia, R.L., Winchester, B., Dwek, R.A. and Butters, T.D. (1997) Prevention of lysosomal storage in Tay-Sachs mice treated with N- butyldeoxynojirimycin. *Science* 276: 428-431.

Poenaru, L. (1996) Particularities of lysosomal storage diseases gene therapy. *Gene Therapy* 3: 1039-1041.

Pohlmann, R., Boeker, M.W. and von Figura, K. (1995) The two mannose 6-phosphate receptors transport distinct complements of lysosomal proteins. *J Biol Chem* 270: 27311-27318.

Potier, M., Yan, D. and Womack, J. (1979) Neuraminidase deficiency in the mouse. *FEBS Lett* 108: 345-348.

Raben, N., Nagaraju, K., Lee, E., Ward, J., Byrne, B., Kessler, P., Sauer, B. and Plotz, P. (1998a) Modulation of disease severity in knockout mice with targeted disruption of the acid α-glucosidase gene. *Am J Hum Genet* **64 suppl**: A381.

Raben, N., Nagaralu, K., Lee, E., Kessler, P., Byrne, B., Lee, L., LaMarca, M., King, C., Ward, J., Sauer, B. and Plotz, P. (1998b) Targeted disruption of the acid α-glucosidase gene in mice causes an illness with critical features of both infantile and adult human glycogen storage disease type II. *J Biol Chem* **273**: 19086-19092.

Rajagopalan, S., Xu, Y. and Brenner, M.B. (1994) Retention of unassembled components of integral membrane proteins by calnexin. *Science* **263**: 387-390.

Rapoport, T.A., Jungnickel, B. and Kutay, U. (1996) Protein transport across the eukaryotic endoplasmic reticulum and bacterial inner membranes. *Annu Rev Biochem* **65**: 271-303.

Reitman, M.L. and Kornfeld, S. (1981) UDP-N-acetylglucosamine:glycoprotein N-acetylglucosamine-1-phosphotransferase. Proposed enzyme for the phosphorylation of the high mannose oligosaccharide units of lysosomal enzymes. *J Biol Chem* **256**: 4275-4281.

Reuser, A.J.J., Kroos, M.A., Visser, W.J. and Willemsen, R. (1994) Lysosomal storage diseases: cellular pathology, clinical and genetic heterogeneity, therapy. *Ann Biol Clin* **52**: 721-728.

Rijnkels, M., Kooiman, P.M., Krimpenfort, P.J., de Boer, H.A. and Pieper, F.R. (1995) Expression analysis of the individual bovine β-, $α_{S2}$- and κ-casein genes in transgenic mice. *Biochem J* **311**: 929-937.

Rosen, J.M., Li, S., Raught, B. and Hadsell, D. (1996) The mammary gland as a bioreactor: factors regulating the efficient expression of milk protein-based transgenes. *Am J Clin Nutr* **63**: 627S-632S.

Rosenthal, D.I., Doppelt, S.H., Mankin, H.J., Dambrosia, J.M., Xavier, R.J., McKusick, K.A., Rosen, B.R., Baker, J., Niklason, L.T., Hill, S.C. and et al. (1995) Enzyme replacement therapy for Gaucher disease: skeletal responses to macrophage-targeted glucocerebrosidase. *Pediatrics* **96**: 629-637.

Roth, W., Deussing, J., Von Figura, K., Anton-Lamprecht, I., Schmahl, W., Schmidt, P., Hafner, A. and Peters, C. (1997) Cathepsin L knockout mice present periodic loss of hair, hyperkeratosis and acanthosis as do furless mice carrying a missense mutation in the cathepsin L gene. *ESGLD Workshop*, Bad Deutsch-Altenburg, Austria: 75.

Rottier, R.J., Bonten, E. and d'Azzo, A. (1998) A point mutation in the neu-1 locus causes the neuraminidase defect in the SM/J mouse. *Hum Mol Genet* **7**: 313-321.

Rozmahel, R., Wilschanski, M., Matin, A., Plyte, S., Oliver, M., Auerbach, W., Moore, A., Forstner, J., Durie, P., Nadeau, J., Bear, C. and Tsui, L.C. (1996) Modulation of disease severity in cystic fibrosis transmembrane conductance regulator deficient mice by a secondary genetic factor. *Nat Genet* **12**: 280-287.

Russell, S.J. (1997) Science, medicine, and the future. *Brit Med J* **315**: 1289-1292.

Saftig, P., Hetman, M., Schmahl, W., Weber, K., Heine, L., Mossmann, H., Köster, A., Hess, B., Evers, M., von Figura, K. and Peters, C. (1995) Mice deficient for the lysosomal proteinase cathepsin D exhibit progressive atrophy of the intestinal mucosa and profound destruction of lymphoid cells. *EMBO J* **14**: 3599-3608.

Saftig, P., Hartmann, D., Lullmann-Rauch, R., Wolff, J., Evers, M., Köster, A., Hetman, M., von Figura, K. and Peters, C. (1997) Mice deficient in lysosomal acid phosphatase develop lysosomal storage in the kidney and central nervous system. *J Biol Chem* **272**: 18628-18635.

Saftig, P., Hunziker, E., Wehmeyer, O., Jones, S., Boyde, A., Rommerskirch, W., Moritz, J.D., Schu, P. and von Figura, K. (1998) Impaired osteoclastic bone resorption leads to osteopetrosis in cathepsin-K-deficient mice. *Proc Natl Acad Sci USA* **95**: 13453-13458.

Salvetti, A., Heard, J.M. and Danos, O. (1995a) Gene therapy of lysosomal storage disorders. *Brit Med Bull* **51**: 106-122.

Salvetti, A., Moullier, P., Cornet, V., Brooks, D., Hopwood, J.J., Danos, O. and Heard, J.M. (1995b) *In vivo* delivery of human α-L-iduronidase in mice implanted with neo-organs. *Hum Gene Ther* **6**: 1153-1159.

Sandoval, I.V. and Bakke, O. (1994) Targeting of membrane proteins to endosomes and lysosomes. *Trends in Cell Biol* **4**: 292-297.

Sands, M.S., Barker, J.E., Vogler, C., Levy, B., Gwynn, B., Galvin, N., Sly, W.S. and Birkenmeier, E. (1993) Treatment of murine mucopolysaccharidosis type VII by syngeneic bone marrow transplantation in neonates. *Lab Invest* **68**: 676-686.

Sands, M.S. and Birkenmeier, E.H. (1993) A single-base-pair deletion in the β-glucuronidase gene accounts for the phenotype of murine mucopolysaccharidosis type VII. *Proc Natl Acad Sci USA* **90**: 6567-6571.

Sands, M.S., Vogler, C., Kyle, J.W., Grubb, J.H., Levy, B., Galvin, N., Sly, W.S. and Birkenmeier, E.H. (1994) Enzyme replacement therapy for murine mucopolysaccharidosis type VII. *J Clin Invest* **93**: 2324-2331.

Sands, M.S., Vogler, C., Torrey, A., Levy, B., Gwynn, B., Grubb, J., Sly, W.S. and Birkenmeier, E.H. (1997a) Murine mucopolysaccharidosis type VII: Long term therapeutic effects of enzyme replacement and enzyme replacement followed by bone marrow transplantation. *J Clin Invest* **99**: 1596-1605.

Sands, M.S., Wolfe, J.H., Birkenmeier, E.H., Barker, J.E., Vogler, C., Sly, W.S., Okuyama, T., Freeman, B., Nicholes, A., Muzyczka, N., Chang, P.L. and Axelrod, H.R. (1997b) Gene therapy for murine mucopolysaccharidosis type VII. *Neuromuscul Disord* **7**: 352-360.

Sango, K., McDonald, M.P., Crawley, J.N., Mack, M.L., Tifft, C.J., Skop, E., Starr, C.M., Hoffmann, A., Sandhoff, K., Suzuki, K. and Proia, R.L. (1996) Mice lacking both subunits of lysosomal β-hexosaminidase display gangliosidosis and mucopolysaccharidosis. *Nat Genet* **14**: 348-352.

Sango, K., Yamanaka, S., Hoffmann, A., Okuda, Y., Grinberg, A., Westphal, H., McDonald, M.P., Crawley, J.N., Sandhoff, K., Suzuki, K. and Proia, R.L. (1995) Mouse models of Tay-Sachs and Sandhoff diseases differ in neurologic phenotype and ganglioside metabolism. *Nat Genet* **11**: 170-176.

Schiedner, G., Morral, N., Parks, R.J., Wu, Y., Koopmans, S.C., Langston, C., Graham, F.L., Beaudet, A.L. and Kochanek, S. (1998) Genomic DNA transfer with a high-capacity adenovirus vector results in improved *in vivo* gene expression and decreased toxicity. *Nat Genet* **18**: 180-183.

Schiffmann, R., Murray, G., Treco, D., Daniel, P., Myers, M., Quirk, J., Zirzow, G., Oliver, K., Kreps, C., Gunter, K., Selden, R. and Brady, R.O. (1999) A phase I trial of enzyme replacement therapy for Fabry disease. *5th Symposium on Mucopolysaccharide and related diseases*, Vienna, Austria: 110.

Schmid, S.L. (1997) Clathrin-coated vescicle formation and protein sorting: an integrated process. *Annu Rev Biochem* **66**: 511-548.

Scott, H.S., Bunge, S., Gal, A., Clarke, A., Morris, C.P. and Hopwood, J.J. (1996). Molecular genetics of mucopolysaccharidosis type I: Diagnostic, clinical, and biological implications. *Hum Mutat* **6**: 288-302.

Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D., (Eds.) (1995). Part 12. Lysosomal enzymes, *In: The metabolic and molecular bases of inherited disease II*, McGraw-Hill, Inc.: 2527-2979.

Shull, R.M., Breider, M.A. and Constantopoulos, G.C. (1988) Long-term neurological effects of bone marrow transplantation in a canine lysosomal storage disease. *Ped Res* **24**: 347-352.

Shull, R.M., Hastings, N.E., Selcer, R.R., Jones, J.B., Smith, J.R., Cullen, W.C. and Constantopoulos, G. (1987) Bone marrow transplantation in canine mucopolysaccharidosis I. Effects within the central nervous system. *J Clin Invest* **79**: 435-443.

Shull, R.M., Kakkis, E.D., McEntee, M.F., Kania, S.A., Jonas, A.J. and Neufeld, E.F. (1994) Enzyme replacement in a canine model of Hurler syndrome. *Proc Natl Acad Sci USA* **91**: 12937-12941.

Sibilia, M. and Wagner, E.F. (1995) Strain-dependent epithelial defects in mice lacking the EFG receptor. *Science* **269**: 234-238.

Silberstein, S. and Gilmore, R. (1996) Biochemistry, molecular biology, and genetics of the oligosaccharyltransferase. *Faseb J* **10**: 849-858.

Snyder, E.Y., Taylor, R.M. and Wolfe, J.H. (1995) Neural progenitor cell engraftment corrects lysosomal storage throughout the MPS VII mouse brain. *Nature* **374**: 367-370.

Steed, P.M., Lasala, D., Liebman, J., Wigg, A., Clark, K. and Knap, A.K. (1998) Characterization of recombinant human cathepsin B expressed at high levels in baculovirus. *Protein Sci* **7**: 2033-2037.

Suzuki, K., Proia, R.L. and Suzuki, K. (1998) Mouse models of human lysosomal diseases. *Brain Pathol* **8**: 195-215.

Suzuki, Y., Sakuraba, H. and Oshima, A. (1995) β-Galactosidase deficiency (β-galactosidosis): GM1 gangliosidosis and Morquio B disease. *In: The metabolic and molecular bases of inherited disease.* Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. (Eds) New York, McGraw-Hill, Inc. **II**: 2785-2824.

Tager, J.M., Hooghwinkel, G.J.M. and Daems, W.T. (1974). *Enzyme therapy in lysosomal storage diseases.* Amsterdam, North Holland Publishing Company.

Taylor, R.M., Farrow, B.R. and Stewart, G.J. (1992) Amelioration of clinical disease following bone marrow transplantation in fucosidase-deficient dogs. *Am J Med Genet* **42**: 628-632.

Taylor, R.M. and Wolfe, J.H. (1997) Decreased lysosomal storage in the adult MPS VII mouse brain in the vicinity of grafts of retroviral vector-corrected fibroblasts secreting high levels of β-glucuronidase. *Nat Med* **3**: 771-774.

Threadgill, D.W., Dlugosz, A.A., Hansen, L.A., Tennenbaum, T., Lichti, U., Yee, D., LaMantia, C., Mourton, T., Herrup, K., Harris, R.C. and et al. (1995) Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. *Science* **269**: 230-234.

Tilkorn, A.C., Merkel, M., Greten, H. and Ameis, D. (1999) High-level baculoviral expression of lysosomal acid lipase. *Methods Mol Biol* **109**: 177-185.

Trombetta, E.S. and Helenius, A. (1998) Lectins as chaperones in glycoprotein folding. *Curr Opin Struct Biol* **8**: 587-592.

Tybulewicz, V.L.J., Tremblay, M.L., LaMarca, M.E., Willemsen, R., Stubblefield, B.K., Winfield, S., Zablocka, B., Sidransky, E., Martin, B.M., Huang, S.P., Mintzer, K.A., Westphal, H., Mulligan, R.C. and Ginns, E.I. (1992) Animal model of Gaucher's disease from targeted disruption of the mouse glucocerebrosidase gene. *Nature* **357**: 407-410.

Unger, E.G., Durrant, J., Anson, D.S. and Hopwood, J.J. (1994) Recombinant α-L-iduronidase: characterization of the purified enzyme and correction of mucopolysaccharidosis type I fibroblasts. *Biochem J* **304**: 43-49.

Van der Ploeg, A.T. (1989) Glycogenosis type II. A study on clinical heterogeneity and enzyme replacement therapy. *Thesis.*

Van Hove, J.L.K., Yang, H.W., Wu, J.Y., Brady, R.O. and Chen, Y.T. (1996) High-level production of recombinant human lysosomal acid α-glucosidase in Chinese hamster ovary cells which targets to heart muscle and corrects glycogen accumulation in fibroblasts from patients with Pompe disease. *Proc Natl Acad Sci USA* **93**: 65-70.

Verma, I.M. and Somia, N. (1997) Gene therapy- promises, problems and prospects. *Nature* **389**: 239-242.

Vogler, C., Sands, M.S., Levy, B., Galvin, N., Birkenmeier, E.H. and Sly, W.S. (1996) Enzyme replacement with recombinant β-glucuronidase in murine mucopolysaccharidosis type VII: impact of therapy during the first six weeks of life on subsequent lysosomal storage, growth, and survival. *Pediatr Res* **39**: 1050-1054.

Vogler, C., Sands, M.S., Galvin, N., Levy, B., Thorpe, C., Barker, J. and Sly, W.S. (1998) Murine mucopolysaccharidosis type VII: the impact of therapies on the clinical course and pathology in a murine model of lysosomal storage disease. *J Inherit Metab Dis* **21**: 575-586.

Von Figura, K. and Hasilik, A. (1986) Lysosomal enzymes and their receptors. *Ann Rev Biochem* **55**: 167-193.

Walkley, S.U., Thrall, M.A., Dobrenis, K., Huang, M., March, P.A., Siegel, D.A. and Wurzelmann, S. (1994) Bone marrow transplantation corrects the enzyme defect in neurons of the central nervous system in a lysosomal storage disease. *Proc Natl Acad Sci USA* **91**: 2970-2974.

Walkley, S.U. (1998) Cellular pathology of lysosomal storage disorders. *Brain Pathol* **8**: 175-193.

Wall, R.J., Kerr, D.E. and Bondioli, K.R. (1997) Trangenic diary cattle: genetic engineering on a large scale. *J Dairy Sci* **80**: 2213-2224.

Wang, A.M., Stewart, C.L. and Desnick.R.J. (1993) α-N-Acetyl-galactosaminidase: characterization of the murine cDNA and genomic sequences and generation of the mice by targeted gene disruption. *Am J Hum Genet* **53 suppl**: 99.

Wang, Z.Q., Fung, M.R., Barlow, D.P. and Wagner, E.F. (1994) Regulation of embryonic growth and lysosomal targeting by the imprinted Igf2/Mpr gene. *Nature* **372**: 464-467.

Wang, W., Nebes, V.L., Schmidt, M.C. and Barranger, J.A. (1996) Expression of human lysosomal α-mannosidase activity in transfected murine cells and human α-mannosidase deficient fibroblasts. *Biochem Biophys Res Comm.*

Watson, G.L., Sayles, J.N., Chen, C., Elliger, S.S., Elliger, C.A., Raju, N.R., Kurtzman, G.J. and Podsakoff, G.M. (1998) Treatment of lysosomal storage disease in MPS VII mice using a recombinant adeno-associated virus. *Gene Ther* **5**: 1642-1649.

Weber, B., Blanch, L., Clements, P.R., Scott, H.S. and Hopwood, J.J. (1996) Cloning and expression of the gene involved in Sanfilippo B syndrome (mucopolysaccharidosis III B). *Hum Mol Genet* **5**: 771-777.

Wijgerde, M., Grosveld, F. and Fraser, P. (1995) Transcription complex stability and chromatin dynamics in vivo. *Nature* **377**: 209-213.

Willemsen, R., Tybulewicz, V., Sidransky, E., Eliason, W.K., Martin, B.M., LaMarca, M.E., Reuser, A.J., Tremblay, M., Westphal, H., Mulligan, R.C. and et, a. (1995) A biochemical and ultrastructural evaluation of the type 2 Gaucher mouse. *Mol Chem Neuropathol* **24**: 179-192.

Winfield, S.L., Tayebi, N., Martin, B.M., Ginns, E.I. and Sidransky, E. (1997) Identification of three additional genes contiguous to the glucocerebrosidase locus on chromosome 1q21: Implications for Gaucher disease. *Genome Res* **7**: 1020-1026.

Wolf, D.P., Meng, L., Ely, J.J. and Stouffer, R.L. (1998) Recent progress in mammalian cloning. *J Assist Reprod Genet* **15**: 235-239.

Wolfe, J.H., Sands, M.S., Barker, J.E., Gwynn, B., Rowe, L.B., Vogler, C.A. and Birkenmeier, E.H. (1992) Reversal of pathology in murine mucopolysaccharidsosis type VII by somatic cell gene transfer. *Nature* **360**: 749-753.

Wu, J.Y., Van Hove, J.L.K., Huang, Y.S. and Chen, Y.T. (1996) Expression of catalytically active human multifunctional glycogen-debranching enzyme and lysosomal acid α-glucosidase in insect cells. *Biochem Mol Biol Int* **39**: 755-764.

Wynshaw-Boris, A. (1996) Model mice and human disease. *Nat Genet* **13**: 259-260.

Yamanaka, S., Johnson, M.D., Grinberg, A., Westphal, H., Crawley, J.N., Taniike, M., Suzuki, K. and Proia, R.L. (1994) Targeted disruption of the Hexa gene results in mice with biochemical and pathologic features of Tay-Sachs disease. *Proc Natl Acad Sci USA* **91**: 9975-9979.

Yeager, A.M., Brennan, S., Tiffany, C., Moser, H.W. and Santos, G.W. (1984) Prolonged survival and remyelination after hematopoietic cell transplantation in the twitcher mouse. *Science* **225**: 1052-1054.

Yogalingam, G., Crawley, A., Hopwood, J.J. and Anson, D.S. (1999) Evaluation of fibroblast-mediated gene therapy in a feline model of mucopolysaccharidosis type VI. *Biochim Biophys Acta* **1453**: 284-296.

Zhou, X.Y., Morreau, H., Rottier, R., Davis, D., Bonten, E., Gillemans, N., Wenger, D., Grosveld, F.G., Doherty, P., Suzuki, K., Grosveld, G.C. and d'Azzo, A. (1995) Mouse model for the lysosomal disorder galactosialidosis and correction of the phenotype with overexpressing erythroid precursor cells. *Genes Dev* **9**: 2623-2634.

Zhou, X.Y., Van der Spoel, A., Rottier, R., Hale, G., Willemsen, R., Berry, G.T., Strisciuglio, P., Andria, G. and d'Azzo, A. (1996). Molecular and biochemical analysis of protective protein/cathepsin A mutations: correlation with clinical severity in galactosialidosis. *Hum Mol Genet* **5**: 1977-1987.

Chapter 2

Glycogen storage disease type II

Clinical aspects of glycogen storage disease type II

Generalized vacuolar storage of glycogen was first reported by Pompe (Pompe 1932) in a 7-month-old infant, who died with idiopathic hypertrophy of the heart. Membrane bound vacuoles containing a variety of acid hydrolases were identified in 1955 and were given the name "lysosomes" (De Duve *et al.* 1955). The molecular basis of glycogen storage disease type II (GSDII) was revealed in 1963 when Hers and co-workers demonstrated that acid α-glucosidase, normally present in the lysosomes, was absent in infants with GSDII (Hers 1963; Lejeune *et al.* 1963). Besides the classic infantile variant of the disease (often described as Pompe's disease), milder, late-onset, variants were described forming together a clinical spectrum with differences in age of onset and clinical course (Engel *et al.* 1973; Loonen 1979; Hirschhorn 1995; Reuser *et al.* 1995). The disease is inherited as an autosomal recessive trait. The incidence of the disease varies between different ethnic groups and for different clinical forms, and the estimated frequency ranges from 1/300,000 to 1/40,000 (Lin *et al.* 1987; Kroos *et al.* 1995; Ausems *et al.* 1998; Martiniuk *et al.* 1998).

The clinical picture of the most severe infantile form of GSDII is rather uniform and the course is predictable. The first symptoms present from birth to seven months of age. Affected infants have no residual acid α-glucosidase activity. The clinical presentation is that of a floppy baby with generalized muscular weakness and cardiomegaly. The electrocardiogram shows a shortened PR interval and large QRS complexes, and echocardiography reveals a markedly enlarged heart with increased thickness of the walls of both ventricles and of the interventricular septum with marked diminution in size of the ventricular cavities. The cardiac enlargement and muscular weakness are progressive. Feeding difficulties and respiratory problems, often complicated by pulmonary infection, are frequently encountered. Hepatomegaly and enlargement of the tongue may develop. Mental development is apparently normal. Patients die usually within the first two years of life from cardio-respiratory failure.

Patients with late-onset juvenile and adult forms of GSDII have muscle weakness or respiratory problems as first clinical symptom (Rosenow and Engel 1978; Moufarrej and Bertorini 1993; Felice *et al.* 1995). The time of presentation is quite variable from the first to the seventh decade. These patients have residual acid α-glucosidase activities from 3-25 % of the mean normal value (Reuser *et al.* 1995). The disease progression is slower than in the infantile form of GSDII. Usually, there is only involvement of skeletal muscle. Both muscle hypertrophy and hypotrophy c.q. atrophy have been seen. The latter can be masked by adiposity (Engel 1970; Bertagnolio *et al.* 1978). Not all muscle groups are involved to the same extent. In general, the proximal muscles are more severely affected than the distal muscles, and there is greater involvement of the lower than the upper limbs. Muscle computed tomography (CT) and magnetic resonance imaging (MRI) scans show abnormalities probably caused by glycogen storage. Scans may help to assess muscle involvement and to select the site of diagnostic biopsy (Kretzschmar *et al.* 1990; Arai *et al.* 1993; De Jager *et al.* 1998). Biopsy of muscles containing little or no vacuoles may impede correct diagnosis (Arai *et al.* 1993; De Jager *et al.* 1998). Patients often have a waddling gait and juvenile patients are at risk for obtaining skeletal deformities in puberty, like scoliosis, lordosis and kyphosis. In an advanced state of the disease

patients may require artificial ventilation during the night and/or day. The disorder can be misdiagnosed as muscular dystrophy or polymyositis. Respiratory insufficiency, with complications, is the most common cause of death (Engel *et al.* 1973; Hirschhorn 1995).

Pathological aspects of glycogen storage disease type II

Morphological changes of skeletal muscle earmark the disease. Light microscopy reveals vacuoles that stain with periodic acid Schiff reagent (indicating the presence of glycogen) and, at the same time, have increased activity of the lysosomal enzyme acid phosphatase, demonstrating their lysosomal origin. Preferential involvement of type I fibers is reported by some authors, but not confirmed by others (Karpati *et al.* 1977; Matsuishi *et al.* 1984; Isaacs *et al.* 1986; Van der Walt *et al.* 1987). In adult onset patients the muscle weakness appears to correlate with the extent of vacuolization, and severely affected muscles have many vacuolated fibers, whereas relatively unaffected muscles have few (Engel 1970; Carrier *et al.* 1975; Bertagnolio *et al.* 1978; Van der Walt *et al.* 1987). Damaged fibers may be replaced by adipose and/or fibrous tissue. The most characteristic changes at the ultrastructural level are the membrane bound vacuoles with β-particles of glycogen (Garancis 1968; Engel *et al.* 1973).

At autopsy of infants with GSDII, glycogen accumulation is present in liver, heart, skeletal muscle, smooth muscle, kidney, skin, endothelial cells, lymphocytes, and in the peripheral and central nervous system (Mancall *et al.* 1965; Cardiff 1966; Hug and Schubert 1967a; Garancis 1968; Hers and De Barsy 1973; Martin *et al.* 1973; Sakurai *et al.* 1974; Sang Hui *et al.* 1985; Hirschhorn 1995). In the nervous system, glycogen accumulation is prominent in Schwann cells, spinal neurons (including anterior horn cells and motor nuclei of the brain stem and spinal ganglia) and glia cells (Gambetti *et al.* 1971; Martin *et al.* 1973).

In late-onset patients reports are usually on skeletal muscle, but glycogen storage is also documented in smooth muscle, especially in blood vessel walls (Mancall *et al.* 1965; Smith *et al.* 1967; Sakurai *et al.* 1974; Martin *et al.* 1976; Sang Hui *et al.* 1985; Van der Walt *et al.* 1987). Glycogen storage and vacuolar changes are seen in smooth muscle cells of the basilar artery, the circle of Willis and small cerebral arteries. Fusiform dilatations and aneurysms of the basilar artery have been described (Makos *et al.* 1985; Miyamoto *et al.* 1985; Braunsdorf 1987; Matsuoka *et al.* 1988). Although not frequently studied, glycogen deposition is reported in at least three late infantile or juvenile patients to occur in glia and some neuronal cells in specific regions of the brain and in anterior horn cells in the spinal cord (Smith *et al.* 1966, 1967; Matsuishi *et al.* 1984).

Structural and functional aspects of acid α-glucosidase

Acid α-glucosidase is a lysosomal enzyme that catalyzes the hydrolysis of α-1,4- and α-1,6-glucosidic linkages at acid pH (Fig. 1). The enzyme degrades the natural substrate glycogen to glucose. The catalytic activity often is assayed with the artificial substrate 4-methylumbelliferyl-α-D-glucopyranoside. The human acid α-glucosidase gene is localized on chromosome 17 in the region q25.2-q25.3, distal to the thymidine kinase gene at the same localization (Kuo *et al.* 1996). The gene is approximately 20 kb long, contains 20 exons, and the promoter has 'house-keeping' features (Hoefsloot *et al.* 1990a, 1990b; Martiniuk *et al.* 1990, 1991; Tzall and
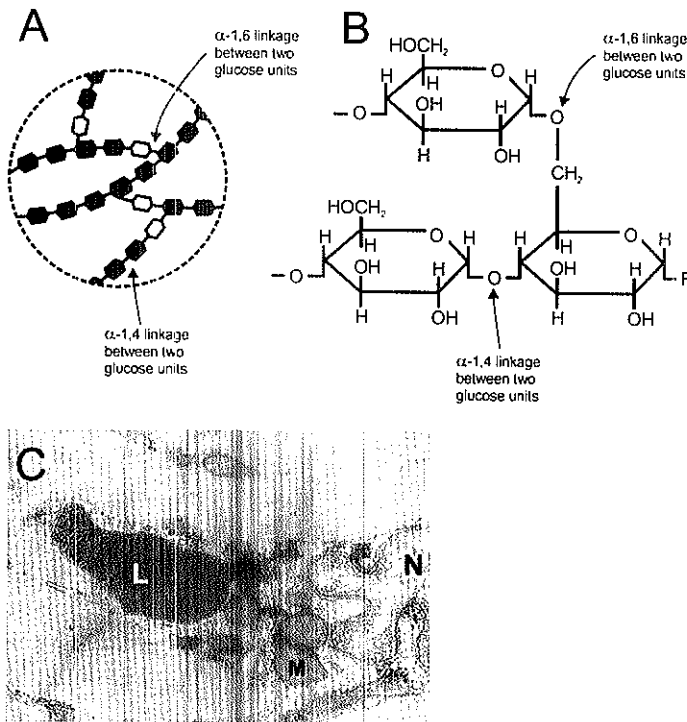
Figure 1. Glycogen is branched polymer of glucose monomers (A) linked to each other with α-1,4- and α-1,6 glycosidic bonds (B). The electronic micrograph (C) shows lysosomal glycogen storage in skeletal muscle of an 8-day-old GSDII knockout mouse. L, lysosome; M, mitochondrion; N, nucleus. (Adapted and modified from Stryer).
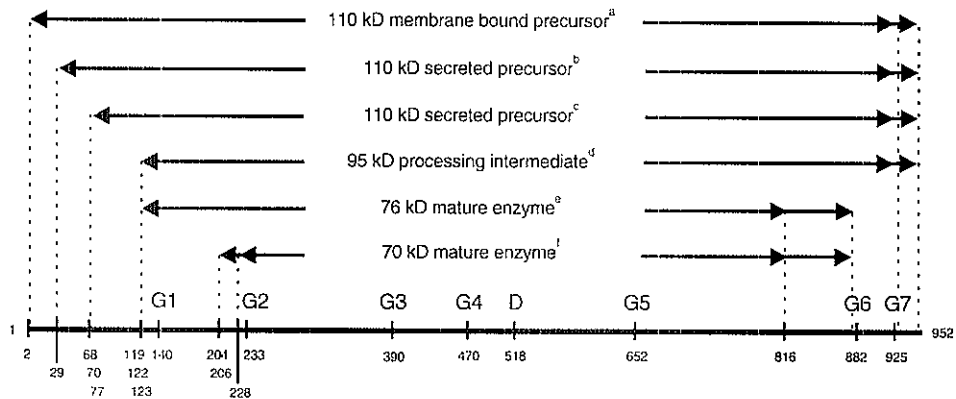
Figure 2. *Post-translational modification and maturation of acid α-glucosidase. The proteolytic cleavage sites used in the process of maturation are given by the amino acid positions. The seven glycosylation sites are marked with the letter G and the catalytic site with the letter D.*
[a]*intracellular in human acid α-glucosidase cDNA transfected COS-1 cells.*
[b]*from human acid α-glucosidase cDNA transfected BHK cells.*
[c]*from human urine, human acid α-glucosidase cDNA transfected CHO cells and milk of mice carrying a human acid α-glucosidase transgene.*
[d]*from human placenta and from human acid α-glucosidase cDNA transfected BHK cells.*
[e]*from human placenta and from human acid α-glucosidase cDNA transfected BHK cells, CHO cells and from milk of mice carrying a human acid α-glucosidase transgene.*
[f]*from human placenta and from human acid α-glucosidase cDNA transfected BHK cells.*
(Adapted and modified from Reuser et al. 1995).

Martiniuk 1991). The cDNA is about 3.6 kb with 2859 coding nucleotides, predicting a protein of 952 amino acids (Martiniuk *et al.* 1986, 1990; Hoefsloot *et al.* 1988). The amino acid sequence predicts seven potential glycosylation sites (Asn-X-Ser/Thr; excluding X=Pro) at codons 140, 233, 470, 652, 882 and 925. The catalytic site of the enzyme was determined both by binding of conduritol β-epoxide as well as by sequence homology and is assigned to codons 513-524 (Hermans *et al.* 1991). The primary translation product, the acid α-glucosidase precursor, has a molecular mass of 110 kD and undergoes extensive modifications (Fig. 2). Of the seven potential glycosylation sites that are all used, at least two contain high mannose-chains that are phosphorylated (Hermans *et al.* 1993). Most of the oligosaccharide chains are of the simple high-mannose type (Mutsaers *et al.* 1987; Hermans *et al.* 1993).

Initially, the precursor remains membrane-bound via the uncleaved signal-peptide. N-terminal proteolytic cleavage and solubilization occurs in a post ER compartment where, after removal of the signal-peptide, the precursor is further processed in late endosomes and/or lysosomes at both the amino- and carboxy-terminal ends. The major species found in the cell are a processing intermediate of 95 kD and two mature forms of 76 and 70 kD (see Fig. 2). The specific activity for the natural substrate glycogen increases during this maturation process (Oude Elferink *et al.* 1984; Wisselaar *et al.* 1993b). The targeting to the lysosomes occurs by a mannose 6-phosphate-independent pathway as well as by both M6P receptors (Tsuji *et al.* 1988;

Wisselaar *et al.* 1993a). Acid α-glucosidase is not only transported to the lysosomes, but is also found at the apical cell surface of various polarized epithelial cells (Hirschhorn 1995). Part of the newly synthesized precursor is secreted in a 110 kD form, and can for instance be isolated from the urine (Wisselaar *et al.* 1993a).

Acid α-glucosidase is present in all tissues of the body, but, for diagnostic purposes, enzyme activity is determined most reliably in fibroblasts and muscle (Hirschhorn 1995; Reuser *et al.* 1995). In general, patients with the infantile variant of the disease have less than 1-2 % of enzyme activity. Adult patients have up to 25 % residual activity, and juvenile patients have intermediate values. However, there is a significant overlap between the values of juvenile and adult patients. Carrier detection is hampered by the wide range of activities in healthy individuals but may be performed by DNA analysis, in case the mutations of the index patients are known. Prenatal diagnosis can be made by enzyme analysis of amniotic fluid cells or uncultured chorionic villi (Niermeijer *et al.* 1975; Besancon *et al.* 1985; Grubisic *et al.* 1986). By this method, the infantile variant of GSDII can be diagnosed reliably, but it is difficult to discriminate between heterozygous carriers and patients affected with juvenile or adult GSDII. In the latter situation, DNA analysis may be required for confirmation (Kleijer *et al.* 1995).

Lysosomal glycogen storage without acid α-glucosidase deficiency
Several cases of cardiomyopathy with lysosomal storage of glycogen but with normal activity of acid α-glucosidase have been reported (reviewed in Verloes *et al.* 1997). The clinical picture is characterized by severe cardiomyopathy and mild myopathy, prominent arrhythmia with Wolf-Parkinson-White syndrome, and sometimes mental retardation. The mode of inheritance is unclear and the syndrome may be heterogeneous. The underlying molecular defect might be in a heat-stable 25 kD protein, which is reported to function as activator of acid α-glucosidase (Radin *et al.* 1989), or in glycogenin, which functions as a primer for glycogen synthesis (Alonso *et al.* 1995).

Mutation analysis; genotype-phenotype correlation

Several polymorphisms and over 40 mutations have been reported in the acid α-glucosidase gene (an overview of mutations and polymorphisms can be found at http://www.eur.nl/fgg/ch1/pompe/mutation.htm). Single-base-pair changes in the coding region without effect on enzyme function are referred as polymorphisms. These changes can be "silent", but some predict amino acid substitutions. Most of the single-base-pair changes resulting in an amino acid substitution have been tested for their effect on acid α-glucosidase synthesis, processing and activity. The over 40 mutations with a deleterious effect are missense, nonsense and splice site mutations, small deletions and insertions, and large deletions. Most mutations were reported for only one or two patients. Several mutations have a higher frequency in specific ethnic groups. The "leaky" splice site mutation IVS1 (-13T→G) is the most frequent mutation in late-onset GSDII among Caucasians (Huie *et al.* 1994; Kroos *et al.* 1995). In addition, four mutations occur frequently in

the infantile variant: the Asp645Glu mutation in Chinese patients from Taiwan (Shieh and Lin 1998), the Arg845X nonsense mutation in Africans and African Americans (Becker *et al.* 1998) and both the deletion 525T and deletion exon 18 in the Dutch population (Kroos *et al.* 1995). These latter mutations seem to occur less frequently in the rest of the Caucasian population (Hirschhorn and Huie 1999).

In general, a correlation exists between the genotype and the phenotype of a patient. All nonsense, frameshift and many of the missense mutations appear to result in lack of transcription or unstable acid α-glucosidase mRNA because no protein is detectable in patients carrying these mutations. Rarely does a mutation result in a normal amount of enzyme that is catalytically inactive. "Late-onset" mutations are identified by their presence in late-onset variants either in a homozygous form or in combination with a fully deleterious mutation. These mutations cause malfunctioning of acid α-glucosidase or, as is the case in the IVS1 splice mutation, lead to decreased synthesis of normal enzyme. Some mutations have a variable phenotype. The underlying factors may be either environmental or genetic. Among the genetic factors are differences in the genetic background, somatic mosaism, presence of a second mutation or polymorphism in the same allele, and differential "leakage" of splice site mutations. In addition, diet and exercise could play a role (Raben *et al.* 1995, 1996; Reuser *et al.* 1995).

Animal models of GSDII

Several natural animal models of GSDII have been reported; these are a cat, Brahman and Shorthorn cattle, Corriedale sheep, Japanese quail, Lapland dog and Nicholas turkey (reviewed in Walvoort 1983). The most detailed studies were performed on the dog (Mostafa 1970; Walvoort *et al.* 1982, 1984a,b, 1985a,b), both breeds of cattle (Howell *et al.* 1981, 1984; O'Sullivan *et al.* 1981; Healy *et al.* 1987; Reichmann *et al.* 1993) and the Japanese quail (Matsui *et al.* 1983; Nunoya *et al.* 1983; Usuki *et al.* 1986a; Higuchi *et al.* 1987; Fujita *et al.* 1991; Miyagawa-Tomita *et al.* 1996). The study of sheep was restricted to the description of tissue pathology (Manktelow and Hartley 1975). Round hearts and excessive glycogen deposition in heart and skeletal muscle were noted in turkey (Czarnecki *et al.* 1975, 1978). The cat study mentions the pathology and glycogen content of the central nervous system of one single animal (Sandström *et al.* 1969).

There are parallels in pathology and biochemical parameters between the various animal models and the human disease. However, a comparison of clinical parameters is difficult to make due to the great diversity of the species, reflected in differences in physiology, length of gestational periods, time to maturity and average normal life span. In addition, environmental factors influence the progression of the disease and life span, like age at weaning and grazing conditions in cattle (Howell *et al.* 1981; Reichmann *et al.* 1993). Lysosomal glycogen storage in both heart and skeletal muscle is found in all animal models. However, cardiac enlargement, a typical feature of the human infantile form of the disease, is found only in dog, turkey, sheep and a subpopulation of the Shorthorn cattle (Czarnecki *et al.* 1975; Manktelow and Hartley 1975; Howell *et al.* 1981; Robinson *et al.* 1983; Walvoort *et al.* 1984b).

Notably, the pre-symptomatic period (from the one cell stage in utero or in the egg until the onset of symptoms) and the duration of the disease in all animal models are very much the same as in human early onset GSDII. Apparently, the progression of the storage process and its effects on the viability of the organisms are similar in all species.

Lysosomal glycogen storage in neurons and Schwann cells is observed in all species, except turkey (Sandström *et al.* 1969; Manktelow and Hartley 1975; Howell *et al.* 1981; Cook *et al.* 1982; Matsui *et al.* 1983; Walvoort *et al.* 1985a; Reichmann *et al.* 1993). Involvement of smooth muscle occurs in sheep, dog, cattle and quail (Manktelow and Hartley 1975; Richards *et al.* 1977; Howell *et al.* 1981; O'Sullivan *et al.* 1981; Matsui *et al.* 1983; Walvoort *et al.* 1985a). Acid α-glucosidase deficiency has been demonstrated for the dog, Shorthorn and Brahman cattle and Japanese quail, but not for the other species. Affected Shorthorn cattle and Lapland dogs both show about normal quantities of enzyme, but without catalytic activity, as is the case in a minor proportion of human patients with the infantile form of the disease (Walvoort *et al.* 1982, 1984a; Palmer *et al.* 1994; Healy *et al.* 1995). Affected Brahman cattle lack acid α-glucosidase protein and mRNA and have no enzyme activity (Wisselaar *et al.* 1993a; Healy *et al.* 1995). Heterozygous animals of both herds have intermediate activities (40-55 % of normal), although, very low enzyme activities (8-12 % of normal) were observed in some unaffected Brahman animals (Healy *et al.* 1987, 1995). The molecular heterogeneity within the Brahman herd has been confirmed by a breeding experiment with presumed heterozygous cows (with activities between 40-55 %) and two bulls with low enzyme activity (8-12 %) which revealed significantly more progeny with enzyme activity levels between 8-55 % than expected from two heterozygous parents (Healy *et al.* 1987).

Affected Japanese quails have 10-16 % of normal acid α-glucosidase activity in skeletal muscle (Usuki *et al.* 1986a). It is unclear to what extent lysosomal acid α-glucosidase activity contributes to the total measured α-glucosidase activity in the tissues. Antibodies against chicken acid α-glucosidase recognize a mature 98 kD acid α-glucosidase in normal quail but not in affected quail (Suhara *et al.* 1989). However, a 110 kD protein is recognized in both normal and affected quail muscle. The identity of this latter protein is uncertain, it could represent the acid α-glucosidase precursor but also a second acid α-glucosidase. Two acid α-glucosidase cDNAs have been cloned in quail and two age dependent neutral α-glucosidases were identified (Usuki *et al.* 1986b, 1988; Kunita *et al.* 1998). More knowledge about the physiologic significance of the neutral and acid isozymes *in vivo* should facilitate the classification of the quail. By now, it is difficult to categorize the quail as a model of late-onset GSDII by residual acid α-glucosidase activity alone.

The only natural animal models still available are cattle (both Shorthorn and Brahman) and the Japanese quail, however, these models are not ideal for studying the pathological process and testing the effect of therapeutic interventions. Cows are large, have a long generation time and a small littersize. Quails are evolutionary too far distant from human. Therefore, a knockout mouse model of GSDII was made as part of the experimental work described in this thesis

(Chapters 3 and 4). The model was used to test the efficacy of enzyme replacement therapy (Chapter 6 and 7). Similar mouse models were recently reported by others (Raben *et al.* 1998a,b).

Attempts at therapy for GSDII

At present, effective therapy for GSDII is not yet available, but patients with the late-onset disease may benefit from supportive therapy. Ventilatory support via an oscillating bed, cuirass respirator, mouth cap or tracheostomy may improve quality of life (Trend *et al.* 1985; Sivak *et al.* 1987; Demey *et al.* 1989; Wong *et al.* 1991; Kurz *et al.* 1998). Respiratory improvement by inspiratory muscle training was reported for one adult patient (Martin *et al.* 1983). Dietary treatment may help to reduce the increased muscle protein breakdown and improve the net protein balance. The effects of high protein diets and supplementation with branched chain amino acids, sometimes combined with a low carbohydrate diet or the use of ephedrine, are ambiguous (Slonim *et al.* 1983; Isaacs *et al.* 1986; Margolis and Hill 1986; Demey *et al.* 1989; Wong *et al.* 1991; Bodamer *et al.* 1997; Hawley *et al.* 1997). Only 25 % of all reported patients that followed a high-protein diet showed improvement of muscle strength or respiratory function. Difficulties in adherence to the diet may partly explain these results. In addition, the potential benefits are partially lost by the consequent weight gain (Bodamer *et al.* 1997). Objective evaluation of the effects remains difficult.

Enzyme replacement therapy
Enzyme replacement therapy might become feasible for all GSDII subtypes, as suggested by results from *in vitro* and *in vivo* studies. At present, it is unclear whether patients with infantile onset disease are at risk to develop neurological symptoms, when enzyme replacement therapy would prolong their life span. A form of enzyme therapy (circum)passing the blood-brain barrier might be needed.

The first attempts at enzyme replacement therapy for GSDII were performed very soon after the discovery of the enzyme defect, but these attempts were limited in duration and amount of enzyme administered. Patients with the infantile variant of the disease received acid α-glucosidase extracted from the fungus *Aspergillus niger* via intravenous or intramuscular injections (Baudhuin *et al.* 1964; Hug and Schubert 1967b; Lauer *et al.* 1968). The enzyme activity increased in liver, but a decrease of liver glycogen was only observed in two patients who received high enzyme doses over a longer period of time, and no effects were seen on the glycogen storage in muscle (Baudhuin *et al.* 1964; Lauer *et al.* 1968). Moreover, the foreign protein was highly immunogenic so that the treatment had to be terminated (Hug and Schubert 1967b; Hug *et al.* 1968). The few clinical trials with acid α-glucosidase purified from human placentas were unsuccessful (De Barsy *et al.* 1973; De Barsy and Van Hoof 1974). As in the previous trials, an increase in enzyme activity was reported in liver, but no reduction of glycogen storage was obtained.

M6P receptor mediated uptake: The growing understanding of mannose 6-phosphate (M6P) receptor mediated endocytosis and lysosomal targeting stimulated renewed investigations into the possibility of enzyme replacement therapy (see Chapter 1) (Kaplan *et al.* 1977; Sando and Neufeld 1977). Both cardiomyocytes and skeletal muscle cells (the main targets in GSDII) express the M6P receptor on their surface and are targets for M6P containing acid α-glucosidase (Salminen and Marjomaki 1985; Taylor *et al.* 1985). Cultured fibroblasts and muscle cells of patients with GSDII showed increased enzyme activity and decreased glycogen content when they were fed with M6P containing acid α-glucosidase from bovine testis, human urine or the medium of acid α-glucosidase cDNA transfected COS-cells (Reuser *et al.* 1984; Di Marco *et al.* 1985; Howell *et al.* 1985; Van der Ploeg *et al.* 1987, 1988a,b; Hoefsloot *et al.* 1990b). Enzyme uptake could be inhibited by simultaneous addition of free M6P. Acid α-glucosidase derived from human placenta, not having the M6P groups, was unable to correct glycogen storage in cultured GSD II cells (Reuser *et al.* 1984). Perfusion of rat hearts with M6P containing acid α-glucosidase, however, resulted in an increase of enzyme activity in the tissue (Van der Ploeg *et al.* 1990). Furthermore, the acid α-glucosidase activity substantially increased in liver, heart and skeletal muscle of mice after intravenous injections of phosphorylated enzyme, while the uptake of unphosphorylated enzyme was less efficient (Van der Ploeg *et al.* 1991).

Acid α-glucosidase production: Since the cloning of the acid α-glucosidase cDNA and gene, the feasibility of large-scale production of recombinant human acid α-glucosidase has been explored in various systems. Recombinant acid α-glucosidase produced by bacteria and insect cells did not receive the proper "human" post-translational modifications (Martiniuk *et al.* 1992; Wu *et al.* 1996). Overexpression and secretion of recombinant human acid α-glucosidase by CHO cells was realized by transfection of the cells with constructs in which the acid α-glucosidase cDNA was placed under transcriptional control of the human polypeptide elongation factor 1α gene promoter or under control of the cytomegalovirus promoter in combination with the dihydrofolate reducatase gene allowing methotrexate induced amplification (Fuller *et al.* 1995; Van Hove *et al.* 1996). Both systems produce recombinant enzyme with the right "high-uptake" properties. The 110 kD precursor could be purified from the medium and administration to cultured fibroblasts and muscle cells of GSDII patients resulted in correction of the enzyme deficiency and degradation of the lysosomal glycogen (Fuller *et al.* 1995; Van Hove *et al.* 1996; Yang *et al.* 1998). Intravenous administration of the enzyme to guinea pig resulted in increased activity in liver and heart (Van Hove *et al.* 1996). In addition, promising results were obtained with four weeks old quails with GSDII after repeated enzyme doses (Kikuchi *et al.* 1998). The acid α-glucosidase activity increased in most tissues, and the glycogen levels decreased to normal in heart and liver. The tissue morphology improved significantly.

    The feasibility of acid α-glucosidase production in milk of transgenic animals for therapeutic purposes was studied as part of the experimental work described in this thesis (Chapters 5, 6, and 7). This production method was chosen because of the presence of small amounts of endogenous, catalytically active acid α-glucosidase in milk and the promising

technical and economical features of this transgenic production system (see Chapter 1 "Biotechnology for the production of lysosomal proteins").

Bone marrow transplantation

Other therapeutic approaches for GSDII aim at providing a permanent source of enzyme to the patients in order to circumvent the life-long need for enzyme infusion. Bone marrow transplantation (BMT) was tested in humans and cattle with GSDII, but the results were disappointing. No increase of acid α-glucosidase activity was detected in heart and skeletal muscle of infantile-onset patients who underwent BMT and the disease process could not be stopped (Harris *et al.* 1986; Watson *et al.* 1986). In cattle, BMT was naturally obtained by spontaneous exchange of haematopoietic cells in utero between affected and unaffected twin fetuses. However, although the animals were chimeric, there was no effect on the course of the disease (Howell *et al.* 1991).

Gene therapy

Gene therapy aims to provide a permanent internal source of enzyme, both by direct correction of transfected cells as well as by correction of more distant cells and tissues via uptake of secreted enzyme. However, many obstacles have yet to be overcome. For instance, the gene expression is usually transient, the expression level is in general low, and viral vectors, especially the adenovirus-based vectors, evoke severe immune responses (see Chapter 1). However, pilot studies have demonstrated the feasibility of gene therapy. *Ex vivo* gene transfer by retroviruses, which only can infect dividing cells, have been successfully performed for a variety of genes in myoblasts and muscle cells (Partridge and Davies 1995). Moreover, production of biologically active blood clotting factor IX was obtained in mice after transplantation of transfected myoblasts and persisted over six months (Dai *et al.* 1992). The principle of this kind of gene therapy has also been tested for GSDII. The human acid α-glucosidase cDNA was introduced via retroviral infection in cultured fibroblasts and myoblasts of patients, and this resulted in an enzyme activity increase to 30 times the level of normal cells and clearance of glycogen storage. Furthermore, the secreted enzyme could correct the glycogen storage in uninfected, distant cells. Importantly, the transduced myoblasts were able to fuse with deficient myoblasts and provide them with enzyme (Zaretsky *et al.* 1997).

Adenoviruses can transfect non-dividing cells and have the advantage that no cell or tissue transplantation is necessary to deliver the gene to the target. Transfection of various types of cultured cells from GSDII patients, with adenovirus based vectors, has demonstrated correction of acid α-glucosidase deficiency and glycogen storage (Nicolino *et al.* 1998; Pauly *et al.* 1998). *In vivo* action of these vectors was examined by intracardiac and intramuscular injection into newborn rats and intramuscular injections into quails with GSDII. High level expression of acid α-glucosidase was obtained in the area around the injection site (Pauly *et al.* 1998). Local correction of enzyme deficiency and glycogen storage was demonstrated in the GSDII quail, however these effects were only measurable in the first few weeks after virus injection (Tsujino *et al.* 1998).

In addition, correction of the acid α-glucosidase activity and degradation of glycogen was obtained in GSDII knockout mice after intravenous injection of an adenovirus with the human acid α-glucosidase gene (Amalfitano *et al.* 1998). Thus, gene therapy is in principle feasible for the treatment of GSDII, but clinical application seems still far away.

Introduction to the experimental work

The experimental work described in this thesis was based on the three key-findings indicating the feasibility of enzyme replacement therapy in glycogen storage disease type II.

1. The lysosomal system has a role in the uptake and recycling of both intra as well as extracellular material (De Duve *et al.* 1955).
2. Correction of acid α-glucosidase deficiency and glycogen storage was obtained in the liver of a patient with GSDII after intravenous administration of acid α-glucosidase from *Aspergilus niger*, demonstrating the principle of enzyme replacement therapy (De Barsy *et al.* 1973; De Barsy and Van Hoof 1974; Hug and Schubert 1967b).
3. Uptake of acid α-glucosidase in heart and skeletal muscle, *in vivo and in vitro*, is facilitated by targeting towards the M6P receptor (Van der Ploeg *et al.* 1990, 1991).

Further *in vivo* studies on the feasibility of enzyme replacement therapy were impeded by the lack of a suitable laboratory animal model and the lack of a system to produce human acid α-glucosidase in bulk quantity.

Two lines of research were developed towards the realization of enzyme replacement therapy. The first involved the generation and characterization of a knockout mouse model of GSDII (described in detail in Chapters 3 and 4). The second involved the production of recombinant human acid α-glucosidase in the milk of transgenic mammals using mice as a model system (described in detail in Chapters 5 and 6). Low yields of human acid α-glucosidase were obtained in the milk of transgenic mice expressing human acid α-glucosidase cDNA under control of the bovine α_{S1}-casein promoter (Chapter 5). The production level could be increased 1000 fold by using the genomic human acid α-glucosidase sequences (Chapter 6). Based on the promising *in vitro* and *in vivo* uptake studies, the genomic construct was used for the industrial production of human recombinant acid α-glucosidase in the milk of transgenic rabbits. The therapeutic testing of the product from rabbit milk in the knockout mouse is described in Chapter 7. The work described in this thesis had led to the design of a phase II clinical trial of enzyme replacement therapy in patients with GSDII. The trial has meanwhile begun in the Department of Pediatrics of the Sophia Children's Hospital, Rotterdam, the Netherlands.

References

Alonso, M.D., Lomako, J., Lomako, W.M. and Whelan, W.J. (1995). A new look at the biogenesis of glycogen. *Faseb J* 9: 1126-1137.

Amalfitano, A., Hu, H., McVie, A.J., Dawson, T.L., Pennybacker, M., Raben, N., Plotz, P.H. and Chen, Y.T. (1998). A potential paradigm for the treatment of lysosomal disorders via modified adenovirus mediated gene therapy. *Am J Hum Genet* **64 suppl**: A397.

Arai, Y., Osawa, M., Shishikura, K., Suzuki, H., Saito, K., Fukuyama, Y. and Sugie, H. (1993). Computed tomography and magnetic resonance imaging of affected muscle in childhood acid α-glucosidase deficiency: a case report. *Brain Dev* **15**: 147-152.

Ausems, M.G.E.M., Verbiest, J., Hermans, M.M.P., Kroos, M.A., Wokke, J.H.J., Beemer, F.A., Sandkuijl, L.A., Reuser, A.J.J. and Van der Ploeg, A.T. (1998). Studies on the frequency and incidence of glycogen storage disease type II. *Am J Hum Genet* **64 suppl**: A207.

Baudhuin, P., Hers, H.G. and Loeb, H. (1964). An electron microscopic and biochemical study of type II glycogenosis. *Lab Invest* **13**: 1139-1152.

Becker, J.A., Vlach, J., Raben, N., Nagaraju, K., Adams, E.M., Hermans, M.M.P., Reuser, A.J.J., Sklower Brooks, S., Tifft, C.J., Hirschhorn, R., Huie, M.L., Nicolino, M. and Plotz, P. (1998). The African origin of the common mutation in African American patients with glycogen storage disease type II. *Am J Hum Genet* **62**: 991-994.

Bertagnolio, B., Di Donato, S., Peluchetti, D., Rimoldi, M., Storchi, G. and Cornelio, F. (1978). Acid maltase deficiency in adults. Clinical, morphological and biochemical study of three patients. *Eur Neurol* **17**: 193-204.

Besancon, A.M., Castelnau, L., Nicolesco, H., Dumez, Y. and Poenaru, L. (1985). Prenatal diagnosis of glycogenosis type II (Pompe's disease) using chorionic villi biopsy. *Clin Genet* **27**: 479-482.

Bodamer, O.A., Leonard, J.V. and Halliday, D. (1997). Dietary treatment in late-onset acid maltase deficiency. *Eur J Pediatr* **156**: S39-42.

Braunsdorf, W.E. (1987). Fusiform aneurysm of basilar artery and ectatic internal carotid arteries associated with glycogenosis type 2 (Pompe's disease). *Neurosurg* **21**: 748-749.

Cardiff, R.D. (1966). A histochemical and electron microscopic study of skeletal muscle in a case of Pompe's disease (glycogenosis II). *Pediatrics* **37**: 249-259.

Carrier, H., Lebel, M., Mathieu, M., Pialat, J. and Devic, M. (1975). Late familial pseudo-myopathic muscular glycogenosis with α-1,4 glucosidase deficiency. *Path Europ* **10**: 51-59.

Cook, R.D., Howell, J.M., Dorling, P.R. and Richards, R.B. (1982). Changes in nervous tissue in bovine generalized glycogenosis type II. *Neuropathol Appl Neurobiol* **8**: 95-107.

Czarnecki, C.M., Feneau, J.K. and Jankus, E.F. (1975). Blood glucose and tissue glycogen levels in turkey poults with spontaneous round heart disease and furazolicone-induced cardiomyopathy. *Avian Dis* **19**: 773-780.

Czarnecki, C.M., Jegers, A. and Jankus, E.F. (1978). Characterization of glycogen in selected tissues of turkey poults with spontaneous round heart disease and furazolicone-induced cardiomyopathy. *Acta Anat* **102**: 33-39.

Dai, Y., Roman, M., Naviaux, R.K. and Verna, I.M. (1992). Gene therapy via primary myoblasts: long term expression of factor IX protein following transplantation in vivo. *Proc Nat Acad Sci USA* **89**: 10892-10895.

De Barsy, T., Jacquemin, P., Van Hoof, F. and Hers, H.G. (1973). Enzyme replacement in Pompe disease: an attempt with purified human acid α-glucosidase. *Birth Defects* **9**: 184-190.

De Barsy, T. and Van Hoof, F. (1974). Enzyme replacement therapy with purified human acid α-glucosidase in type II glycogenosis. *In: Enzyme therapy in lysosomal storage diseases.* Tager, J.M., Hooghwinkel, G.J.M. and Daems, W.T. (Eds) Amsterdam, North-Holland Publishing Company: 277-279.

De Duve, C., Pressman, B.C., Gianetto, R., Wattiaux, R. and Appelmans, F. (1955). Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem J* **60**: 604-617.

De Jager, A.E.J., van der Vliet, T.M., van der Ree, T.C., Oosterink, B.J. and Loonen, M.C.B. (1998). Muscle computed tomography in adult-onset acid maltase deficiency. *Muscle Nerve* **21**: 398-400.

Demey, H.E., Van Meerbeeck, J.P., Vandewoude, M.F., Prove, A.M., Martin, J.J. and Bossaert, L.L. (1989). Respiratory insufficiency in acid maltase deficiency: the effect of high protein diet. *J Parenter Enteral Nutr* **13**: 321-323.

Di Marco, P.N., Howell, J.M. and Dorling, P.R. (1985). Bovine generalised glycogenosis type II. Uptake of lysosomal α-glucosidase by cultured skeletal muscle and reversal of glycogen accumulation. *FEBS Lett* **190**: 301-304.

Engel, A.G. (1970). Acid maltase deficiency in adults: studies in four cases of a syndrome which may mimic muscular dystrophy or other myopathies. *Brain* **93**: 599-616.

Engel, A.G., Gomez, M.R., Seybold, M.E. and Lambert, E.H. (1973). The spectrum and diagnosis of acid maltase deficiency. *Neurology* 23: 95-106.

Felice, K.J., Alessi, A.G. and Grunnet, M.L. (1995). Clinical variability in adult-onset acid maltase deficiency: report of affected sibs and review of the literature. *Medicine* 74: 131-135.

Fujita, T., Nonaka, I. and Sugita, H. (1991). Japanese quail and human acid maltase deficiency: a comparative study. *Brain Dev* 13: 247-255.

Fuller, M., Van der Ploeg, A., Reuser, A.J.J., Anson, D.S. and Hopwood, J.J. (1995). Isolation and characterisation of a recombinant, precursor form of lysosomal acid α-glucosidase. *Eur J Biochem* 234: 903-909.

Gambetti, P., DiMauro, S. and Baker, L. (1971). Nervous system in Pompe's disease. Ultrastructure and biochemistry. *J Neuropathol Exp Neurol* 30: 412-430.

Garancis, J.C. (1968). Type II Glycogenosis; Biochemical and electron microscopic study. *Americ J Med* 44: 289-299.

Grubisic, A., Shin, Y.S., Meyer, W., Endres, W., Becker, U. and Wischerath, H. (1986). First trimester diagnosis of Pompe's disease (glycogenosis type II) with normal outcome: assay of acid α-glucosidase in chorionic villous biopsy using antibodies. *Clin Genet* 30: 298-301.

Harris, R.E., Hannon, D., Vogler, C. and Hug, G. (1986). Bone marrow transplantation in type IIa glycogen storage disease. *Birth Defects Orig Artic Ser* 22: 119-132.

Hawley, J.A., Palmer, G.S. and Noakes, T.D. (1997). Effects of 3 days of carbohydrate supplementation on muscle glycogen content and utilisation during a 1-h cycling performance. *Eur J Appl Physiol* 75: 407-412.

Healy, P.J., Sewell, C.A., Nieper, R.E., Whittle, R.J. and Reichmann, K.G. (1987). Control of generalised glycogenosis in a Brahman herd. *Aust Vet J* 64: 278-280.

Healy, P.J., Nicholls, P.J., Mariniuk, F., Tzall, S., Hirschhorn, R. and Howell, J.M. (1995). Evidence of molecular heterogeneity for generalised glycogenosis between and within breeds of cattle. *Biochem Biophys Res Commun* 179: 919-926.

Hermans, M.M., Kroos, M.A., van Beeumen, J., Oostra, B.A. and Reuser, A.J. (1991). Human lysosomal α-glucosidase. Characterization of the catalytic site. *J Biol Chem* 266: 13507-13512.

Hermans, M.M.P., Wisselaar, H.A., Kroos, M.A., Oostra, B.A. and Reuser, A.J.J. (1993). Human lysosomal α-glucosidase: functional characterization of the glycosylation sites. *Biochem J* 289: 681-686.

Hers, H.G. (1963). α-Glucosidase deficiency in generalized glycogen storage disease (Pompe's disease). *Biochem J* 86: 11-16.

Hers, H.G. and De Barsy, T. (1973). Type II glycogenosis (acid maltase deficiency). *In: Lysosomes and storage diseases*. Hers H.G. and Van Hoof, F., (Eds) New York, Academic Press: 197-216.

Higuchi, I., Nonaka, I., Usuki, F., Ishiura, S. and Sugita, H. (1987). Acid maltase deficiency in the Japanese quail; early morphological event in skeletal muscle. *Acta Neuropathol* 73: 32-37.

Hirschhorn, R. (1995). Glycogen storage disease type II: acid α-glucosidase (acid maltase) deficiency. *In: The metabolic and molecular bases of inherited disease*. Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D., (Eds) McGraw-Hill, Inc. 1: 2443-2464.

Hirschhorn, R. and Huie, M.L. (1999). Frequency of mutations for glycogen storage disease type II in different populations: the Δ525T and Δexon 18 mutations are not generally "common" in white populations. *J Med Genet* 36: 85-86.

Hoefsloot, L.H., Hoogeveen-Westerveld, M., Kroos, M.A., van Beeumen, J., Reuser, A.J.J. and Oostra, B.A. (1988). Primary structure and processing of lysosomal α-glucosidase; homology with the intestinal sucrase-isomaltase complex. *Embo J* 7: 1697-1704.

Hoefsloot, L.H., Hoogeveen-Westerveld, M., Reuser, A.J.J. and Oostra, B.A. (1990a). Characterization of the human lysosomal α-glucosidase gene. *Biochem J* 272: 493-497.

Hoefsloot, L.H., Willemsen, R., Kroos, M.A., Hoogeveen-Westerveld, M., Hermans, M.M.P., Van der Ploeg, A.T., Oostra, B.A. and Reuser, A.J.J. (1990b). Expression and routeing of human lysosomal α-glucosidase in transiently transfected mammalian cells. *Biochem J* 272: 485-492.

Howell, M.M., Dorling, P.R., Cook, R.D., Robinson, W.F., Bradley, S. and Gawthorne, J.M. (1981). Infantile and late onset form of generalized glycogenosis type II in cattle. *J Pathol* 134: 266-277.

Howell, J.M., Dorling, P.R. and Cook, R.D. (1984). The effect of age on biochemical and morphological changes in the semitendinosus muscle of cattle with generalized glycogenosis type II. *Neuropathol Appl Neurobiol* 10: 255-266.

Howell, J.M., Dorling, P.R. and al, e. (1985). Multiple implantation of normal amnion into cattle with generalized glycogenosis type II. *Neuropathol Appl Neurobiol* 10: 255-266.

Howell, J.M., Dorling, P.R., Shelton, J.N., Taylor, E.G., Palmer, D.G. and Di Marco, P.N. (1991). Natural bone marrow transplantation in cattle with Pompe's disease. *Neuromuscul Disord* 1: 449-454.

Hug, G. and Schubert, W.K. (1967a). Glycogenosis type II. Glycogen distribution in tissues. *Arch Path* 84: 141-152.

Hug, G. and Schubert, W.K. (1967b). Lysosomes in type II glycogenosis. Changes during administration of extract from Aspergillus niger. *J Cell Biol* 35: C1-6.

Hug, G., Schubert, W.K. and Chuck, G. (1968). Type II glycogenosis: Treatment with extract of Aspergillus Niger. Abstr. *Clin Res*: 345.

Huie, M.L., Chen, A.S., Tsujino, S., Shanske, S., DiMauro, S., Engel, A.G. and Hirschhorn, R. (1994). Aberrant splicing in adult onset glycogen storage disease type II (GSDII): molecular identification of an IVS1 (-13T→G) mutation in a majority of patients and a novel IVS10 (+1GT→CT) mutation. *Hum Mol Genet* 3: 2231-2236.

Isaacs, H., Savage, N., Badenhorst, M. and Whistler, T. (1986). Acid maltase deficiency: a case study and review of the pathophysiological changes and proposed therapeutic measures. *J Neurol Neurosurg Psychiatry* 49: 1011-1018.

Kaplan, A., Achord, D.T. and Sly, W.S. (1977). Phosphohexosyl components of a lysosomal enzyme are recognized by pinocytosis receptors on human fibroblasts. *Proc Natl Acad Sci USA* 74: 2026-2030.

Karpati, G., Carpenter, S., Eisen, A., Aube, M. and DiMauro, S. (1977). The adult form of acid maltase (α-1,4-glucosidase) deficiency. *Ann Neurol* 1: 276-280.

Kikuchi, T., Yang, H.W., Pennybacker, M., Ichihara, N., Mizutani, M., Van Hove, J.L.K. and Chen, Y.T. (1998). Clinical and metabolic correction of Pompe disease by enzyme therapy in acid maltase-deficient quail. *J Clin Invest* 101: 827-833.

Kleijer, W.J., van der Kraan, M., Kroos, M.A., Groener, J.E., van Diggelen, O.P., Reuser, A.J.J. and van der Ploeg, A.T. (1995). Prenatal diagnosis of glycogen storage disease type II: enzyme assay or mutation analysis? *Pediatr Res* 38: 103-106.

Kretzschmar, H.A., Wagner, H., Hubner, G., Danek, A., Witt, T.N. and Mehraein, P. (1990). Aneurysms and vacuolar degeneration of cerebral arteries in late-onset acid maltase deficiency. *J Neurol Sci* 98: 169-183.

Kroos, M.A., Van der Kraan, M., Van Diggelen, O.P., Kleijer, W.J., Reuser, A.J.J., Van den Boogaard, M.J., Ausems, M.G.E.M., Ploos van Amstel, H.K., Poenaru, L., Nicolino, M. and Wevers, R. (1995). Glycogen storage disease type II: frequency of three common mutant alleles and their associated clinical phenotypes studied in 121 patients. *J Med Genet* 32: 836-837.

Kunita, R., Nakabyashi, O., Wu, J.Y., Hagiwara, Y., Mizutani, M., Pennybacker, M., Chen, Y.T. and Kikuchi, T. (1998). Molecular cloning of acid α-glucosidase cDNA of Japanese quail (Coturnix coturnix japonica) and the lack of its mRNA in acid maltase deficient quails. *Biochim Biophys Acta* 1362: 269-278.

Kuo, W.L., Hirschhorn, R., Huie, M.L. and Hirschhorn, K. (1996). Localization and ordering of acid α-glucosidase (GAA) and thymidine kinase (TK1) by fluorescence in situ hybridization. *Hum Genet* 97: 404-406.

Kurz, D., Aguzzi, A. and Scherer, T.A. (1998). Decompensated cor pulmonaleas the first manifestation of adult-onset myopathy. *Respiration* 65: 317-319.

Lauer, R.M., Mascarinas, T., Racela, A.S., Diehl, A.M. and Brown, B.I. (1968). Administration of a mixture of fungal glucosidases to a patient with type II glycogenosis (Pompe's disease). *Pediatrics* 42: 672-676.

Lejeune, N., Thinès-Sempoux, D. and Hers, H.G. (1963). Tissue fractionation studies: Intracellular distribution and properties of α-glucosidases in rat liver. *Biochem J* 86: 16-21.

Lin, C.Y., Hwang, B., Hsiao, K.J. and Jin, Y.R. (1987). Pompe's disease in Chinese and prenatal diagnosis by determination of α-glucosidase activity. *J Inherit Metab Dis* 10: 11-17.

Loonen, M.C.B. (1979). The variability of Pompe's disease. A clinical, biochemical and genetic study of glycogen storage disease type II, or acid maltase deficiency. *Thesis*.

Makos, M.M., McComb, R.D., Adickes, E.D. and Bennett, D.R. (1985). Acid maltase deficiency and basilar artery aneurysms: a report of a sibship. *Neurology* 35 suppl 1: 193-194.

Mancall, E.L., Aponte, G.E. and Berry, R.G. (1965). Pompe's disease (diffuse glycogenosis) with neuronal storage. *J Neuropath Exp Neurol* 24: 85-96.

Manktelow, B.W. and Hartley, W.J. (1975). Generalized glycogen storage disease in sheep. *J Comp Pathol* 85: 139-145.

Margolis, M.L. and Hill, A.R. (1986). Acid maltase deficiency in an adult. Evidence for improvement in respiratory function with high-protein dietary therapy. *Am Rev Respir Dis* 134: 328-331.

Martin, J.J., De Barsy, T., Van Hoof, F. and Palladini, G. (1973). Pompe's disease: an inborn lysosomal disorder with storage of glycogen. A study of brain and striated muscle. *Acta Neuropathol* 23: 229-244.

Martin, J.J., de Barsy, T. and den Tandt, W.R. (1976). Acid maltase deficiency in non-identical adult twins. A morphological and biochemical study. *J Neurol* 213: 105-118.

Martin, R.J., Sufit, R.L., Ringel, S.P., Hudgel, D.W. and Hill, P.L. (1983). Respiratory improvement by muscle training in adult-onset acid maltase deficiency. *Muscle Nerve* 6: 201-203.

Martiniuk, F., Mehler, M., Pellicer, A., Tzall, S., La Badie, G., Hobart, C., Ellenbogen, A. and Hirschhorn, R. (1986). Isolation of a cDNA for human acid α-glucosidase and detection of genetic heterogeneity for mRNA in three α-glucosidase-deficient patients. *Proc Natl Acad Sci USA* 83: 9641-9644.

Martiniuk, F., Mehler, M., Tzall, S., Meredith, G. and Hirschhorn, R. (1990). Sequence of the cDNA and 5'-flanking region for human acid α-glucosidase, detection of an intron in the 5' untranslated leader sequence, definition of 18-bp polymorphisms, and differences with previous cDNA and amino acid sequences. *Dna Cell Biol* 9: 85-94.

Martiniuk, F., Bodkin, M., Tzall, S. and Hirschhorn, R. (1991). Isolation and partial characterization of the structural gene for human acid α-glucosidase. *Dna Cell Biol* 10: 283-292.

Martiniuk, F., Tzall, S. and Chen, A. (1992). Recombinant human acid α-glucosidase generated in bacteria: antigenic, but enzymatically inactive. *Dna Cell Biol* 11: 701-706.

Martiniuk, F., Chen, A., Arvanitopoulos, E., Chen, Y., Rom, W.N., Codd, W.J., Hanna, B., Alcabes, P., Raben, N. and Plotz, P. (1998). Carrier frequency for glycogen storage disease type II in New York and estimates of affected individuals born with the disease. *Am J Med Genet* 79: 69-72.

Matsui, T., Kuroda, S., Mizutani, M., Kiuchi, Y., Suzuki, K. and Ono, T. (1983). Generalized glycogen storage disease in Japanese quail (Coturnix coturnix japonica). *Vet Pathol* 20: 312-321.

Matsuishi, T., Yoshino, M., Terasawa, K. and Nonaka, I. (1984). Childhood acid maltase deficiency. A clinical, biochemical, and morphologic study of three patients. *Arch Neurol* 41: 47-52.

Matsuoka, Y., Senda, Y., Hirayama, M., Matsui, T. and Takahashi, A. (1988). Late-onset acid maltase deficiency associated with intracranial aneurysm. *J Neurol* 235: 371-373.

Miyagawa-Tomita, S., Morishima, M., Nakazawa, M., Mizutani, M. and Kikuchi, T. (1996). Pathological study of Japanese quail embryo with acid α-glucosidase deficiency during early development. *Acta Neuropathol* 92: 249-254.

Miyamoto, Y., Etoh, Y., Joh, R., Noda, K., Ohya, I. and Morimatsu, M. (1985). Adult-onset acid maltase deficiency in siblings. *Acta Pathol Jpn* 35: 1533-1542.

Mostafa, I.E. (1970). A case of glycogenic cardiomegaly in a dog. *Acta Vet Scand* 11: 197-208.

Moufarrej, N.A. and Bertorini, T.E. (1993). Respiratory insufficiency in adult-type acid maltase deficiency. *South Med J* 86: 560-567.

Mutsaers, J.H.G.M., Van Halbeek, H., Vliegenthart, J.F.G., Tager, J.M., Reuser, A.J.J., Kroos, M. and Galjaard, H. (1987). Determination of the structure of the carbohydrate chains of acid α-glucosidase from human placenta. *Biochim Biophys Acta* 911: 244-251.

Nicolino, M., Puech, J.P., Kremer, E.J., Reuser, A.J.J., Mbebi, C., Veridière-Sahuqué, M., Kahn, A. and Poenaru, L. (1998). Adenovirus-mediated transfer of the acid α-glucosidase gene into fibroblasts, myoblasts and myotubes from patients with glycogen storage disease type II to high level expression of enzyme and corrects glycogen accumulation. *Hum Mol Genet* 2375: 131695-117028-131141.

Niermeijer, M.F., Koster, J.F., Jahodova, M., Fernandes, J., Heukels-Dully, M.J. and Galjaard, H. (1975). Prenatal diagnosis of type II glycogenosis (Pompe's disease) using microchemical analyses. *Pediatr Res* 9: 498-503.

Nunoya, T., Tajima, M. and Mizutani, M. (1983). A new mutant of Japanese quail (Coturnix coturnix japonica) characterized by generalized glycogenosis. *Lab Anim* 17: 138-142.

O'Sullivan, B.M., Healy, P.J., Fraser, I.R., Nieper, R.E., Whittle, R.J. and Sewell, C.A. (1981). Generalized glycogenosis in Brahman cattle. *Aust Vet J* 57: 227-229.

Oude Elferink, R.P.J., Brouwer-Kelder, E.M., Surya, I., Strijland, A., Kroos, M., Reuser, A.J.J. and Tager, J.M. (1984). Isolation and characterization of a precursor form of lysosomal α-glucosidase from human urine. *Eur J Biochem* 139: 489-495.

Palmer, D.G., Dorling, P.R. and Howell, J.M. (1994). Bovine glycogenosis type II: the molecular defect in Shorthorn cattle. *Neuromuscul Disord* 4: 39-48.

Partridge, T.A. and Davies, K.E. (1995). Myoblast-based gen therapies. *Brit Med Bull* 51: 123-137.

Pauly, D.F., Johns, D.C., Matelis, L.A., Lawrence, J.H., Byrne, B.J. and Kessler, P.D. (1998). Complete correction of acid α-glucosidase deficiency in Pompe disease fibroblasts in vitro, and lysosomally targeted expression in neonatal rat cardiac and skeletal muscle. *Gene Ther* 5: 473-480.

Pompe, J.C. (1932). Over idiopathische hypertrofie van het hart. *Ned Tijdsch Geneesk* **76**: 304-311.

Raben, N., Nichols, R.C., Boerkoel, C. and Plotz, P. (1995). Genetic defects in patients with glycogenosis type II (acid maltase deficiency). *Muscle Nerve* **3**: S70-S74.

Raben, N., Nichols, R.C., Martiniuk, F. and Plotz, P.H. (1996). A model of mRNA splicing in adult lysosomal storage disease (glycogenosis type II). *Hum Mol Genet* **5**: 995-1000.

Raben, N., Nagaraju, K., Lee, E., Ward, J., Byrne, B., Kessler, P., Sauer, B. and Plotz, P. (1998a). Modulation of disease severity in knockout mice with targeted disruption of the acid α-glucosidase gene. *Am J Hum Genet* **64 suppl**: A381.

Raben, N., Nagaralu, K., Lee, E., Kessler, P., Byrne, B., Lee, L., LaMarca, M., King, C., Ward, J., Sauer, B. and Plotz, P. (1998b). Targeted disruption of the acid α-glucosidase gene in mice causes an illness with critical features of both infantile and adult human glycogen storage disease type II. *J Biol Chem* **273**: 19086-19092.

Radin, N.S., Shukla, A., Shukla, G.S. and Sano, A. (1989). Heat-stable protein that stimulates acid α-glucosidase. *Biochem J* **264**: 845-849.

Reichmann, K.G., Twist, J.O. and Thistlethwaite, E.J. (1993). Clinical, diagnostic and biochemical features of generalised glycogenosis type II in Brahman cattle. *Aust Vet J* **70**: 405-408.

Reuser, A.J.J., Kroos, M.A., Ponne, N.J., Wolterman, R.A., Loonen, M.C.B., Busch, H.F.M., Visser, W.J. and Bolhuis, P.A. (1984). Uptake and stability of human and bovine acid α-glucosidase in cultured fibroblasts and skeletal muscle cells from glycogenosis type II patients. *Exp Cell Res* **155**: 178-189.

Reuser, A.J.J., Kroos, M.A., Hermans, M.M.P., Bijvoet, A.G.A., Verbeet, M.P., Van Diggelen, O.P., Kleijer, W.J. and Van der Ploeg, A.T. (1995). Glycogenosis type II (acid maltase deficiency). *Muscle Nerve* **3**: S61-S69.

Richards, R.B., Edwards, J.R., Cook, R.D. and White, R.R. (1977). Bovine generalized glycogenosis. *Neuropathol Appl Neurobiol* **3**: 45-56

Robinson, W.F., Howell, J.M. and Dorling, P.R. (1983). Cardiomyopathy in generalised glycogenosis type II in cattle. *Cardiovasc Res* **17**: 238-242.

Rosenow, E.C.d. and Engel, A.G. (1978). Acid maltase deficiency in adults presenting as respiratory failure. *Am J Med* **64**: 485-491.

Sakurai, I., Tosaka, A., Mori, Y., Imura, S. and Aoki, K. (1974). Glycogenosis type II (Pompe). The fourth autopsy case in Japan. *Acta Pathol Jpn* **24**: 829-846.

Salminen, A. and Marjomaki, V. (1985). Phosphomannosyl receptors of lysosomal enzymes in cardiac and skeletal muscles of young and old mice. *Comp Biochem Physiol* **82**: 259-262.

Sando, G.N. and Neufeld, E.F. (1977). Recognition and receptor-mediated uptake of a lysosomal enzyme, α-L-iduronidase, by cultured human fibroblasts. *Cell* **12**: 619-627.

Sandström, B., Westman, J. and Ockerman, P.A. (1969). Glycogenosis of the central nervous system in the cat. *Acta Neuropathol* **14**: 194-200.

Sang Hui, K., Williams, J.C., Borit, A. and Rosenberg, H.S. (1985). The endocrine glands in Pompe's disease. *Arch Pathol Lab Med* **109**: 921-925.

Shieh, J.J. and Lin, C.Y. (1998). Frequent mutation in Chinese patients with infantile type of GSD II in Taiwan: evidence for a founder effect. *Hum Mutat* **11**: 306-312.

Sivak, E.D., Ahmad, M., Hanson, M.R., Mitsumoto, H. and Wilbourn, A.J. (1987). Respiratory insufficiency in adult-onset acid maltase deficiency. *South Med J* **80**: 205-208.

Slonim, A.E., Coleman, R.A., McElligot, M.A., Najjar, J., Hirschhorn, K., Labadie, G.U., Mrak, R., Evans, O.B., Shipp, E. and Presson, R. (1983). Improvement of muscle function in acid maltase deficiency by high-protein therapy. *Neurology* **33**: 34-38.

Smith, H.R., Amick, L.D. and Sidbury, J.B. (1966). Type II Glycogenosis. Report of a case with four-year survival and absence of acid maltase associated with an abnormal glycogen. *Amer J Dis Child* **111**: 475-481.

Smith, J., Zellweger, H. and Afifi, A.K. (1967). Muscular form of glycogenosis, type II (Pompe). *Neurology* **17**: 537-549.

Stryer, L. (1988) *Biochemistry*, W.H. Freeman company, New York.

Suhara, Y., Ishiura, S., Tsukahara, T. and Sugita, H. (1989). Mature 98,000-dalton acid α-glucosidase is deficient in Japanese quails with acid maltase deficiency. *Muscle Nerve* **12**: 670-678.

Taylor, J.E., Scott, C.D. and Baxter, R.C. (1985). Comparison of receptors for insulin-like growth factor II from various rat tissues. *J Endocr* **115**: 35-41.

Trend, P.S., Wiles, C.M., Spencer, G.T., Morgan-Hughes, J.A., Lake, B.D. and Patrick, A.D. (1985). Acid maltase deficiency in adults. Diagnosis and management in five cases. *Brain* **108**: 845-860.

Tsuji, A., Omura, K. and Suzuki, Y. (1988). Intracellular transport of acid α-glucosidase in human fibroblasts: evidence for involvement of phosphomannosyl receptor-independent system. *J Biochem* 104: 276-278.

Tsujino, S., Kinoshita, N., Tashiro, T., Ikeda, K., Ichihara, N., Kikuchi, H., Hagiwara, Y., Mizutani, M., Kikuchi, T. and Sakuragawa, N. (1998). Adenovirus-mediated transfer of human acid maltase gene reduces glycogen accumulation in skeletal muscle of Japanese quail with acid maltase deficiency. *Hum Gene Ther* 9: 1609-1616.

Tzall, S. and Martiniuk, F. (1991). Identification of the promoter region and gene expression for human acid α-glucosidase. *Biochem Biophys Res Commun* 176: 1509-1515.

Usuki, F., Ishiura, S. and Sugita, H. (1986a). Developmental study of α-glucosidases in Japanese quails with acid maltase deficiency. *Muscle Nerve* 9: 537-543.

Usuki, F., Ishiura, S. and Sugita, H. (1986b). Isolation and characterization of three α-glucosidases from the Japanese quail. *J Biochem* 99: 985-988.

Usuki, F., Ishiura, S., Higuchi, I. and Sugita, H. (1988). Reappearance of embryonic neutral α-glucosidase isoenzyme in acid maltase-deficient muscle of Japanese quail. *Exp Neurol* 100: 394-402.

Van der Ploeg, A.T., Kroos, M., Van Dongen, J.M., Visser, W.J., Bolhuis, P.A., Loonen. M.C. and Reuser, A.J.J. (1987). Breakdown of lysosomal glycogen in cultured fibroblasts from glycogenosis type II patients after uptake of acid α-glucosidase. *J Neurol Sci* 79: 327-336.

Van der Ploeg, A.T., Bolhuis, P.A., Wolterman, R.A., Visser, J.W., Loonen, M.C., Busch, H.F. and Reuser, A.J. (1988a). Prospect for enzyme therapy in glycogenosis II variants: a study on cultured muscle cells. *J Neurol* 235: 392-396.

Van der Ploeg, A.T., Loonen, M.C.B., Bolhuis, P.A., Busch, H.M.F., Reuser, A.J.J. and Galjaard, H. (1988b). Receptor-mediated uptake of acid α-glucosidase corrects lysosomal glycogen storage in cultured skeletal muscle. *Pediatr Res* 24: 90-94.

Van der Ploeg, A.T., Van der Kraaij, A.M.M., Willemsen, R., Kroos, M.A., Loonen, M.C.B., Koster, J.F. and Reuser, A.J.J. (1990). Rat heart perfusion as model system for enzyme replacement therapy in glycogenosis type II. *Pediatr Res* 28: 344-347.

Van der Ploeg, A.T., Kroos, M.A., Willemsen, R., Brons, N.H.C. and Reuser, A.J.J. (1991). Intravenous administration of phosphorylated acid α-glucosidase leads to uptake of enzyme in heart and skeletal muscle of mice. *J Clin Invest* 87: 513-518.

Van der Walt, J.D., Swash, M., Leake, J. and Cox, E.L. (1987). The pattern of involvement of adult-onset acid maltase deficiency at autopsy. *Muscle Nerve* 10: 272-281.

Van Hove, J.L.K., Yang, H.W., Wu, J.Y., Brady, R.O. and Chen, Y.T. (1996). High-level production of recombinant human lysosomal acid α-glucosidase in Chinese hamster ovary cells which targets to heart muscle and corrects glycogen accumulation in fibroblasts from patients with Pompe disease. *Proc Natl Acad Sci USA* 93: 65-70.

Verloes, A., Massin, M., Lombet, J., Grattagliano, B., Soyeur, D., Rigo, J., Koulischer, L. and Van Hoof, F. (1997). Nosology of lysosomal glycogen storage diseases without in vitro acid maltase deficiency. Delineation of a neonatal form. *Am J Med Genet* 72: 135-142.

Walvoort, H.C., Slee, R.G. and Koster, J.F. (1982). Canine glycogen storage disease type II. A biochemical study of an acid α-glucosidase-deficient Lapland dog. *Biochim Biophys Acta* 715: 63-69.

Walvoort, H.C. (1983). Glycogen storage diseases in animals and their potential value as models of human disease. *J Inherit Metab Dis* 6: 3-16.

Walvoort, H.C., Slee, R.G., Sluis, K.J., Koster, J.F. and Reuser, A.J. (1984a). Biochemical genetics of the Lapland dog model of glycogen storage disease type II (acid α-glucosidase deficiency). *Am J Med Genet* 19: 589-598.

Walvoort, H.C., Van Nes, J.J., Stokhof, A.A. and Wolvekamp, W.T.C. (1984b). Canine glycogen storage disease type II (GSDII): a clinical study of four affected Lapland dogs. *J Am Anim Hosp Assoc* 20: 279-286.

Walvoort, H.C., Dormans, J.A.M.A. and Van den Ingh, T.S.G.A.M. (1985a). Comparative pathology of the canine model of glycogen storage disease type II (Pompe's disease). *J Inherit Metab Dis* 8: 38-46.

Walvoort, H.C., Koster, J.F. and Reuser, A.J. (1985b). Heterozygote detection in a family of Lapland dogs with a recessively inherited metabolic disease: canine glycogen storage disease type II. *Res Vet Sci* 38: 174-178.

Watson, J.G., Gardner-Medwin, D., Goldfinch, M.E. and Pearson, A.D. (1986). Bone marrow transplantation for glycogen storage disease type II (Pompe's disease). *N Engl J Med* 314: 385.

Wisselaar, H.A., Hermans, M.M.P., Visser, W.J., Kroos, M.A., Oostra, B.A., Aspden, W., Harrison, B., Hetzel, D.J.S., Reuser, A.J.J. and Drinkwater, R.D. (1993a). Biochemical genetics of glycogenosis type II in brahman cattle. *Biochem Biophys Res Com* **190**: 941-947.

Wisselaar, H.A., Kroos, M.A., Hermans, M.M.P., van Beeumen, J. and Reuser, A.J.J. (1993b). Structural and functional changes of lysosomal acid α-glucosidase during intracellular transport and maturation. *J Biol Chem* **268**: 2223-2231.

Wong, K.S., Lai, C. and Ng, H.K. (1991). Late-onset acid maltase deficiency in a Chinese girl. *Clin Exp Neurol* **28**: 210-218.

Wu, J.Y., Van Hove, J.L.K., Huang, Y.S. and Chen, Y.T. (1996). Expression of catalytically active human multifunctional glycogen-debranching enzyme and lysosomal acid α-glucosidase in insect cells. *Biochem Mol Biol Int* **39**: 755-764.

Yang, H.W., Kikuchi, T., Hagiwara, Y., Mizutani, M., Chen, Y.T. and Van Hove, J.L.K. (1998). Recombinant human acid α-glucosidase corrects acid α-glucosidase deficient human fibroblasts, quail fibroblasts, and quail myoblasts. *Pediastr Res* **43**: 374-380.

Zaretsky, J.Z., Candotti, F., Boerkoel, C., Adams, E.M., Yewdell, J.W., Blaese, R.M. and Plotz, P.H. (1997). Retroviral transfer of acid α-glucosidase to enzyme deficient myoblasts results in phenotypic spread of the genotypic correction by both secretion and fusion. *Hum Gene Ther* **8**: 1555-1563

Chapter 3

Generalized glycogen storage and cardiomegaly in a knockout mouse model of Pompe disease.

Generalized glycogen storage and cardiomegaly in a knockout mouse model of Pompe disease

Agnes G.A. Bijvoet[1,2], Esther H.M. van de Kamp[2], Marian A. Kroos[1], Jia-Huan Ding[3,+], Bing Z. Yang[3,+], Pim Visser[4], Cathy E. Bakker[1], Martin Ph. Verbeet[5], Ben A. Oostra[1], Arnold J.J. Reuser[1,*] and Ans T. van der Ploeg[2]

[1]Department of Clinical Genetics and [4]Department of Cell Biology and Genetics, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands, [2]Department of Paediatrics, Sophia Children's Hospital, P.O. Box 2060, 3000 CB Rotterdam, The Netherlands, [3]Department of Pediatrics, Division of Genetics and Metabolism, Duke University Medical Centre, P.O. Box 14991, Durham, NC 27709, USA and [5]Metalloprotein and Protein Engineering Group, Leiden Institute of Chemistry, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands

Glycogen storage disease type II (GSDII; Pompe disease), caused by inherited deficiency of acid α-glucosidase, is a lysosomal disorder affecting heart and skeletal muscles. A mouse model of this disease was obtained by targeted disruption of the murine acid α-glucosidase gene (*Gaa*) in embryonic stem cells. Homozygous knockout mice (*Gaa* –/–) lack *Gaa* mRNA and have a virtually complete acid α-glucosidase deficiency. Glycogen-containing lysosomes are detected soon after birth in liver, heart and skeletal muscle cells. By 13 weeks of age, large focal deposits of glycogen have formed. Vacuolar spaces stain positive for acid phosphatase as a sign of lysosomal pathology. Both male and female knockout mice are fertile and can be intercrossed to produce progeny. The first born knockout mice are at present 9 months old. Overt clinical symptoms are still absent, but the heart is typically enlarged and the electrocardiogram is abnormal. The mouse model will help greatly to understand the pathogenic mechanism of GSDII and is a valuable instrument to explore the efficacy of different therapeutic interventions.

INTRODUCTION

Heart disease and skeletal muscle disorders are life threatening and often incurable. They have become the subject of a gamut of therapeutic studies in which animal models play a crucial role, be it for the development of drug, transplantation, enzyme or gene therapy. Here, we report on the generation of a knockout mouse model of glycogen storage disease type II (GSDII; Pompe disease) with obvious involvement of heart and skeletal muscle.

GSDII is a fatal disorder with a characteristic progressive loss of skeletal and/or heart muscle function (1). Early and late onset subtypes are distinguished. The infantile or generalized form of the disease presents in the first few months of life with cardiomyopathy and hypotonia. There is moderate enlargement of the liver, without serious impairment of liver function. Patients die before the age of 2 years by cardiorespiratory failure (2,3). Milder forms of the disease are characterized by skeletal muscle weakness in the absence of cardiac symptoms and are easily misdiagnosed as muscular dystrophy or limb girdle dystrophy (4). Most patients become wheelchair bound and/or dependent on artificial ventilation. There is no effective therapy for any form of this disease.

GSDII is inherited in an autosomal recessive mode. At the very basis of this disease are mutations in the acid α-glucosidase gene (*GAA*) (5,6) that fully or in part prohibit the biosynthesis of acid α-glucosidase and thereby the degradation of lysosomal glycogen. The clinical phenotype is largely determined by the combination of mutant alleles and the resulting level of residual acid α-glucosidase activity (7,8). Infantile patients show virtually no activity, whereas older and more mildly affected patients have enzyme activity levels up to 25% of the control value (8).

The extent of lysosomal glycogen storage is concordantly different. Infantile patients have massive storage in many tissues including heart, skeletal muscle, smooth muscle, liver, kidney and the peripheral and central nervous system (1,3,9–12). In late onset GSDII, there is minimal to no storage of glycogen in tissues other than skeletal muscle (13).

Despite the fact that this disease has been known for many years, questions concerning its pathogenesis have remained. This was one reason for us to invest in the generation of a laboratory mouse model. A second reason was the need to have a versatile animal model for testing the efficacy of innovative therapeutic developments such as enzyme and gene therapy.

*To whom correspondence should be addressed. Tel: +31 10 4087153; Fax: +31 10 4362536; Email: reuser@ikg.fgg.eur.nl
+Present address: Kimberly H. Courtwright & Joseph W. Summers Institute of Metabolic Disease, Baylor University Medical Centre, 3812 Elm Street, Dallas, TX 75226, USA

RESULTS

Targeted disruption of the *Gaa* gene and generation of *Gaa* (–/–) mice

The rationale for making a mouse model of GSDII via targeted disruption of the acid α-glucosidase (*Gaa*) gene is the 84% identity of the murine and human acid α-glucosidase amino acid sequences (14) indicating conservation of enzyme function. For this purpose, a 6.8 kb *Asp*718 fragment containing exon 5–14 of the murine *Gaa* gene was isolated from a 129 genomic library. To disrupt the murine *Gaa* gene, a *neo* cassette was inserted in the unique *Eag*I site in exon 13 of the genomic fragment. The *tk* cassette for counter selection was positioned at the 5′ end. Both cassettes are orientated in the same direction as the *Gaa* gene (Fig. 1A). The construct was introduced into the E14 embryonic stem (ES) cell line by electroporation, and 137 clones were picked after positive and negative selection. Twenty-two clones were positive for homologous recombination according to PCR analysis as described in Materials and Methods (targeting frequency of 16%). Seven of these were tested further by Southern blotting, after *Nco*I digestion. The blot was hybridized with the *neo* sequence as probe resulting in a 2.6 kb fragment indicative of homologous recombination (data not shown). The clones were karyotyped. Three clones were euploid and had correct homologous recombination. Two of these (15.1D and 17.5D) were injected into blastocysts. Six chimeric mice were identified by coat colour, five males and one female. The males were mated with C57BL/6 and FVB females. Four males appeared to be germline transmitters. The heterozygous offspring were intercrossed, and their offspring were tested by PCR analysis with primers m8–m9 and nested PCR to discriminate between homozygous knockout (–/–), heterozygous knockout (+/–) and wild-type (+/+) offspring (Fig. 1B). Genotyping of 274 offspring from heterozygous crosses revealed a frequency of 22.6% for homozygous knockout mice compatible with the expected Mendelian frequency (25%). The α-glucosidase knock-out mice, both females and males, are fertile and produce normal litter sizes. No differences were observed between mice derived from the two different clones or the different inbred strains.

Analysis of gene products of GSDII knockout mice

No correct *Gaa* mRNA is produced by cultured cells of homozygous knockout mice as illustrated by reverse transcription-PCR analysis using primer set m8–m4 (Fig. 1C). Instead, there is an abnormal messenger in which acid α-glucosidase sequences are linked to sequences of the *neo* cassette (Fig. 1C; primer set n2–m4). RT-PCR with primers RT2 in exon 4 and n1 in the *neo* cassette revealed expression of a correctly spliced recombinant messenger (data not shown). For each RT reaction, the amplification of α-actin mRNA was performed as control. In accordance with the absence of a normal acid α-glucosidase transcript, the enzyme activity is virtually fully deficient in the cultured cells and in the organs of knockout mice, the exception is the intestine due to the sucrase–isomaltase activity in this tissue (Table 1). Cells and tissues of heterozygous mice have intermediate activity levels. The data in Table 1 are activities measured with the artificial substrate 4-methylumbelliferyl-α-D-glucopyranoside (4MU) at a suboptimal pH of 3.6 (instead of 4.3) to reduce the interference of neutral α-glucosidase isozymes. The deficiency of acid α-glucosidase in organs of homozygous knockout mice was

confirmed by Western blotting. Figure 1D shows expression of a 65 kDa acid α-glucosidase (arrow) in all tissues of wild-type mice and an additional 70 kL _ form in skeletal muscle, brain and intestine. No trace of acid α-glucosidase is detectable in tissues of homozygous knockout mice, while the method is sensitive enough to detect 3% of wild-type activity. The cultured cells are also devoid of acid α-glucosidase, as demonstrated by immunofluorescence microscopy (Fig. 2A and B).

Table 1. Acid α-glucosidase activity in tissues of wild-type, heterozygous and knockout mice

Tissue	Acid α-glucosidase activity (nmol 4MU/mg/h)		
	Wild-type	Heterozygote	Knockout
Cultured cells	129.0	73.2	2.5 (1.9)
Liver	29.2	13.7	0.6 (2.0)
Spleen	7.4	3.5	0.2 (2.6)
Kidney	31.9	19.5	0.6 (1.9)
Intestine	70.1	53.0	51.9 (74.0)
Thymus	9.8	3.4	0.3 (2.1)
Lung	8.4	3.0	0.3 (3.6)
Heart	5.6	1.8	0.2 (3.8)
Triceps	8.7	4.7	0.2 (1.8)
Femoral muscle	7.8	4.5	0.2 (2.6)
Sural muscle	6.2	4.8	0.2 (2.6)
Tongue	10.3	6.3	0.1 (1.3)
Cerebellum	39.6	17.7	0.1 (0.4)
Cerebrum	47.0	21.0	0.2 (0.5)

Enzyme activity was assayed in cell lysates and tissue homogenates with the artificial 4MU substrate at pH 3.6 and expressed per mg protein. Measurements were performed in duplicate, and figures represent the mean of two independent experiments. The figures in parentheses are the activities in knockout mice as a percentage of wild-type mice.

Phenotype of the GSDII knockout mice

Clinical signs. At birth, the homozygous knockout mice were indistinguishable from their normal littermates in terms of physical appearance. Up until the present time (9 months after birth), this situation has not changed. The mice still lack overt clinical symptoms, but there is evidence of a developing cardiomyopathy. Several knockout mice had an abnormal electrocardiogram at 32 weeks of age with a high voltage QRS complex, as seen in human infantile GSDII (not shown). Radioscopy revealed cardiac enlargement which was confirmed at autopsy (Fig. 3). The heart of a knockout mouse (right) is round, swollen and pale compared with the smaller, oval and darker stained heart of an unaffected littermate (left). A dilatation of both ventricles was observed in cross-section. The pale colour of the heart was described earlier for Corriedale sheep with GSDII (15). The condition is called round heart disease in affected Nicholas turkeys (16).

Tissue pathology. Animals were sacrificed at 8 days, 6 weeks and 13 weeks after birth to search for pathological changes. No macroscopic abnormalities of organs were noted at these time points, but microscopical changes were seen early after birth. At 8 days, the femoral muscle is morphologically unaffected (Fig. 2C; stained with methylene blue), but some muscle fibres contain groups of
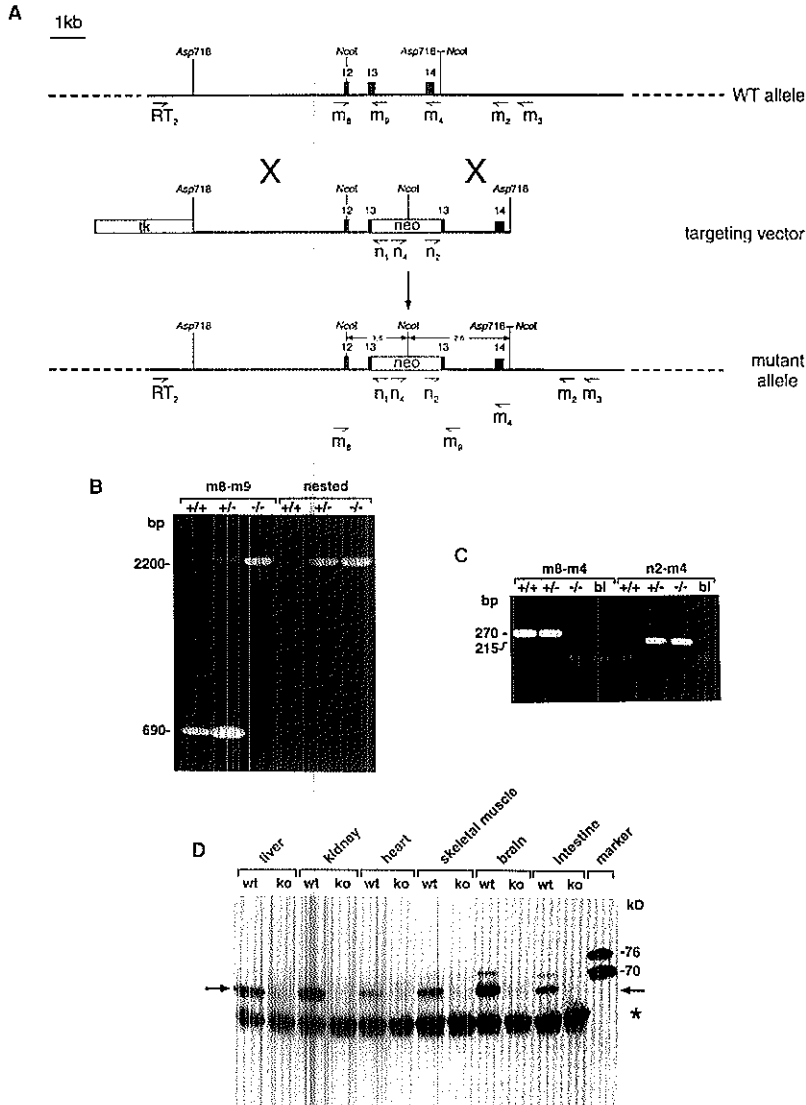
Figure 1. Generation of α-glucosidase-deficient mice by homologous recombination. (A) Partial structure of the murine *Gaa* gene (top), the targeting vector (middle) and the predicted structure of the homologous recombined locus (bottom). *Asp*718 and *Nco*I are the relevant restriction sites. The primer positions are indicated. (B) Analysis of genomic DNA from tail. The left panel with the primer combination m8–m9 discriminates between wild-type (+/+), heterozygous (+/–) and homozygous knockout (–/–) mice, containing respectively only the wild-type (690 bp), the wild-type and targeted (2200 bp) and only the targeted allele. The nested PCR (right panel) with n4–m3 as the first and n2–m2 as the second primer set identifies the targeted allele (2200 bp) in the heterozygous (+/–) and homozygous (–/–) knockout mice. (C) RT-PCR analysis of mRNA extracted from cultured cells of wild-type (+/+), heterozygous (+/–) and homozygous knockout (–/–) mice using primer sets m8–m4 and n2–m4. The first primer set shows the absence of normal mRNA in homozygous knockout mice (–/–), the second the presence of a fusion product of *neo* and acid α-glucosidase sequences. (D) Western blot analysis of *Gaa* expression. Acid α-glucosidase was immunoprecipitated from tissue homogenates with rabbit anti-human acid α-glucosidase antibodies and applied to SDS–PAGE. After subsequent blotting, acid α-glucosidase was visualized with the same antibodies and chemiluminescence staining. Human placental acid α-glucosidase was applied as molecular marker. Arrows indicate the position of (cross-reacting) murine acid α-glucosidase in wild-type (wt) tissues. Tissues of knockout mice (ko) are deficient. The immunoglobulin used for immunoprecipitations stains in all lanes (*).
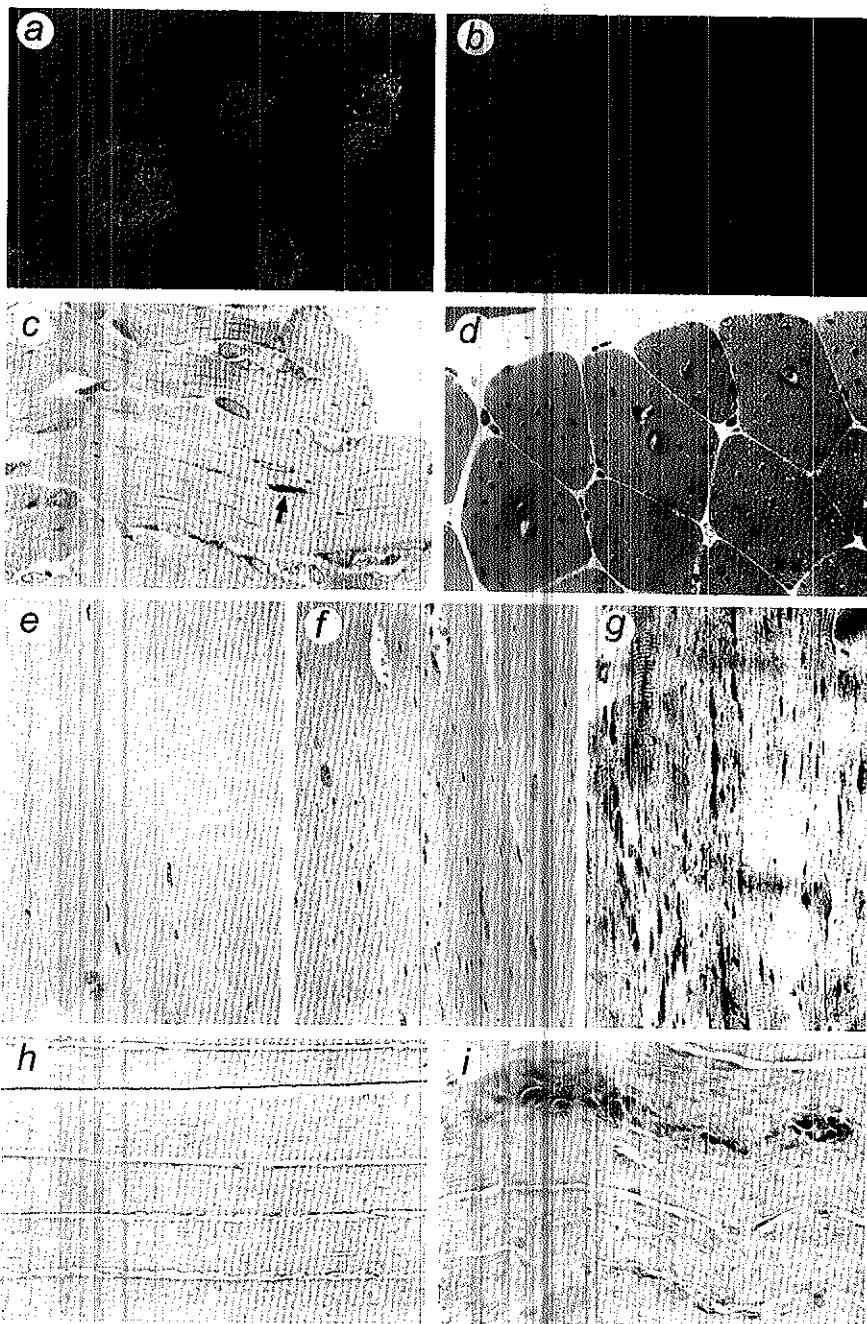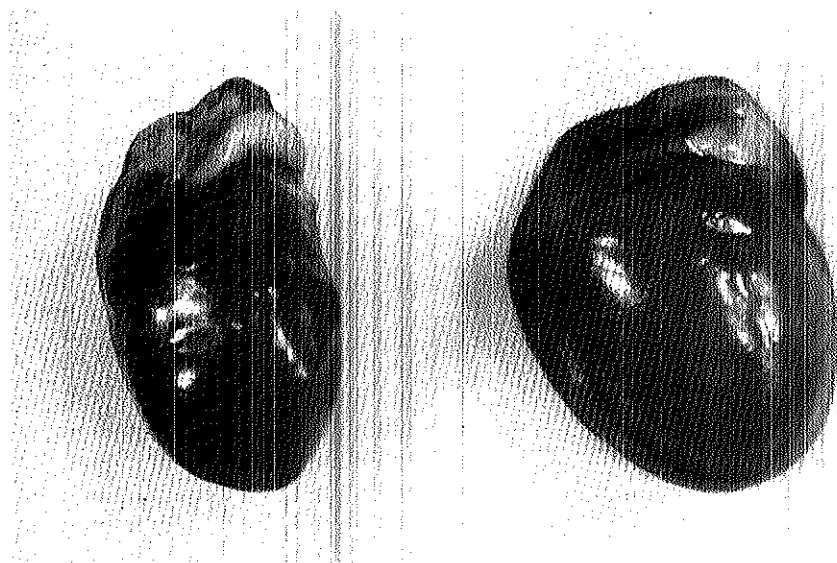
Figure 3. Comparative morphology of the hearts of wild-type (left) and knockout (right) littermates at 32 weeks of age. Note the ventricular hypertrophy and the pale colour of the heart of the afflicted animal.

dark granules (arrow) not present in muscle fibres of wild-type mice of the same age. Similar granules were observed by Cardiff *et al.* (9) in muscle of a patient with GSDII. Conclusive information on the lysosomal accumulation of glycogen was obtained by electron microscopy. Figure 4A shows glycogen deposits surrounded by a membrane in femoral muscle of 8-day-old mice. At 6 weeks after birth, the majority of fibres contains numerous glycogen deposits, as illustrated in cross-section (Fig. 2D). Large vacuoles are seen in the centre of some fibres. Electron microscopy performed at 6 weeks confirms this picture (not shown). The progressive nature of glycogen storage is also illustrated in Figure 2F and G showing femoral muscle stained with periodic acid–Schiff (PAS) reagent. Arrays of PAS-positive material are observed 6 weeks after birth, in between and parallel to the fibrils (Fig. 2F). At 13 weeks of age, the PAS-positive arrays are more numerous and start to form a continuity along the fibrils. Muscle fibres from wild-type animals do not stain with PAS (Fig. 2E).

As another sign of progressive cellular pathology, the muscle fibres of the homozygous knockout mice become positive for acid phosphatase, a lysosomal marker with increased activity in various lysosomal storage disorders and commonly used as diagnostic marker for GSDII (13). Figure 2I illustrates this phenomenon at 13 weeks of age, but it is already notable at 6

weeks. Similar observations were made in sural muscle, triceps and skeletal muscle of the tongue.

Pathological signs are also manifest in heart muscle of knockout mice. At 8 days after birth, small fields with glycogen-containing lysosomes are observed by electron microscopy (Fig. 4B and C). Loss of organized structure of heart muscle cells is seen at 13 weeks of age in knockout mice (Fig. 5B) compared with wild-type (Fig. 5A). Large vacuoles have formed inside the cells. The vacuoles are PAS positive, indicating that they are filled with glycogen (Fig. 5C), and acid phosphatase-positive (red colour), indicating that they are actually lysosomes (Fig. 5D).

Other tissues and cells also show lysosomal glycogen storage. This was observed in liver of 8-day-old mice using transmission electron microscopy (not shown). PAS-positive granules were seen in smooth muscle cells of blood vessels (Fig. 5E), in Schwann cells (Fig. 5F) and in anterior horn cells (Fig. 5G).

DISCUSSION

Animal models of human diseases have proven their value for obtaining insight into pathogenic mechanisms and for testing therapeutic drugs and innovative treatment protocols. It was for use in these two applications that we set out to generate a mouse model of GSDII by targeted disruption of the acid α-glucosidase

Figure 2. Cultured cells from the tail of wild-type (A) and knockout mice (B) stained for the presence of acid α-glucosidase using rabbit anti-human acid α-glucosidase antibodies. (C) Methylene blue staining of a semi-thin (1 µm) longitudinal section of femoral muscle of an 8-day-old knockout mouse. (D) Same staining of a semi-thin cross-section of the same type of muscle of a knockout mouse at 6 weeks of age. (E and F) PAS staining of longitudinal section of GMA-embedded femoral muscle of a wild-type (E) and knockout mouse (F) at 6 weeks of age. (G) PAS staining of a longitudinal cryo-section of femoral muscle of a 13-week-old knockout mouse. (H and I) Acid phosphatase staining of the same muscle of wild-type (H) and knockout (I) mice at 13 weeks of age.
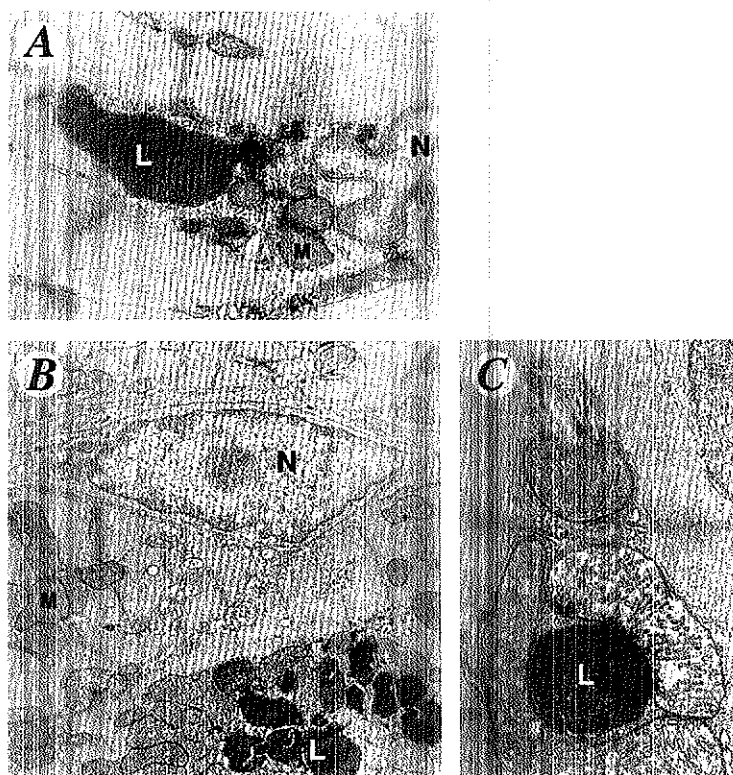
Figure 4. Electron micrographs showing lysosomal glycogen storage in an 8-day-old knockout mouse. (A) Femoral muscle fibre. (B and C) Heart muscle fibre. Glycogen particles are dark and densely packed in lysosomes, and freely dispersed in cytoplasm. (L) lysosomes, (M) mitochondria, (N) nucleus.

gene. The naturally occurring animal models of GSDII are less suitable for this purpose because of the physical dimension of the animal, the long generation time and the small littersize (cattle) (17–19) or the evolutionary distance from humans (quail) (20). Strains of sheep (15), dogs (21,22), cats (23) and turkeys (16) with the disease have to our knowledge not been established.

Evidence that the knockout mouse described in this study stands as a model for human GSDII is based on the following findings. Analysis of genomic DNA demonstrates the presence of the disrupted *Gaa* gene and the absence of the normal gene. The normal messenger is not present either. Instead, there is a messenger in which *neo* sequences are linked to acid α-glucosidase sequences. Furthermore, tissues and cultured cells of homozygous knockout mice are fully deficient in acid α-glucosidase as judged by immunoblotting or immunocytochemistry. Acid α-glucosidase activity is profoundly deficient in all organs except intestine. The residual activity in the latter organ is explained by the presence of the intestinal enzymes sucrase and isomaltase, which share structural homology with acid α-glucosidase. They act on the same artificial substrate with maximal activity at neutral pH, but exhibit residual activity at pH 3.6 (24). Heterozygous knockout

mice have 50% of the normal acid α-glucosidase activity, as expected for an autosomal recessive trait. The best evidence that this knockout mouse represents a model of human GSDII is the progressive lysosomal glycogen storage and the ensuing cellular pathology.

There are several arguments to classify this mouse model as representative for the early-onset infantile form of GSDII. mRNA synthesis is not detectable, the acid α-glucosidase deficiency is virtually complete and the heart muscle is evidently involved. The tissue pathology of the mice also points to infantile GSDII. Knockout mice have, from birth onwards, a generalized and progressive lysosomal storage of glycogen. At 13 weeks of age, many muscle fibres show structural abnormalities. Large PAS-positive vacuoles have formed between the contractile elements. These vacuoles are in essence expanded lysosomes as evidenced by the fact that they are acid phosphatase positive. Moreover, there is accumulation of glycogen in motor neurons and Schwann cells, as only observed in tissue specimens of patients with infantile GSDII (11–13). However, in contrast to most babies born with infantile GSDII, the knockout mice do not develop overt clinical symptoms before adulthood.
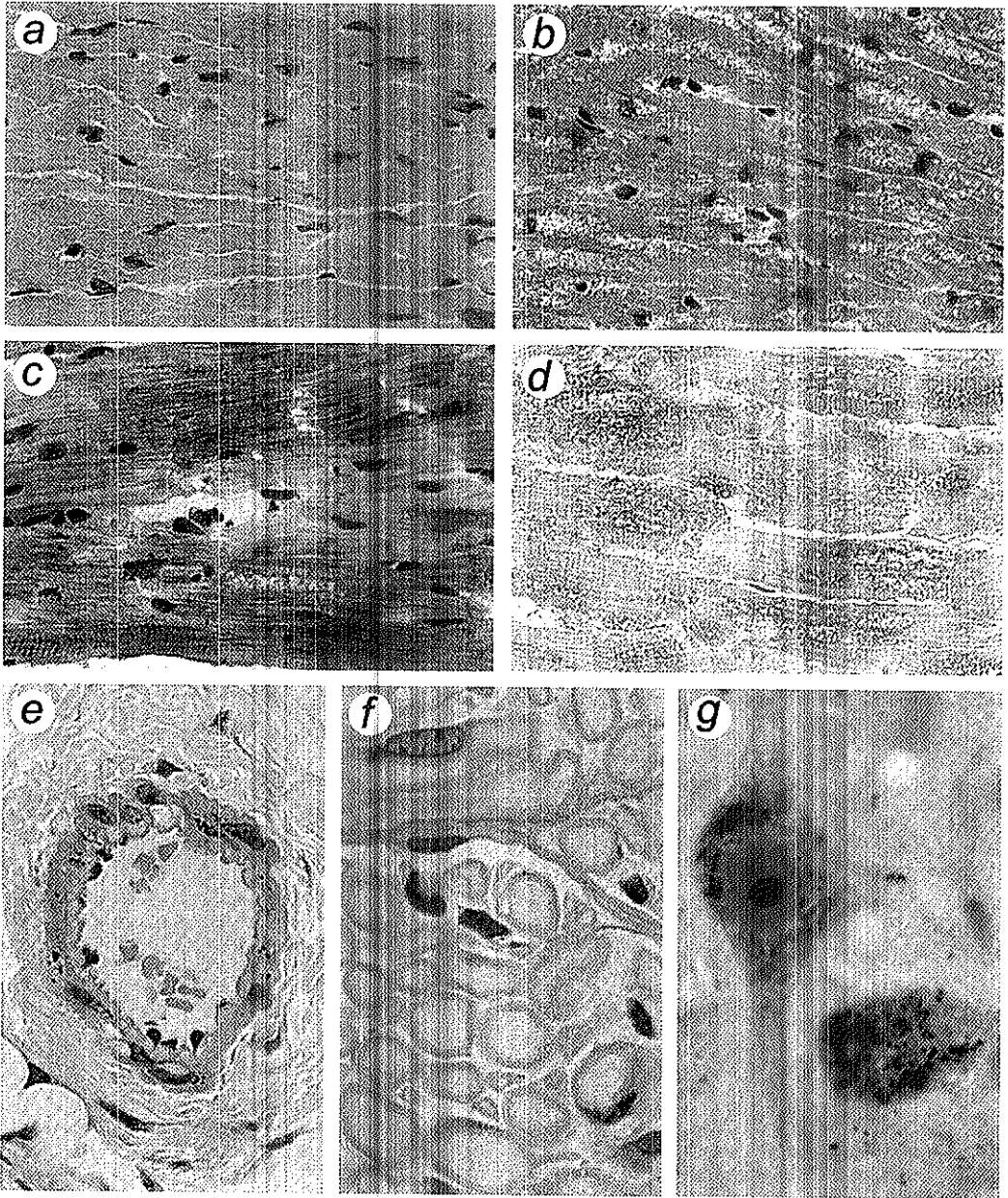
Figure 5. Tissue pathology and glycogen storage in knockout mice. (A-D) Heart muscle of 13-week-old mice. Haematoxylin-eosin staining shows fibre damage in tissue of the knockout (B), but not the wild-type (A) mouse. Vacuoles in between the muscle fibres of the knockout mouse are PAS- (D) positive. (E-G) Smooth muscle cells of blood vessels at 6 weeks of age (E). Schwann cells of peripheral nerves at 6 weeks of age and anterior horn cells at 3 weeks of age (G) also contain PAS-positive granules.

67

The knockout mouse model resembles in biochemical, pathological and clinical aspects the naturally occurring animal models of GSDII. Afflicted Brahman and Shorthorn cattle (17–19), Corriedale sheep (15), Lapland dog (21,22,25), Japanese quail (20,26,27) and Nicholas turkey (16) have both skeletal muscle and heart muscle involvement, like the knockout mice. Also, lysosomal glycogen storage in neurons and Schwann cells was described for all species, except turkey (28). Biochemical data are only available for cattle, dog and quail. Afflicted cattle and dogs have a complete acid α-glucosidase deficiency like the knockout mouse model (29,30). For quail, it is unclear to what extent lysosomal acid α-glucosidase activity contributes to the total measured α-glucosidase activity (31–33).

Clinical parameters are more difficult to compare due to the great diversity of the species, but it is interesting to compare the clinical course of the disease in relation to the duration of the gestation period, the time to maturity and the normal life span of the species (Table 2). There is evidently no correlation between the length of the clinical course and the time to maturity or the life span. However, there is a striking similarity between all species including humans, with regard to the length of the pathologic process, which in Table 2 is defined as the arithmetic sum of the duration of the gestation period and the life expectancy of the affected individual. Perhaps this is not completely unexpected if one considers that the pathological process starts for inherited lysosomal storage disease in principle at the (one cell) embryonic stage. If the absolute rather than the relative age of the species is counted when comparing clinical phenotypes, it can be concluded that all animal models resemble the human infantile form of GSDII. This principle may hold in a similar way for animal models of other lysosomal storage diseases, with a comparatively late onset of symptoms (34–36). For our *Gaa* knockout mouse model, it implies that clinical symptoms are likely to emerge at around 1 year of age.

Meanwhile, the mouse model can be used for the development of therapeutic regimens. Approaches to consider are diet, drugs, transplantation, enzyme and gene therapy. The combination of easy to measure glycogen concentration and acid α-glucosidase activity gives the GSDII mouse model a wider applicability. It can serve to test the effectiveness of various gene targeting vectors for diseases of heart and skeletal muscle in general.

MATERIALS AND METHODS

Construction of targeting vector

The mouse *Gaa* cDNA (GenBank accession No. U449351) was isolated from a mouse liver cDNA library using human acid α-glucosidase cDNA as probe. With the murine cDNA as probe, 11 kb of the mouse *Gaa* gene consisting of exons 3–16 was isolated subsequently from a mouse 129 genomic library. A 6.8 kb *Asp*718 genomic fragment, including exons 5–14, was then cloned into the *Kpn*I site of pBluescript with an adapted polylinker, containing *Cla*I, *Sal*I, *Kpn*I, *Cla*I, *Sac*I restriction sites. A *neo* expression cassette with a length of 1.6 kb was isolated from pPGKneopA (37) by digestion with *Eco*RI and *Xho*I, blunt ends were generated and the fragment was inserted in the blunted unique *Eag*I site in exon 13 of the *Gaa* gene. The

HSV-tk expression cassette, isolated from pHA140 (38) by digestion with *Sal*I, was introduced in the *Sal*I site 5' of the construct. Both cassettes were inserted in the sense orientation with respect to the transcriptional orientation of the *Gaa* gene. The structure of the targeting vector is shown in Figure 1A.

Gene targeting in embryonic stem cells

After *Cla*I digestion, the targeting construct (15–20 μg) was separated from the vector by agarose gel electrophoresis and isolated via electroelution. It was introduced into E14 ES cells by electroporation, essentially as described by Bakker *et al.* (39). Briefly, transfected cells were cultured in conditioned medium, and double selection with G418 (200 μg/ml) and FIAU (2 μM) was started 24 h after electroporation. Clones were picked 10–14 days after electroporation and screened for homologous recombination by nested PCR amplification. Two primer sets were used to amplify a 1.6 kb fragment that includes the 3' recombination junction. The first PCR (35 cycles, each consisting of 45 s at 94°C, 1 min at 59°C and 2.5 min at 68°C) was performed with primers n4 (sense, GACGAGTTCTTCTGAGGGGATCA), complementary to a sequence in the *neo* cassette, and m3 (antisense, GCCTTCCTCATGGCCTGCTG), complementary to a sequence in exon 15 immediately downstream of the *Asp*718 restriction site. In the nested PCR (25 cycles, with the same temperature program) primers n2 (sense, GTGGGCTCTATGG-CTTCTGAGG) and m2 (antisense, GCTGAACCTGTACGGC-TCCTG) were used (Fig. 1A). Correctly recombined clones were karyotyped according to standard procedures and tested by Southern blotting. Genomic DNA was digested with *Nco*I and a *neo* fragment was used as probe.

Generation of chimeric and acid α-glucosidase (–/–) mice (GSDII knockout mice)

Two positive clones were injected into C57BL/6 blastocysts. Chimeric mice were bred to C57BL/6 and FVB females, and the offspring with agouti and grey coats were tested for the transmission of the disrupted allele by the PCR described above. Homozygous mice (–/–) were obtained by interbreeding the heterozygotes (+/–). DNA from the tail was analysed using a PCR (35 cycles, following the same temperature program as described above) with primer m8 (sense, CCTCACTGAAGCTATCGCC-TCC) in exon 12 and m9 (antisense, GCAAGATGCTCCCAAG-AGCTCC) in exon 13 in combination with the nested PCR described above.

Cell culture from murine tail

Tails were cut into small pieces, and the tissue was dissociated for 15 min at 37°C in 10 mM sodium phosphate, 150 mM NaCl (pH 7.1), containing 0.05% trypsin and 0.02% EDTA. After low speed centrifugation, a fresh solution of trypsin/EDTA was added for another 15 min. Cells and softened tissue were then resuspended in Dulbecco's modified Eagle's medium (DMEM) with 15% fetal calf serum (FCS) and antibiotics and seeded in 25 cm² tissue culture flask, pre-coated with FCS and 1 ml of medium. Clonal outgrowth of cells was observed 3 weeks later, and stable cell lines were obtained after 2 months of growth.

Table 2. Normal development and clinical course of GSDII in humans and in animal models

Species	Normal development			Clinical course of GSDII		
	Gestation period	Time to maturity	Average life span	Onset of clinical symptoms	Death	Duration of the pathologic process[a]
Human[b]	9 m	12–15 y	75 y	3 m	6–18 m	15–27 m
Brahman	9 m	2 y	15–20 y	3 m	8–9 m	17–18 m
Shorthorn	9 m	2 y	15–20 y	3 m	7–16 m	16–25 m
Sheep	5 m	8 m	15–20 y	<6 m	6–12 m	11–17 m
Dog	2 m	12 m	15–20 y	4 m	18 m	20 m
Mouse	0.7 m	2 m	2–3 y	>9 m	>9 m	>10 m
Quail	0.5 m	2 m	7–8 y	3–8 m	18 m	19 m

[a]Time span from the one-cell embryonic stage to death.
[b]The infantile form.
m stands for months, y for years.

Analysis of RNA

RNA was isolated from cultured cells with RNAzol· according to the manufacturer's directions (Tel-Test). cDNA synthesis was carried out using the Superscript℗ pre-amplification system and random hexamer-primers (Gibco BRL). The cDNA was PCR-amplified with several primer combinations: m8 with m4 (antisense, TGGGTCCAGCGCACACACAGCT in exon 14) amplifying a fragment including the *neo* cassette in the knockout allele, n2 in the *neo* cassette in combination with m4 amplifying the 3′ junction of the *neo* cassette and the *Gaa* gene, RT2 (GAACCAGTTCCTTCAGCTGT sense in exon 4) in combination with n1 (GTGTAGCGCCAAGTGCCAGCG antisense, in the *neo* cassette). Primers AGGTCATCACCATCGGCAATG (sense) and CGTCGTACTCCTGCTTGGTG (antisense) were used for amplifying α-actin, a non-relevant control gene.

Acid α-glucosidase activity and protein assays

Mouse tissues were homogenized in phosphate-buffered saline using an ultra turrax (TP 18–10, 20 000 Upm; 170 W, Janke & Kunkel KG). After removal of the large debris at 10 000 *g* twice for 15 min, the supernatant was stored at −20°C. The supernatants were assayed for α-glucosidase activity with 4MU as substrate (40). The protein content of the supernatant was determined using the bicinchoninic acid (BCA) protein assay (Pierce).

Western blotting

Acid α-glucosidase was immunoprecipitated from the homogenates with rabbit antibodies raised against human placental acid α-glucosidase complexed to protein A–Sepharose 4B as described (41). The Sepharose beads with the enzyme–immunoglobulin complex bound to them were boiled in sample buffer and applied to 10% SDS–PAGE. The proteins subsequently were blotted onto nitrocellulose filters and visualized on these filters with rabbit anti-human placental acid α-glucosidase antibodies using the ECL detection kit (Amersham).

Immunocytochemistry

Acid α-glucosidase in cultured cells was visualized with rabbit polyclonal antiserum raised against human placental acid α-glucosidase in combination with goat anti rabbit IgG conjugated to fluorescein isothiocyanate (FITC).

Histology

For routine histological examination, tissues were either frozen in 2-methylbutane chilled by liquid nitrogen or fixed in 2.5% glutaraldehyde in 0.15 M cacodylate buffer and embedded in glycolmethacrylate (GMA). Cryostat sections (6 μm) were stained with haematoxylin–eosin (HE), PAS and acid phosphatase. Alternatively, sections (2 μm) from GMA-embedded tissues were PAS stained. For electron microscopy, glutaraldehyde-fixed tissues specimens were post-fixed in 1% OsO_4 with $K_3Fe(CN)_6$ in 0.1 M cacodylate buffer according to De Bruijn (42). Standard procedures were used for epon embedding. Semi-thin (1 μm) and ultrathin sections were cut on an LKB Nova ultratome and stained with methylene blue (semi-thin) and uranylactate in combination with Sato's lead citrate (43) (ultrathin). Ultrathin sections were examined in a Philips CM100 electron microscopy.

Cardiology

Mice were anaesthetized with ether and pulsed radioscopies were made (25/s) with a Siemens radiography apparatus. A surface electrocardiogram (standard Einthoven) was obtained by subcutaneous placement of 27-gauge needles in two front and one hind limb. A 300 Hz filter was used. Data were recorded, digitized on-line using a data acquisition program (WinDaq/ex; Dataq Instrument, Inc., USA) and stored for post-acquisition off-line analysis. The mice were killed with an overdose of hypnodil (Janssen Pharmaceutica B.V.) for *ex vivo* comparison of gross cardiac morphology.

technology sharing. This study was supported by grants from the Prinses Beatrix Fonds and the Sophia Foundation for Medical Research.

REFERENCES

1. Hirschhorn, R. (1995) In Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. (eds), *The Metabolic and Molecular Basis of Inherited Disease*. McGraw-Hill, New York, Vol. 1, pp. 2443–2464.
2. Hers, H.G. (1963) α-Glucosidase deficiency in generalized glycogen storage disease (Pompe's disease). *Biochem. J.*, 86, 11–16.
3. Hers, H.G. and De Barsy, T. (1973) In Hers, H.G. and Van Hoof, F. (eds), *Lysosomes and Storage Diseases*. Academic Press, New York, pp. 197–216.
4. Engel, A.G. (1970) Acid maltase deficiency in adults: studies in four cases of a syndrome which may mimic muscular dystrophy or other myopathies. *Brain*, 93, 599–616.
5. Hoefsloot, L.H., Hoogeveen-Westerveld, M., Reuser, A.J.J. and Oostra, B.A. (1990) Characterization of the human lysosomal α-glucosidase gene. *Biochem. J.*, 272, 493–497.
6. Martiniuk, F., Bodkin, M., Tzall, S. and Hirschhorn, R. (1991) Isolation and partial characterization of the structural gene for human acid α-glucosidase. *DNA Cell Biol.*, 10, 283–292.
7. Raben, N., Nichols, R.C., Boerkoel, C. and Plotz, P. (1995) Genetic defects in patients with glycogenosis type II (acid maltase deficiency). *Muscle Nerve*, 3, S70–S74.
8. Reuser, A.J.J., Kroos, M.A., Hermans, M.M.P., Bijvoet, A.G.A., Verbeet, M.P., Van Diggelen, O.P., Kleijer, W.J. and Van der Ploeg, A.T. (1995) Glycogenosis type II (acid maltase deficiency). *Muscle Nerve*, 3, S61–S69.
9. Cardiff, R.D. (1966) A histochemical and electron microscopic study of skeletal muscle in a case of Pompe's disease (glycogenosis II). *Pediatrics*, 37, 249–259.
10. Hug, G. and Schubert, W.K. (1967) Glycogenosis type II. Glycogen distribution in tissues. *Arch. Pathol.*, 84, 141–152.
11. Gambetti, P., DiMauro, S. and Baker, L. (1971) Nervous system in Pompe's disease. Ultrastructure and biochemistry. *J. Neuropathol. Exp. Neurol.*, 30, 412–430.
12. Martin, J.J., De Barsy, T., Van Hoof, F. and Palladini, G. (1973) Pompe's disease: an inborn lysosomal disorder with storage of glycogen. A study of brain and striated muscle. *Acta Neuropathol.*, 23, 229–244.
13. Engel, A.G., Gomez, M.R., Seybold, M.E. and Lambert, E.H. (1973) The spectrum and diagnosis of acid maltase deficiency. *Neurology*, 23, 95–106.
14. Ding, J.H., Yang, B.Z., Liu, H.M. and Reuser, A.J.J. (1994) Cloning the mouse homologue of the human lysosomal acid α-glucosidase gene. *Am. J. Hum. Genet.*, 55, A325.
15. Manktelow, B.W. and Hartley, W.J. (1975) Generalized glycogen storage disease in sheep. *J. Comp. Pathol.*, 85, 139–145.
16. Czarnecki, C.M. and Jankus, E.F. (1974) Observations on cardiac glycogen in spontaneous round heart disease. *Avian Dis.*, 18, 614–618.
17. Jolly, R.D., Van-de-Water, N.S., Richards, R.B. and Dorling, P.R. (1977) Generalized glycogenosis in beef shorthorn cattle—heterozygote detection. *Aust. J. Exp. Biol. Med. Sci.*, 55, 141–150.
18. Richards, R.B., Edwards, J.R., Cook, R.D. and White, R.R. (1977) Bovine generalized glycogenosis. *Neuropathol. Appl. Neurobiol.*, 3, 45–56.
19. O'Sullivan, B.M., Healy, P.J., Fraser, I.R., Nieper, R.E., Whittle, R.J. and Sewell, C.A. (1981) Generalised glycogenosis in Brahman cattle. *Aust. Vet. J.*, 57, 227–229.
20. Murakami, H., Takagi, A., Nanaka, S., Ishiura, S. and Sugita, H. (1980) Glycogenosis II in Japanese quails. *Exp. Anim.*, 29, 475–485.
21. Mostafa, I.E. (1970) A case of glycogenic cardiomegaly in a dog. *Acta Vet. Scand.*, 11, 197–208.
22. Walvoort, H.C., Slee, R.G. and Koster, J.F. (1982) Canine glycogen storage disease type II. A biochemical study of an acid α-glucosidase-deficient Lapland dog. *Biochim. Biophys. Acta*, 715, 63–69.
23. Sandstrom, B., Westman, J. and Ockerman, P.A. (1969) Glycogenosis of the central nervous system in the cat. *Acta Neuropathol.*, 14, 194–200.
24. Hoefsloot, L.H., Hoogeveen-Westerveld, M., Kroos, M.A., Van Beeumen, J., Reuser, A.J.J. and Oostra, B.A. (1988) Primary structure and processing of lysosomal α-glucosidase; homology with the intestinal sucrase-isomaltase complex. *EMBO J.*, 7, 1697–1704.
25. Walvoort, H.C., Dormans, J.A.M.A. and van den Ingh, T.S.G.A.M. (1985) Comparative pathology of the canine model of glycogen storage disease type II (Pompe's disease). *J. Inherit. Metab. Dis.*, 8, 38–46.
26. Nunoya, T., Tajima, M. and Mizutani, M. (1983) A new mutant of Japanese quail (*Coturnix coturnix japonica*) characterized by generalized glycogenosis. *Lab. Anim.*, 17, 138–142.
27. Matsui, T., Kuroda, S., Mizutani, M., Kiuchi, Y., Suzuki, K. and Ono, T. (1983) Generalized glycogen storage disease in Japanese quail (*Coturnix coturnix japonica*). *Vet. Pathol.*, 20, 312–321.
28. Walvoort, H.C. (1983) Glycogen storage diseases in animals and their potential value as models of human disease. *J. Inherit. Metab. Dis.*, 6, 3–16.
29. Wisselaar, H.A., Hermans, M.M.P., Visser, W.J., Kroos, M.A., Oostra, B.A., Aspden, W., Harrison, B., Hetzel, D.J.S., Reuser, A.J.J. and Drinkwater, R.D. (1993) Biochemical genetics of glycogenosis type II in Brahman cattle. *Biochem. Biophys. Res. Commun.*, 190, 941–947.
30. Healy, P.J., Nicholls, P.J., Martiniuk, F., Tzall, S., Hirschhorn, R. and Howell, J.M. (1995) Evidence of molecular heterogeneity for generalized glycogenosis between and within breeds of cattle. *Aust. Vet. J.*, 72, 309–311.
31. Usuki, F., Ishiura, S. and Sugita, H. (1986) Isolation and characterization of three alpha-glucosidases from the Japanese quail. *J. Biochem.*, 99, 985–988.
32. Usuki, F., Ishiura, S., Higuchi, I. and Sugita, H. (1988) Reappearance of embryonic neutral α-glucosidase isoenzyme in acid maltase deficient muscle of Japanese quail. *Exp. Neurol.*, 100, 394–402.
33. Suhara, Y., Ishiura, S., Tsukahara, T. and Sugita, H. (1989) Mature 98,000 dalton acid α-glucosidase is deficient in Japanese quails with acid maltase deficiency. *Muscle Nerve*, 12, 670–678.
34. Zhou, X.Y., Morreau, H., Rottier, R., Davis, D., Bonten, E., Gillemans, N., Wenger, D., Grosveld, F.G., Doherty, P., Suzuki, K., Grosveld, G.C. and d'Azzo, A. (1995) Mouse model for the lysosomal disorder galactosialidosis and correction of the phenotype with overexpressing erythroid precursor cells. *Genes Dev.*, 9, 2623–2634.
35. Hess, B., Saftig, P., Hartmann, D., Coenen, R., Lullmann-Rauch, R., Goebel, H.H., Evers, M., von Figura, K., D'Hooge, R., Nagels, G., De Deyn, P., Peters, C. and Gieselmann, V. (1996) Phenotype of arylsulfatase A-deficient mice: relationship to human metachromatic leukodystrophy. *Proc. Natl Acad. Sci. USA*, 93, 14821–14826.
36. Hahn, C.N., del Pilar Martin, M., Schröder, M., Vanier, M.T., Hara, Y., Suzuki, K., Suzuki, K. and d'Azzo, A. (1997) Generalized CNS disease and massive G_{M1}-ganglioside accumulation in mice defective in lysosomal acid β-galactosidase. *Hum. Mol. Genet.*, 6, 205–211.
37. Soriano, P., Montgomery, C., Geske, R. and Bradley, A. (1991) Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. *Cell*, 64, 693–702.
38. Clarke, A.R., Robanus Maandag, E., Van Roon, M., Van der Lugt, N.M.T., Van der Valk, M., Hooper, M.L., Berns, A. and Te Riele, H. (1992) Requirement for a functional Rb-1 gene in murine development. *Nature*, 359, 328–330.
39. Bakker, C.E., Verheij, C., Willemsen, R., Van der Helm, R., Oerlemans, F., Vermey, M., Bygrave, A., Hoogeveen, A.T., Oostra, B., Reyniers, E., De Boulle, K., D'Hooge, R., Cras, P., Van Vlezen, S., Nagels, G., Marin, J.J., De Deyn, P.P., Darby, J.K. and Willems, P.J. (1994) Fmr1 knockout mice; a model to study fragile X mental retardation. *Cell*, 78, 23–33.
40. De Jonge, A.J.R., De Smit, S., Kroos, M.A. and Reuser, A.J.J. (1985) Cotransfer of syntenic human genes into mouse cells using isolated metaphase chromosomes or cellular DNA. *Hum. Genet.*, 69, 32–38.
41. Bijvoet, A.G.A., Kroos, M.A., Pieper, F.R., De Boer, H.A., Reuser, A.J.J., Van der Ploeg, A.T. and Verbeet, M.P. (1996) Expression of cDNA-encoded human acid α-glucosidase in milk of transgenic mice. *Biochim. Biophys. Acta*, 1308, 93–96.
42. De Bruijn, W.C. (1973) Glycogen, its chemistry and morphologic appearance in the electron microscope. I. A modified OsO_4 fixative which selectively contrasts glycogen. *J. Ultrastruct. Res.*, 42, 29–50.
43. Hanaichi, T., Sato, T., Iwamoto, T., Malavasi-Yamashiro, J., Hoshino, M. and Mizuno, N. (1986) A stable lead by modification of Sato's method. *J. Electron Microsc.*, 35, 304–306.

Chapter 4

Pathologic features of glycogen storage disease type II highlighted in the knockout mouse model.

Pathologic features of glycogen storage disease type II highlighted in the knockout mouse model.

Agnes G.A. Bijvoet, Hans Van Hirtum, Marcel Vermey, Dik Van Leenen, Ans T. Van der Ploeg, Wolter J. Mooi and Arnold J.J. Reuser.

Summary

Glycogen storage disease type II (GSDII; Pompe's disease) is an autosomal recessive disease caused by lysosomal α-glucosidase deficiency. Skeletal muscle weakness is the most conspicuous clinical symptom of patients suffering from GSDII, and skeletal muscle also is prominently involved in the knockout mouse model of this disease. Thus far, little detailed information has been published on the pathologic changes of other mouse tissues. This paper aims to provide these data and gives a record of the clinical course of the mouse model over a two-year period. Four months old affected mice perform in a running wheel worse than their unaffected littermates, but do not yet display other clear symptoms. The lysosomal glycogen storage, already evident at birth, aggravates in time, leads to muscle wasting by 9 to 10 months of age, and then starts to cause limb girdle weakness and kyphosis. The disease does not shorten the animal's life span dramatically despite the serious tissue pathology which is not limited to heart and skeletal muscle, but also includes smooth muscle of blood vessels, pulmonary, digestive, and urogenital tract. In addition, the mice have lysosomal glycogen storage in liver, kidney, spleen, and salivary gland, in Schwann cells of the peripheral nerves and in a subset of neurons in the central nervous system. By pathologic criteria the knockout mouse model parallels the human infantile form of GSDII and is attractive for studying the possible reversion of tissue pathology and symptomatology under different therapeutic regimens.

Introduction

Glycogen storage disease type II (GSDII; Pompe's disease; acid maltase deficiency) is an autosomal recessive lysosomal disorder caused by the deficiency of acid α-glucosidase. The most conspicuous clinical symptom is skeletal muscle weakness, but many different cell types manifest progressive lysosomal glycogen storage[1]. There is a spectrum of disease severity with marked differences in age of onset and rate of progression[2]. The clinical picture of the most severe infantile form of GSDII, without residual acid α-glucosidase activity[3], is rather uniform, and the course is predictable. The first symptoms present within the first seven months of life and patients usually die from cardio-respiratory failure within two years. The presenting clinical picture is that of a floppy baby with generalized muscle weakness and cardiomegaly. Drinking and respiratory problems are frequently encountered. Often the liver and tongue are enlarged. Morphologic changes of skeletal muscle earmark the disease, but glycogen accumulation also occurs in other tissues[1,4-8].

Patients with late onset juvenile and adult forms of GSDII have residual acid α-glucosidase activity[3]. Muscle weakness or respiratory problems may arise from the first to the seventh decade. The disease progression is slower than in infantile GSDII. Respiratory insufficiency is the most common cause of death[1]. Both muscle hypertrophy and atrophy have been reported[9,10]. In general, proximal muscles are more severely affected than distal muscles. Patients often have a waddling gait. Juvenile patients are at risk for obtaining skeletal deformities in puberty, like scoliosis, lordosis, and kyphosis. The pathologic reports focus mainly on liver, heart and skeletal muscle obtained by biopsy or post mortem[1].

Mouse models of GSDII were recently developed to obtain better insight in the pathologic process and to study the effect of therapeutic interventions[11,12]. In a first description of our knockout (KO) mouse model, we have reported on the genetic and biochemical characteristics. Furthermore, we have demonstrated the progressive nature of the lysosomal glycogen storage in heart, skeletal muscle and liver, of affected animals up to 13 weeks of age[11]. Here, we present a detailed pathologic report of the mice at 12-16 months of age, together with an inventory of clinical parameters.

Results

In a previous report, we have described in brief the pathology of our GSDII KO mice at six and thirteen weeks of age[11]. The KO mice lack *Gaa* mRNA and have a virtually complete acid α-glucosidase deficiency. Glycogen-containing lysosomes were detected soon after birth in liver, heart, and skeletal muscle, and the storage was progressive. In this article we present the clinical course and describe in detail the tissue pathology of older KO mice.

At birth, the KO mice were indistinguishable from their wild type (wt) and heterozygous littermates. From about six months on, some KO mice displayed subtle symptoms, such as spreading of the hind limbs when placed on a smooth solid surface, and diminished spinal muscle tone when held upside-down by the tail. A further clinical sign was back hunching. By one year of age, these symptoms had aggravated as shown in Figs. 1A-C. The mice sat and walked with splayed legs and developed thoracic kyphosis.

In addition to these symptoms, there were differences in weight gain (Fig. 2). During the first 10 months, the average weight of affected and unaffected mice followed the same curve. Thereafter, the average weight of KO mice stayed more or less constant, whereas there was further gain of weight of healthy mice. This phenomenon was observed in females and males. Remarkably, however, some affected males were heavier than normal. The loss of weight-gain was in part attributable to loss of muscle mass as illustrated in Fig. 1D. KO mice were less active than their normal littermates in a running wheel (Fig. 3) used for measuring muscle strength and endurance.

While monitoring the mice, we observed a substantial variation between littermates and between mouse strains. It is our impression that KO mice with a C57/Bl6 background show symptoms earlier than mice with FVB background.
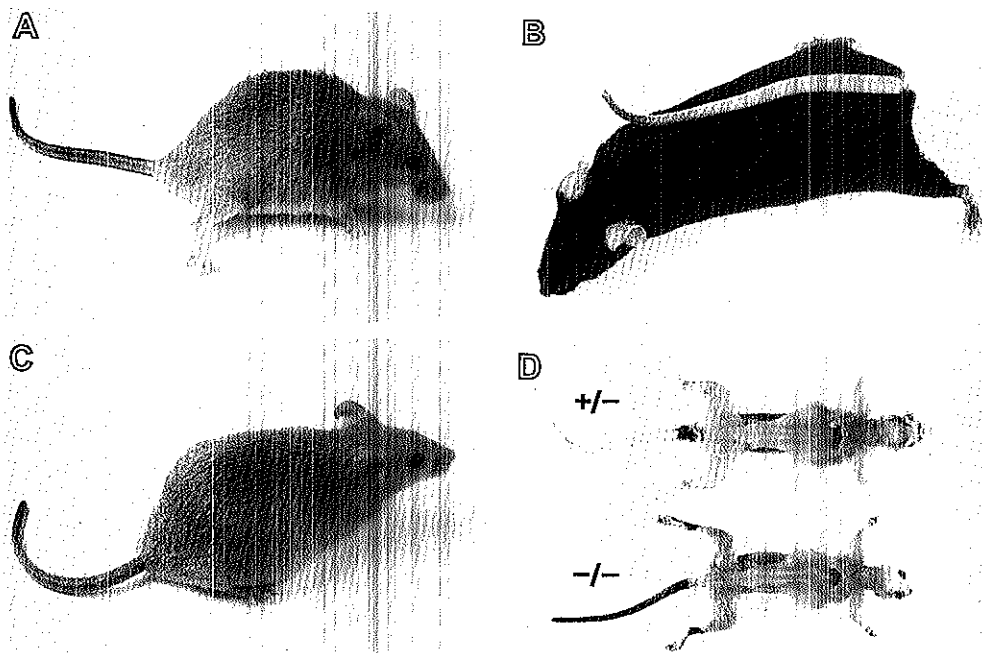
Fig. 1. Clinical signs of muscle weakness and wasting. The KO females in picture A (FVB; 17 months old) and B (C57/Bl6; 15 months old) show kyphosis and splayed posture. C. is a 15 months old wild type female (FVB). D. illustrates wasting of the proximal muscles of a 12 months old KO (-/-) mouse; (+/-) is a heterozygous female.

Macroscopy

Three KO mice and five normal (heterozygous and wild type) mice aged 12 to 16 months were killed in order to evaluate macroscopic and microscopic pathology. In addition, over 30 other mice were investigated that had died spontaneously or that were sacrificed in other experimental designs.

At autopsy the lack of adipose tissue, especially in the abdominal region and between the scapulae, was striking in most KO mice. Interestingly, this was not the case in KO mice with normal weight. The muscle mass of the KO mice was evidently less than of wt and heterozygous littermates (Fig. 1D). This gross observation was confirmed by dissection and weighing of individual muscles (Fig. 4). The cardiac enlargement reported earlier for 32 weeks old mice was frequently seen in mice older than a year[11]. One mouse had intestinal obstruction and several had a large gallbladder. Another mouse, killed because of paralysis of the lower back and hind limbs (Fig. 5), had a haematoma in the spinal cord, compressing the nerves. Among the mice that had died spontaneously, one (12 months of age) had food in the trachea and another (10 months) had a haemothorax. Both these findings are possibly disease-related (see discussion).
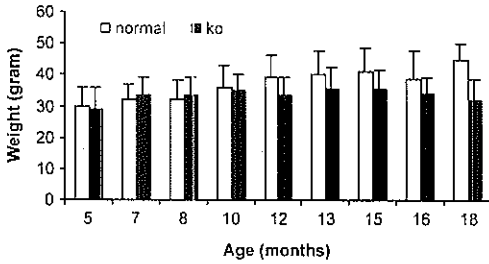
Fig. 2. *The average weight of normal and KO females at different ages. Each bar represents the average weight of 8 to 26 female mice (±SD).*
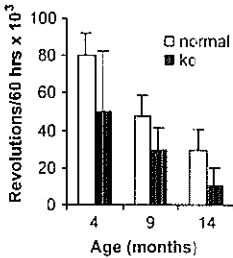
Fig. 3. *Performance of normal and KO females in a running wheel. The voluntary activity was measured over a 60 hours period including three nights. Each bar represents the average performance of 3 to 7 female mice (±SD).*
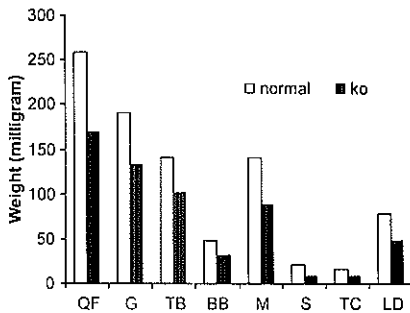
Fig. 4. *The muscle mass of female littermates (two normal and one KO) at 12 months of age. QF: quadriceps femoralis; G: gastrocnemius; TB: triceps brachii; BB: biceps brachii; M: masseter; S: sternomastoideus; TC: trapezius clavicularis; LD: latissimus dorsi.*
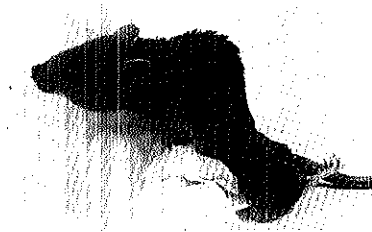
Fig. 5. *The posture of a KO mouse with a sudden paralysis of the lower back and hind limbs at 17 months of age.*

Light microscopy

Skeletal and heart muscle: All skeletal muscles showed lysosomal glycogen storage. The presence of glycogen-filled vacuoles in the sarcoplasm is demonstrated best with periodic acid-Schiff (PAS) staining (Figs. 6A-D and F), and is confirmed by disappearance of staining after diastase pre-treatment (not shown). Haematoxylin azofloxin (HA) staining demonstrates the cellular damage more clearly (Fig. 6E). In longitudinal sections, fields of glycogen and smaller
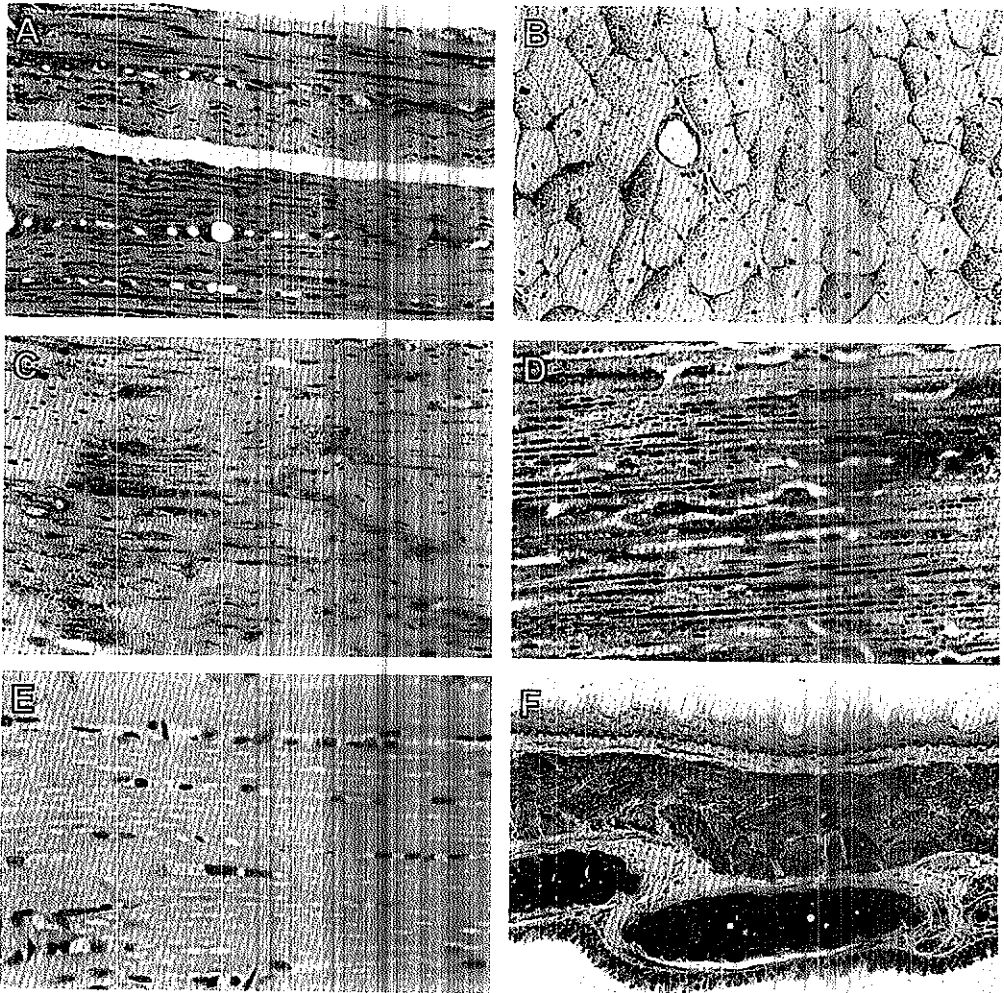
Fig. 6. *Skeletal muscle pathology. A longitudinal (A) and cross section (B) of the quadriceps femoralis; longitudinal sections of the pectoralis major (C), the diaphragm (D) and the masseter (E). A section shows the wall of the oesophagus (F; top) and the trachea including cartilage rings (F; bottom). Sections A-D and F were stained with periodic acid-Schiff (PAS) for demonstrating glycogen storage. Section E was stained with haematoxylin azofloxin. The tissues in panels A-E were taken from 15.5 months old mice, the section in panel F from a 12 months old mouse.*

granular deposits were present throughout the fibres and in longitudinal arrays between adjacent myofibrils. In cross-sections, an excessive variation in fibre size was noticed, as illustrated in Figs. 6B and F for the quadriceps femoralis and the striated muscles of the oesophagus, respectively. In the pectoralis major, the muscle fibres had a decreased diameter (not shown).

Not all muscles were equally involved. The diaphragm, which was one of the most severely affected muscles (Fig. 6D), showed myofibrils separated by large pools of glycogen (fibre
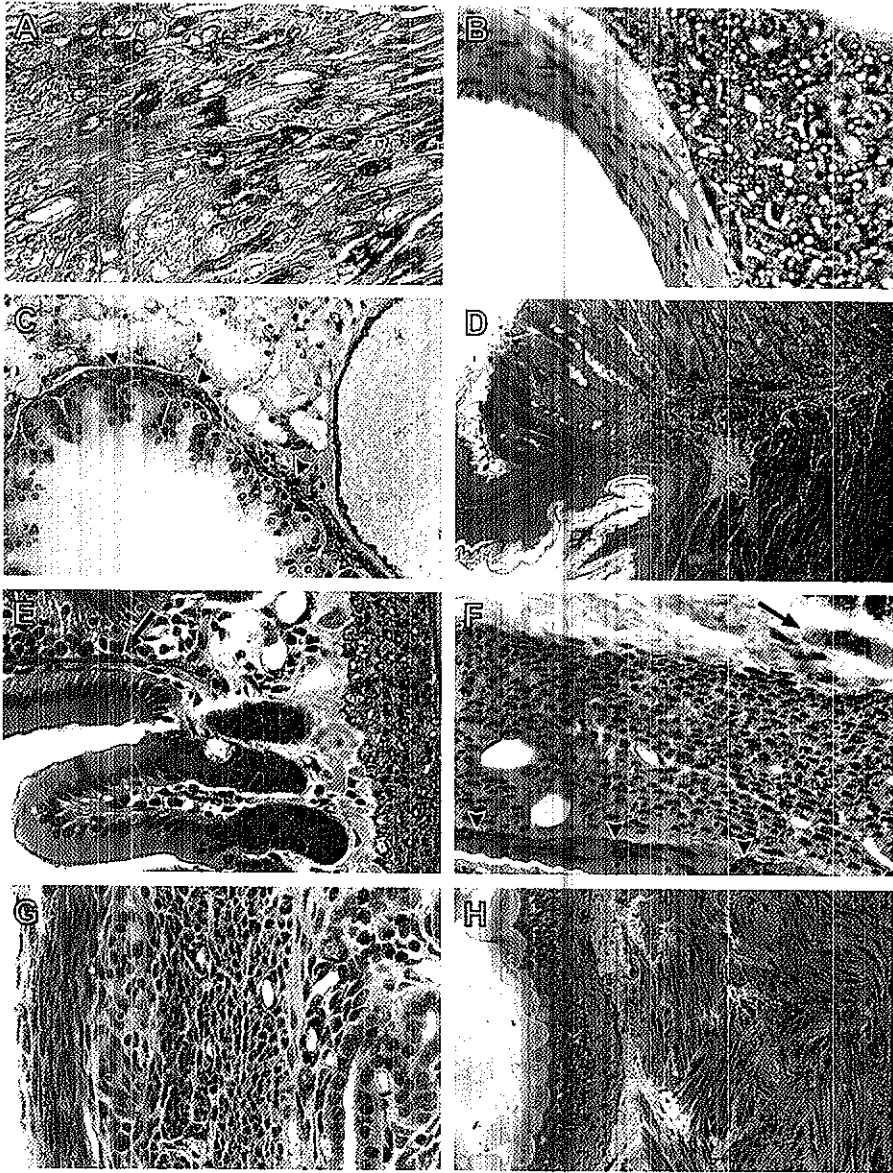
Fig. 7. *Pathologic changes in heart and smooth muscle tissue. A. Loss of cardiomyocytes and formation of fibrous tissue in the heart (Goldner's trichrome staining at 12 months of age). Sections B-H were stained with PAS, to demonstrate the presence of glycogen. B. Minor accumulation of glycogen in the wall of a large artery, major storage in adipose tissue. Smooth muscle is strongly positive in the wall of (C) a smaller vein and in the bronchiolus (arrowheads), (D) in the stomach, (E) small intestine, (F) colon, (G) uterus, and (H) bladder. The arrow in E points to the muscularis mucosae in the villus. The arrowheads in F point to the plexus of Auerbach and the arrow to the plexus of Meissner, where lysosomal glycogen storage occurs in ganglion cells. Sections B-G were taken at 15 months of age and section H at 12 months.*
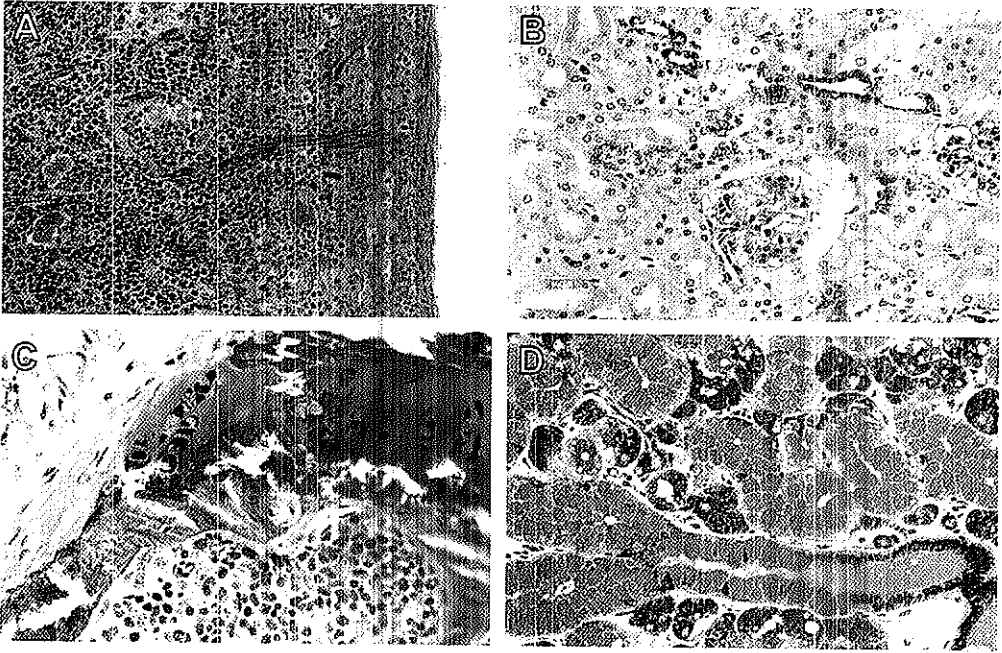
Fig. 8. *Pathology in other organs. (A) involvement of capsule and trabeculae of the spleen, (B) blood vessels, proximal and distal tubules of the kidney, (C) cartilage of the sternum and (D) serous glands and ducts in the salivary gland. Sections A-C are from 15 months old mice and section D from a 12 months old animal. All sections were PAS stained.*

splitting). The pectoralis major (Fig. 6C) was less severely affected. Dispersed glycogen granules and more dense deposits were present, but the organization of the myofibrils was still intact. Fibre splitting was seen occasionally. Arrays of round nuclei in the center of the fibres were a sign of muscle regeneration (Fig. 6E). Fig. 6B illustrates this phenomenon in cross section.

Heart muscle cells of KO mice had vacuoles in the center, resulting in a lace-work pattern typical of Pompe's disease (not shown). In several KO mice we observed foci of fibrous tissue replacing damaged cardiomyocytes (demonstrated by Goldner's trichrome staining; Fig. 7A). Wt mice of the same age did not show this pathologic phenomenon. In addition, fibrous tissue was present to varying extent in skeletal muscles (not shown).

Smooth muscle: Lysosomal glycogen storage was prominently present in smooth muscle at a variety of sites, including blood vessels (both arteries and veins), bronchioli, digestive tract, uterus, urinary bladder, urethra, and gallbladder. The PAS-positive lysosomes were mainly located in the perinuclear region. Glycogen storage in the arteries and veins is illustrated in Figs. 6B, 7C, E, G, and 8B and was usually more abundant in the smaller muscular arteries than in the larger, elastic ones (Fig. 7B). Capillaries, lacking smooth muscle, were PAS negative (Figs. 7F and G). Involvement of the digestive tract is illustrated in Fig. 7D (junction of the

Table 1. Distribution of glycogen storage (PAS staining).

Liver	Hepatocytes around central veins	++[a]
Spleen	Capsule (smooth muscle)	++
	Trabeculae (smooth muscle)	+++
Kidney	Proximal tubules (epithelium)	+
	Distal tubules (epithelium)	++
	Loop of Henle (epithelium)	++
	Collecting ducts (epithelium)	++
	Capsule of Bowman (epithelium)	±
Testis	Epididymus (epithelium, smooth muscle)	++
Skin	Hair root (epithelium)	+
	Outer hair shaft (epithelium)	+
	Fibroblasts	+
	Sebaceous glands	-
Adipose tissue	Brown	+++
	White	+
Tendons	Fibrocytes	-
Cartilage	Chondrocytes	+++
	Chondroblasts	+++
Bone	Osteocytes	-
Bone marrow		-
Salivary gland	Mucous epithelium	±
	Serous epithelium	+++
	Ducts (epithelium)	+++
Pancreas	Endocrine and exocrine	-
Adrenal gland	Cortex	+
	Medulla	++
Peripheral nerve bundles	Schwann cells	+
Ganglion cells	Plexus of Meissner	+
	Plexus of Auerbach	+
Central nervous system	Subpopulations of neurons	+++
	Non-neuronal cells	+++

[a]*Staining intensity was assessed semi-quantitatively: -, no staining; ±, weak; +, moderate; ++, strong; +++, very strong.*

forestomach and fundic stomach), E (small intestine) and F (colon). Smooth muscle cells of the muscularis mucosae as well as the tunica muscularis were involved. Notably, also the tiny bundles of the muscularis mucosae branching in the folds of the lamina propria stained positive (Fig. 7E). Storage in smooth muscle of the uterus and the bladder is illustrated in Figs. 7G and H, respectively.

Other organs and tissues: The glycogen storage is truly generalized and seen in many organs. Distribution and severity are depicted semi-quantitatively in Table 1. Fig. 8 illustrates the storage in spleen (A), kidney (B), cartilage (C) and salivary gland (D). Involvement of adipose tissue is shown in Fig. 7B and storage in ganglion cells of the plexus of Meissner and Auerbach in Fig. 7F. The central nervous system was not investigated in detail, but there was evidently storage in different subpopulations of neurons and non-neuronal cells (not shown).

Many tissues had inflammation infiltrates; although this is normal for older mice, they occurred with higher frequency in KO mice.

Electron microscopy

The light microscopic findings were confirmed by electron microscopy. Membrane-bound glycogen in vesicles of variable size and shape were present in many cell types. In skeletal muscle, there were large fields of glycogen deposited in between the myofibrils. Sometimes there was obvious loss of myofibrils, and the fibre structure was disorganized. Not all muscle fibres were involved to the same extent. Some fibres contained numerous large and densely packed lysosomes. Other fibres contained a large number of smaller lysosomes, while a third group of fibres was hardly involved. The fourth group of fibres contained a collection of autophagic vacuoles with heterogeneous content. Free glycogen was detected between these vacuoles (Fig. 9A). The glycogen storage in the blood vessel walls was restricted to the smooth muscle cells (Fig. 9B). In the kidney, the glycogen was localized in epithelial cells of the proximal and distal tubules, loop of Henle, and collecting ducts, without disturbing the cellular morphology (Fig. 9B). Fig. 9C illustrates the involvement of smooth muscle in the bladder.

Fig. 9. Electron microscopy. Autophagy in affected striated muscle (A), lysosomal glycogen storage in (B) epithelial cells of a proximal tubule and in smooth muscle cells of a small artery in the kidney, and (C) in smooth muscle cells of the bladder, at 9 months. Bar represents 1 μm (A) or 2 μm (B and C).
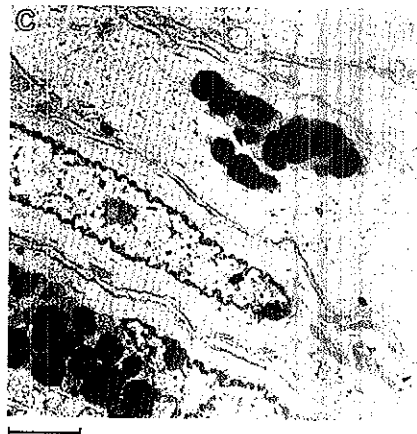
Discussion

KO mouse models of GSDII were made to obtain an easy to breed and easy to handle laboratory animal for studies on pathogenesis and therapy of this disease. Thus far, three mouse models have been described in general terms[11,12]. That is, the strategy for gene disruption was presented, the acid α-glucosidase deficiency was demonstrated and the heart and skeletal muscle pathology developing in the first four months of life was documented. Comparative clinical and pathologic data on the three published models are still scarce, which makes conclusions about potential differences between the models preliminary and speculative. To facilitate comparison and to collect further information on the pathogenesis of the disease, we have followed the disease process of the oldest mouse model of GSDII over a period of two years. This clinical and pathologic evaluation reveals remarkable parallels between the mouse models, the natural animal models, and the human condition.

Clinical aspects

Skeletal muscle weakness and cardiomegaly are prominent manifestations in the human infantile condition[1] and in all animal models with a fully deleterious acid α-glucosidase defect[14-16]. Our KO mice did not show obvious symptoms before six months of age, but objective measures of voluntary activity in a running wheel already demonstrate clinical signs at four months of age. Nine months olds KO mice start to display clearly problems of posture and gait. When sitting, but more apparent when walking, the hind limbs are in splayed position. It reminds of the waddling gait of affected cow and dog and the uncoordinated walking of affected sheep[14,16,17]. Mice, still alive at 18 months, all have muscle wasting and most have decreased weight, apparently related to loss of total muscle mass and adipose tissue. It is notable that some of the KO mice remain on the normal weight curve. In contrast to the majority of affected mice, these animals are rich in adipose tissue, which seems to compensate the loss of muscle mass. The fat cells themselves are severely affected, showing a massive load of glycogen. The parallel with the human condition is that also patients tend to loose weight and that hypotrophy of the muscles can be masked by adiposity[9,10]. Since all mice have an identical acid α-glucosidase deficiency, receive the same diet and are exposed to identical environmental factors, it seems likely that the different genetic background of the individual mice (which are not yet inbred) determines loss versus gain of adipose tissue.

Skeletal muscle involvement

In the mouse model, we see the same generalized involvement of skeletal muscle as in the infantile form of GSDII. When the mice are still young, the extent of lysosomal glycogen storage in different muscles is variable; however, almost all muscles are severely affected after one year. The weakness of the muscles of the proximal hind limbs and the long extensor of the back is clinically most obvious. The diaphragm is extremely pathologic and can cause respiratory insufficiency. Whether this occurs in KO mice can be tested with a spirometer specifically developed for small laboratory animals, but we did not have this instrument available. The decreased performance in the running wheel may result from respiratory insufficiency, but may as well be due to weakness of the skeletal muscles, to cardiac

insufficiency or to inferior blood vessels and circulation. Of note, respiratory insufficiency has been described in the dog model of GSDII, and cardiomegaly is a characteristic feature of all animal models with early onset disease[14,15,18].

The most detailed reports are of dog, quail and sheep[14,16,19,20]. The common denominator is a critical decline of the animal's condition due to generalized muscle weakness and probably cardiac insufficiency. In affected Lapland dogs, feeding problems were reported to contribute to the catabolic state. Chewing, swallowing and drinking were compromised by the involvement of the skeletal muscles of the upper digestive tract, and the oesophagus was dilated, causing reflux. In parallel, we see in our KO mice excessive glycogen storage in the muscles of the tongue, masseter and oesophagus. One mouse that had died spontaneously at one year of age had food residues in the trachea. This finding suggests suffocation as cause of death and may relate to the pathologic findings. Human patients also have feeding problems and are at risk for entering a catabolic state[21,22]. They may benefit from a high protein diet or a gastrojejunal feeding tube[1].

Smooth muscle involvement

The pathologic findings in our KO mouse model underscore the involvement of smooth muscle in GSDII. Very intense PAS positive staining is seen in the smooth muscle layers along the digestive tract (stomach, coecum, and intestines). More so, obstruction of the intestine might have been the cause of death of one mouse. Pathologic changes in the wall of the bladder are further evidence for participation of smooth muscle in the disease process. Urinary tract infections, incontinence and reflux are possibly related complications in late onset GSDII[23]. With respect to involvement of the vascular system, there is again a significant parallel between the mouse and the human condition. There are several reports on the pathologic changes of blood vessel walls, but this phenomenon receives in general little attention[6-8,24-27]. More specifically, glycogen storage and vacuoles have been seen in smooth muscle cells of the basilar artery, the circle of Willis and small cerebral arteries. More than once there is mention of fusiform dilatation and aneurysms of the basilar artery[28-30]. Given the above observations, aneurysm of a major thoracic artery is therefore a plausible cause of death of one of our KO mice.

Other tissues

The effect of lysosomal glycogen storage on cardiomyocytes is devastating. In some older mice, we observed loss of muscle tissue and outgrowth of loose connective tissue. This supports the notion that the cardiomegaly, characteristic for human infantile GSDII, is not only caused by hypertrophy but also by loss of cardiomyocyte function. Involvement of liver, kidney, and spleen is in accordance with the pathologic features known for humans and animal models. Less anticipated was the accumulation of glycogen in adipose tissue, salivary gland and cartilage, although a literature study led us to find some reports mentioning glycogen storage in these tissues of patients with Pompe's disease and of affected animals.

Finally, the involvement of the peripheral and central nervous system deserves attention. Some clinical findings in KO mice may have a neurogenic component. For instance, the intestinal obstruction may relate to the glycogen storage in ganglion cells of the plexi of

Meissner and Auerbach. Clearly, the motor horn cells and neurons in the cerebrum and cerebellum participate in the storage process. This holds for the mouse model as well as for other animal models (dog, bovine, and sheep)[14,16,17] and for the human classical infantile form of GSDII[1]. When enzyme therapy for GSDII reaches the stage of practical application, potential CNS-related problems need special attention, since the blood-brain barrier is likely to prevent effective treatment of the CNS.

In conclusion our KO mouse model of GSDII parallels closely the clinical and pathologic features of human infantile GSDII and highlights some less well-known but potentially important pathology of smooth muscle in a wide variety of organs.

Materials and methods
Mice: The KO mice used in this study were obtained by targeted disruption of the *Gaa* gene and have a complete deficiency of acid α-glucosidase[11]. The mice were backcrossed for two generations in either FVB or C57/BJ6 background. They were genotyped by PCR analysis and characterized biochemically. The mice were housed in a controlled facility and fed regular chow *ad libitum*. Mice were weighted and inspected at fixed hours. The voluntary activity of mice was measured with a running wheel as described by Horton et al.[13] with software developed by Van Leenen (personal communication). The mice were trained for five days before data collection was started.

Histology: Mice were starved over night to deplete the cytoplasmic glycogen store. The circulation was perfused with PBS under anaesthesia, followed by perfusion with 4 % glutaraldehyde or 4 % paraform-aldehyde. Tissues were collected and processed according to standard procedures. Glutaraldehyde-fixed tissue specimens were embedded in glycol-methacrylate (GMA) and tissue sections (4 μm) were stained with haematoxylin azofloxin (HA) or periodic acid-Schiff (PAS). Paraformaldehyde tissues were embedded in paraffin, and sections (4 μm) were stained with haematoxylin eosin (HE), PAS or Goldner's trichrome. As a control, paraffin sections were pre-incubated with diastase (to digest glycogen) before PAS staining.
For electron microscopy, glutaraldehyde-fixed tissues were post-fixed in 1 % OsO_4 with $K_3Fe(CN)_6$ in 0.1 M cacodylate as described[11]. Standard procedures were used for epon embedding. Semi-thin (1 μm) and ultrathin (60 nm) sections were cut on an LKB Nova ultratome and stained with methylen blue (semi-thin) or contrasted with uranylacetate in combination with Sato's lead citrate (ultrathin). Ultrathin sections were examined in a Philips CM100 electron microscopy (80 kV).

References
1. Hirschhorn R. Glycogen storage disease type II: acid α-glucosidase (acid maltase) deficiency. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. The metabolic and molecular bases of inherited disease. McGraw-Hill, Inc., 1995; 2443-2464.

2. Engel AG, Gomez MR, Seybold ME, Lambert EH. The spectrum and diagnosis of acid maltase deficiency. *Neurology* 1973; **23**, 95-106.

3. Reuser AJJ, Kroos MA, Hermans MMP *et al.* Glycogenosis type II (acid maltase deficiency). *Muscle Nerve* 1995; **3**, S61-S69.

4. Hers HG, De Barsy T. Type II glycogenosis (acid maltase deficiency). In: Hers HG, Van Hoof F, eds. Lysosomes and storage diseases, eds. New York. Academic Press: 197-216.

5. Hug G, Schubert WK. Glycogenosis type II. Glycogen distribution in tissues. *Arch Path* 1967; **84**, 141-152.

6. Martin JJ, De Barsy T, Van Hoof F, Palladini G. Pompe's disease: an inborn lysosomal disorder with storage of glycogen. A study of brain and striated muscle. *Acta Neuropathol* 1973; **23**, 229-244.

7. Sakurai I, Tosaka A, Mori Y, Imura S, Aoki K. Glycogenosis type II (Pompe). The fourth autopsy case in Japan. *Acta Pathol Jpn* 1974; **24**, 829-46.

8. Sang Hui K, Williams JC, Borit A, Rosenberg HS. The endocrine glands in Pompe's disease. *Arch Pathol Lab Med* 1985; **109**, 921-925.

9. Engel AG. Acid maltase deficiency in adults: studies in four cases of a syndrome which may mimic muscular dystrophy or other myopathies. *Brain* 1970; **93**, 599-616.

10. Bertagnolio B, Di Donato S, Peluchetti D *et al.* Acid maltase deficiency in adults. Clinical, morphological and biochemical study of three patients. *Eur Neurol* 1978; **17**, 193-204.

11. Bijvoet AGA, Van de Kamp EHM, Kroos MA *et al.* Generalized glycogen storage and cardiomegaly in a knockout mouse model of Pompe disease. *Hum Mol Genet* 1998; **7**, 53-62.

12. Raben N, Nagaralu K, Lee E *et al.* Targeted disruption of the acid α-glucosidase gene in mice causes an illness with critical features of both infantile and adult human glycogen storage disease type II. *J Biol Chem* 1998; **273**, 19086-19092.

13. Horton RM, Karachunski PI, Kellermann SA. Conti-Fine BM. Simple, inexpensive computerized rodent activity meters. *Biotechniques* 1995; **19**, 594-597.

14. Manktelow BW, Hartley WJ. Generalized glycogen storage disease in sheep. *J Comp Pathol* 1975; **85**, 139-145.

15. Howell MM, Dorling PR, Cook RD *et al.* Infantile and late onset form of generalized glycogenosis type II in cattle. *J Pathol* 1981; **134**, 266-277.

16. Walvoort HC, Dormans JAMA. Van den Ingh TSGAM. Comparative pathology of the canine model of glycogen storage disease type II (Pompe's disease). *J Inherit Metab Dis* 1985; **8**, 38-46.

17. O'Sullivan BM, Healy PJ, Fraser IR *et al.* Generalized glycogenosis in Brahman cattle. *Aust Vet J* 1981; **57**, 227-229.

18. Walvoort HC, Van Nes JJ, Stokhof AA, Wolvekamp WTC. Canine glycogen storage disease type II (GSDII): a clinical study of four affected Lapland dogs. *J Am Anim Hosp Assoc* 1984; **20**, 279-286.

19. Matsui T, Kuroda S, Mizutani M *et al.* Generalized glycogen storage disease in Japanese quail (Coturnix coturnix japonica). *Vet Pathol* 1983; **20**, 312-321.

20. Fujita T, Nonaka I, Sugita H. Japanese quail and human acid maltase deficiency: a comparative study. *Brain Dev* 1991; **13**, 247-55.

21. Slonim AE, Coleman RA, McElligot MA *et al.* Improvement of muscle function in acid maltase deficiency by high-protein therapy. *Neurology* 1983; **33**, 34-38.

22. Bodamer OA, Leonard JV, Halliday D. Dietary treatment in late-onset acid maltase deficiency. *Eur J Pediatr* 1997; **156**, S39-42 .

23. Chancellor AM, Warlow CP, Webb JN *et al.* Acid maltase deficiency presenting with a myopathy and exercise induced urinary incontinence in a 68 year old male. *J Neurol Neurosurg Psychiatry* 1991; **54**, 659-660.

24. Martin JJ, De Barsy T, De Schrijver LJG, Palladini G. Acid maltase deficiency (type II glycogenosis). Morphological and biochemical study of a childhood phenotype. *J Neurol Sci* 1976; **30**, 155-166.

25. Smith J, Zellweger H, Afifi AK. Muscular form of glycogenosis, type II (Pompe). *Neurology* 1967; **17**, 537-549.

26. Van der Walt JD, Swash M. Leake J, Cox EL. The pattern of involvement of adult-onset acid maltase deficiency at autopsy. *Muscle Nerve* 1987; **10**, 272-281.

27. Mancall EL, Aponte GE, Berry RG. Pompe's disease (diffuse glycogenosis) with neuronal storage. *J Neuropath Exp Neurol* 1965; **24**, 85-96.

28. Makos MM, McComb RD, Adickes ED, Bennett DR. Acid maltase deficiency and basilar artery aneurysms: a report of a sibship. *Neurology* 1985; **35 (Suppl. 1)**, 193-194.

29. Braunsdorf WE. Fusiform aneurysm of basilar artery and ectatic internal carotid arteries associated with glycogenosis type 2 (Pompe's disease). *Neurosurg* 1987; **21**, 748-749.

30. Matsuoka Y, Senda Y, Hirayama M, Matsui T, Takahashi A. Late-onset acid maltase deficiency associated with intracranial aneurysm. *J Neurol* 1988; **235**, 371-373.

Chapter 5

Expression of cDNA-encoded human acid α-glucosidase in milk of transgenic mice

Expression of cDNA-encoded human acid α-glucosidase in milk of transgenic mice

Agnes G.A. Bijvoet [a], Marian A. Kroos [a], Frank R. Pieper [b], Herman A. de Boer [c], Arnold J.J. Reuser [a], Ans T. van der Ploeg [d,*], Martin Ph. Verbeet [c]

[a] *Department of Clinical Genetics, Erasmus University and Sophia Children's Hospital, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands*
[b] *Pharming B.V., Niels Bohrweg 11–13, 2333 CA Leiden, The Netherlands*
[c] *Medical Biotechnology, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands*
[d] *Department of Paediatrics, Erasmus University and Sophia Children's Hospital, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands*

Abstract

Enzyme replacement therapy is at present the option of choice for treatment of lysosomal storage diseases. To explore the feasibility of lysosomal enzyme production in milk of transgenic animals, the human acid α-glucosidase cDNA was placed under control of the α_{S1}-casein promoter and expressed in mice. The milk contained recombinant enzyme at a concentration up to 1.5 μg/ml. Enzyme purified from milk of transgenic mice was internalized via the mannose 6-phosphate receptor and corrected enzyme deficiency in fibroblasts from patients. We conclude that transgenically produced human acid α-glucosidase meets the criteria for therapeutic application.

Transgenic production of therapeutic proteins in mammalian milk can be an attractive strategy to provide low cost treatment for rare diseases [1,2]. We have investigated whether this strategy, originally designed for the production of secretory proteins, is also suitable for the manufacturing of lysosomal enzymes to be used for enzyme replacement therapy for lysosomal storage diseases. The production of human acid α-glucosidase was taken as prototype. Deficiency of acid α-glucosidase (acid maltase; EC 3.2.1.3) leads to glycogen storage disease type II (GSD II; Pompe disease; acid maltase deficiency), an autosomal recessive disorder with an estimated incidence of 1 in 100 000 in the Caucasian population and 1 in 50 000 in southern China and Taiwan [3]. The disease is clinically and genetically heterogeneous. The common clinical feature is generalized muscle weakness. In the severe early onset cases also the heart is involved.

A therapy for GSD II is not available yet, but there is

optimism about the applicability of receptor-mediated enzyme replacement therapy [4–9]. The receptor aimed at is the mannose 6-phosphate receptor which is crucial for the intracellular targeting of most lysosomal enzymes and for the endocytosis and lysosomal delivery of exogenous mannose 6-phosphate containing (lysosomal) proteins [10]. In the past, we have demonstrated correction of lysosomal glycogen storage in cultured fibroblasts and muscle cells of GSD II patients after uptake of mannose 6-phosphate containing acid α-glucosidase via this receptor [4,5]. Moreover, uptake was demonstrated in heart and skeletal muscle of mice receiving acid α-glucosidase intravenously [7]. Similarly, administration of mannose 6-phosphate containing β-glucuronidase [11] and α-L-iduronidase [12] to animals deficient in these enzymes remedied associated clinical features. Most of all, we were encouraged by the therapeutic efficacy of enzyme replacement therapy for Gaucher disease type I aimed at the mannose-receptor [13]. Thus, we have set out to develop methods for production of human recombinant, mannose 6-phosphate containing, acid α-glucosidase [8,9].

We present a first evaluation of transgenic production

* Corresponding author. Fax: +31 10 4636811.

of human mannose 6-phosphate containing lysosomal enzymes in milk. The method takes advantages of an expression vector developed to target expression of foreign secretory proteins to the mammary glands of mice, rabbits, sheep, goats and pigs [1,2]. For our pilot studies we have used mice as model system.

To obtain lactation-specific expression of a human acid α-glucosidase transgene, we inserted the human acid α-glucosidase cDNA [14] in an expression cassette derived from the regulatory sequences of the bovine α_{S1}-casein gene [15,16]. The 25.5-kb NotI expression module (Fig. 1) excised from plasmid p16.8αglu was microinjected into pronuclei of fertilized mouse oocytes (CBA/BrA × C57Bl/6), according to Hogan et al. and Platenburg et al. [16,17]. Using Southern blot analysis of genomic DNA prepared from tail biopsies of the newborn mice, 11 transgenic mice were identified. Ten of these founder mice transmitted their transgenes in a Mendelian fashion, one did not. The copy number of the transgene in the 10 mouse lines was estimated to vary from five to twenty, comparing the intensities of the hybridization signals on mouse-tail DNA-blots using the human acid α-glucosidase cDNA as probe (data not shown).

The presence of human recombinant acid α-glucosidase in the milk of transgenic mice was demonstrated with a mouse polyclonal antiserum recognizing specifically the human and not the murine isoform [18]. Of the ten lines tested (Table 1), three were positive (#1672, #1673 and #1676). Line #1672 had the highest activity of 446 nmol MU/ml/h at midlactation, equivalent to 1.5 μg acid α-glucosidase/ml. Expression levels were not related to the copy-number of the transgene, which suggests integration site-dependent transcription. In agreement with the activity data, human recombinant acid α-glucosidase was detected in the milk of lines #1672, #1673 and #1676 by immunoprecipitation followed by immunoblotting (Fig. 2). Panel A shows specifically the human isoforms, whereas both human and murine isoforms are shown in panel B.

Table 1

The activity of human recombinant acid α-glucosidase in the milk of transgenic and non transgenic animals

Line	α-Glucosidase activity [a] (nmol MU/ml/h)	(n)	Estimated amount [b] (μg/ml)
1672	446	(5)	1.5
1673	162	(3)	0.5
1676	28	(6)	0.09
others	≤ 20	(21)	--
n.t.	≤ 20	(5)	–

[a] Milk samples were collected with a hand-made milking apparatus 10 minutes after subcutaneous injection of 1 unit oxytocin (PitonS, Organon) and stored at −20°C until use. The acid α-glucosidase activity in the milk samples was determined with the artificial substrate 4-methylumbelliferyl-α-D-glucopyranoside [18]. Milk samples were adjusted to an activity of 10 nmol 4 methylumbelliferone (MU)/h in a final volume of 200 μl, by adding 10 mM sodiumphosphate pH 6.6, containing 0.9% NaCl and 1 mg/ml bovine serum albumin (PBS), and were incubated with protein A Sepharose beads (Pharmacia) to pre-absorb immunoglobulins. Thereafter, the recombinant acid α-glucosidase was precipitated with antibodies raised in mice and coupled to protein A Sepharose beads, precipitating human but not murine acid α-glucosidase. The activity of human acid α-glucosidase in the milk was calculated from the activity measured on the Sepharose beads with the artificial substrate as described [7,18]. [b] The amount of human acid α-glucosidase in the mouse milk was calculated based on a specific enzyme activity of 300 μmol MU/mg/h [19]. (n): number of milk samples; others: includes transgenic lines #1671, 1675, 1677, 1678, 1679, 1680 and 1692; n.t.: non-transgenic lines.

The human acid α-glucosidase in the mouse milk is present in two isoforms with an apparent molecular mass of 110 and 76 kDa, like the precursor and mature forms of acid α-glucosidase present in human tissues. The murine isoforms are 95 and 73 kDa (Fig. 2B).

Expression of the human acid α-glucosidase transgene was mammary gland-specific and lactation-dependent as illustrated in Fig. 3 for the mammary gland, spleen, heart and skeletal muscle. Also no expression of the transgene was detected in liver, kidney, brain, lacrimal glands, sali-
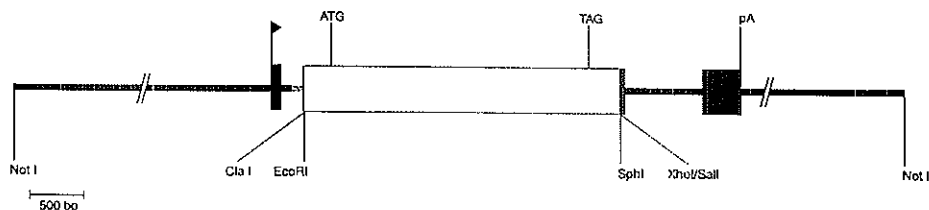

Fig. 1. Physical map of the 25.5 kb mammary gland-specific expression module for human acid α-glucosidase. To make this module, the acid α-glucosidase cDNA was excised from 2pSHAG2 [14] with EcoRI and SphI and subcloned in pKUN7ΔC, a pKUN1 derivative [22]. From this plasmid the 3.3-kb cDNA-fragment was excised with ClaI and XhoI and inserted into the ClaI site and XhoI-compatible SalI site of the plasmid p16.8hll3ks replacing the lactoferrin cDNA [16]. The resulting plasmid p16.8αglu consists of the human acid α-glucosidase cDNA flanked upstream by 14.2 kb of sequences, containing the promoter and untranslated first exon of the bovine α_{S1}-casein gene, and a hybrid α_{S1}-casein/immunoglobulin G intron [23]. The cDNA is flanked downstream by 8 kb of 3′ bovine α_{S1}-casein sequences including the polyadenylation signal. Black boxes represent non-translated bovine α_{S1}-casein exons. Other bovine α_{S1}-casein sequences are indicated with a bold line. The hatched line and box represent immunoglobulin G intron and exon sequences. The open box represents the human acid α-glucosidase cDNA. The transcription initiation site (flag), the translational start site (ATG), the stop codon (TAG), and the polyadenylation site (pA) are indicated.
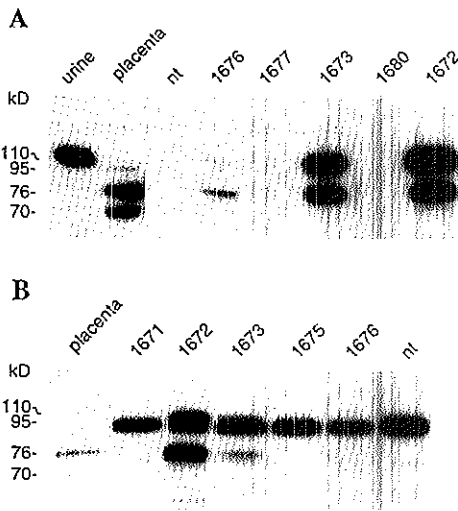
A

B

Table 2
Uptake of acid α-glucosidase purified from the milk of transgenic mice by enzyme-deficient fibroblasts

Cell line	Additions enzyme	M6P	Acid α-glucosidase activity (nmol MU/mg/hr)	(n)
Normal	–	–	145	(12)
Deficient	–	–	≤ 0.1	(4)
Deficient	+	–	676	(8)
Deficient	+	+	5.5	(4)

Acid α-glucosidase was isolated from the milk of transgenic mice. The enzyme was added to the culture medium of enzyme deficient fibroblasts in a concentration equivalent to 1.4 μmol MU/h in 1 ml of Ham's F10 medium supplemented with 10% fetal bovine serum, 3 mM PIPES and antibiotics (as described [24]), in the presence or absence of 5 mM mannose 6-phosphate (M6P). The cells were harvested after 22 h and homogenized. The acid α-glucosidase activity was determined with the artificial substrate 4-methylumbelliferyl-α-D-glucopyranoside. Protein concentrations were determined using the BCA protein assay (Pierce). (n) = number of assays.

Fig. 2. Immunoblot analysis of acid α-glucosidase from milk of transgenic mice. The milk samples were adjusted to an acid α-glucosidase activity of 100 nmol MU/h in a final volume of 1 ml by adding 10 mM PBS and were cleared of immunoglobulins (see the legend of Table 1 for details). Acid α-glucosidase was subsequently precipitated with antibodies raised against human placental acid α-glucosidase complexed to protein A Sepharose beads. The Sepharose beads with the enzyme-immunoglobulin complex bound to it were boiled in sample buffer and applied to SDS-PAGE as described [19]. The proteins were subsequently blotted onto nitrocellulose filters and acid α-glucosidase was visualized on X-ray film after incubating the blots with rabbit anti-human placental acid α-glucosidase antibodies coupled to ^{125}I-protein A. Acid α-glucosidase purified from human urine (110 kDa) and human placenta (76 and 70 kDa) served as molecular mass markers. Panel A shows the human acid α-glucosidase isoforms immunoprecipitated with antibodies raised in mouse, not recognizing the mouse isoforms. Panel B shows both the human and the mouse isoforms immunoprecipitated with antibodies raised in rabbit, recognizing the human as well as the murine acid α-glucosidase isozymes. The numbers above the lanes refer to the transgenic lines from which the samples were taken; n.t. is a sample from a non-transgenic mouse. The non-expressing lines were chosen randomly.
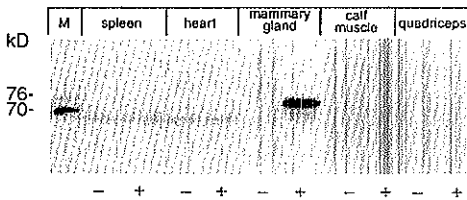
Fig. 3. Immunoblot analysis of tissue and lactation-specific expression of the human acid α-glucosidase transgene. Various tissues from a lactating (8 days post-partum) (+) and a non lactating (–) mouse of line #1672 were homogenized as described [7], and the human acid α-glucosidase isoforms were immunoprecipitated with antiserum raised in mice against human placental acid α-glucosidase as described in the legends of Fig. 2. Acid α-glucosidase purified from human placenta was applied in the first lane (M) to serve as molecular mass marker.

vary glands, and lung (data not shown). The molecular mass of the recombinant enzyme in the mammary gland is the same as of mature lysosomal acid α-glucosidase in human tissues (76 kDa). All transgenic mice appeared healthy and reproduced normally.

To test the applicability of the recombinant acid α-glucosidase for enzyme replacement therapy, the enzyme was purified from transgenic mouse milk, essentially as described before [19], and added to culture medium of acid α-glucosidase deficient fibroblasts. Table 2 shows that the acid α-glucosidase activity of the deficient fibroblasts increased above control level after overnight incubation. Endocytosis was mediated by the mannose 6-phosphate receptor, since no corrective effect was obtained when the milk enzyme was administered in the presence of 5 mM mannose 6-phosphate.

In conclusion, the human recombinant acid α-glucosidase produced in the mammary gland of transgenic mice exhibits the proper characteristics for use in enzyme replacement therapy for GSD II and is an alternative for enzyme production in CHO-cells [8,9]. It is obvious, however, that higher expression levels of the recombinant enzyme in milk are needed for the industrial production of therapeutic enzyme. A way to increase the expression level is to use genomic rather than cDNA constructs [20]. Our preliminary results indicate that the expression can be upgraded by at least 100-fold by using a genomic construct [21]. We are encouraged to fully explore the possibilities to produce mannose 6-phosphate containing lysosomal enzymes in the milk of transgenic animals for treatment of lysosomal storage disease.

References

[1] Hennighausen, L. (1992) J. Cell. Biochem. 49, 325–332.

[2] Lee, S.H. and de Boer, H.A. (1994) J. Control. Release 29, 213–221.

[3] Hirschhorn, R. (1995) in The metabolic and molecular bases of inherited disease Scriver (C.R., Beaudet, A.L., Sly, W.S. and Valle, D., Eds.), pp. 2443–2464.

[4] Van der Ploeg, A.T., Bolhuis, P.A., Wolterman, R.A., Visser, J.W., Loonen, M.C.B., Busch, H.F.M. and Reuser, A.J.J. (1988) J. Neurol. 235, 392–396.

[5] Van der Ploeg, A.T., Loonen, M.C.B., Bolhuis, P.A., Busch, H.M.F., Reuser, A.J.J. and Galjaard, H. (1988) Pediatr. Res. 24, 90–94.

[6] Van der Ploeg, A.T., Van der Kraaij, A.M.M., Willemsen, R., Kroos, M.A., Loonen, M.C.B., Koster, J.F. and Reuser, A.J.J. (1990) Pediatr. Res. 28, 344–347.

[7] Van der Ploeg, A.T., Kroos, M.A., Willemsen, R., Brons, N.H. and Reuser, A.J.J. (1991) J. Clin. Invest. 87, 513–518.

[8] Fuller, M., Van der Ploeg, A., Reuser, A.J.J., Anson, D.S. and Hopwood, J.J. (1995) Eur. J. Biochem. 234, 903–909.

[9] Van Hove, J.L.K., Wang, H.W., Wu, J.-Y., Brady, R.O. and Chen, Y.-T. (1996) Proc. Natl. Acad. Sci. USA 93, 65–70.

[10] Kornfeld, S. (1992) Annu. Rev. Biochem. 61, 307–330.

[11] Sands, M.S., Vogler, C., Kyle, J.W., Grubb, J.H., Levy, B., Galvin, N., Sly, W.S. and Birkenmeier, E.H. (1994) J. Clin. Invest. 93, 2324–2331.

[12] Shull, R.M., Kakkis, E.D., McEntee, M.F., Kania, S.A., Jonas, A.J. and Neufeld, E.F. (1994) Proc. Natl. Acad. Sci. USA 91, 12937–12941.

[13] Brady, R.O. and Barton, N.W. (1994) Biochem. Med. Metab. Biol. 52, 1–9.

[14] Hoefsloot, L.H., Willemsen, R., Kroos, M.A., Hoogeveen-Westerveld, M., Hermans, M.M., Van der Ploeg, A.T., Oostra, B.A. and Reuser, A.J. (1990) Biochem. J. 272, 485–492.

[15] Meade, H., Gates, L., Lacy, E. and Lonberg, N. (1990) Bio/Technology 8, 443–446.

[16] Platenburg, G.J., Kootwijk, E.P.A., Kooiman, P.M., Woloshuk, S.L., Nuijens, J.H., Krimpenfort, P.J.A., Pieper, F.R., de Boer, H.A. and Strijker, R. (1994) Transgenic Res. 3, 99–108.

[17] Hogan, B., Beddington, R., Costantini, F. and Lacy, E. (1994) Manipulating the Mouse Embryo: A Laboratory Manual, 2nd. edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

[18] De Jonge, A.J.R., De Smit, S., Kroos, M.A. and Reuser, A.J.J. (1985) Hum. Genet. 69, 32–38.

[19] Reuser, A.J.J., Kroos, M., Oude Elferink, R.P.J. and Tager, J.M. (1985) J. Biol. Chem. 260, 8336–8341.

[20] Palmiter, R.D., Sandgren, E.P., Avarbock, M.R., Allen, D.D. and Brinster, R.L. (1991) Proc. Natl. Acad. Sci. USA 88, 478–482.

[21] Bijvoet, A.G.A., Kroos, M.A., Van de Kamp, E.H.M., Oostra, B.A., Verbeet, M.P., Van der Ploeg, A.T. and Reuser, A.J.J. (1995) Am. J. Hum. Genet. 57, A176.

[22] Konings, R.N.H., Luiten, R.G.M. and Peeters, B.P.H. (1986) Gene 46, 269–276.

[23] Choi, T., Huang, M., Gorman, C. and Jaenisch, R. (1991) Mol. Cell. Biol. 11, 3070–3074.

[24] Reuser, A.J.J., Kroos, M.A., Ponne, N.J., Wolterman, R.A., Loonen, M.C.B., Busch, H.F.M., Visser, W.J. and Bolhuis, P.A. (1984) Exp. Cell. Res. 155, 178–189.

Chapter 6

**Recombinant human acid α-glucosidase
high level production in mouse milk,
biochemical characteristics,
correction of enzyme deficiency in GSDII KO mice**

Recombinant human acid α-glucosidase: high level production in mouse milk, biochemical characteristics, correction of enzyme deficiency in GSDII KO mice

Agnes G. A. Bijvoet[1,2], Marian A. Kroos[1], Frank R. Pieper[3], Martin Van der Vliet[4], Herman A. De Boer[5], Ans T. Van der Ploeg[2], Martin Ph. Verbeet[6] and Arnold J. J. Reuser[1,*]

[1]Department of Clinical Genetics, Erasmus University, PO Box 1738, 3000 DR Rotterdam, The Netherlands, [2]Department of Paediatrics, Sophia Children's Hospital, PO Box 2060, 3000 CB Rotterdam, The Netherlands, [3]Pharming BV, Niels Bohrweg 11–13, 2333 CA Leiden, The Netherlands, [4]BioCell Technology, Weserstraat 3, 2207 CK Spijkenisse, The Netherlands and [5]Medical Biotechnology and [6]Metalloprotein and Protein Engineering Group, Leiden Institute of Chemistry, Leiden University, PO Box 9502, 2300 RA Leiden, The Netherlands

Glycogen storage disease type II (GSDII) is caused by lysosomal acid α-glucosidase deficiency. Patients have a rapidly fatal or slowly progressive impairment of muscle function. Enzyme replacement therapy is under investigation. For large-scale, cost-effective production of recombinant human acid α-glucosidase in the milk of transgenic animals, we have fused the human acid α-glucosidase gene to 6.3 kb of the bovine α_{S1}-casein gene promoter and have tested the performance of this transgene in mice. The highest production level reached was 2 mg/ml. The major fraction of the purified recombinant enzyme has a molecular mass of 110 kDa and resembles the natural acid α-glucosidase precursor from human urine and the recombinant precursor secreted by CHO cells, with respect to pH optimum, K_m, V_{max}, N-terminal amino acid sequence and glycosylation pattern. The therapeutic potential of the recombinant enzyme produced in milk is demonstrated *in vitro* and *in vivo*. The precursor is taken up in a mannose 6-phosphate receptor-dependent manner by cultured fibroblasts, is converted to mature enzyme of 76 kDa and depletes the glycogen deposit in fibroblasts of patients. When injected intravenously, the milk enzyme corrects the acid α-glucosidase deficiency in heart and skeletal muscle of GSDII knockout mice.

INTRODUCTION

Investigations into the production of pharmaceutical proteins in genetically engineered cells and organisms are a major activity in the biotechnology field. The application of biotechnology instead of classical production methods often has an economical advantage, but becomes mandatory when natural product sources are extremely scarce. The latter situation exists for lysosomal enzymes. There are >40 of these enzymes constitutively expressed in almost all cell types of the human body, where they act in concert to degrade and recycle a variety of macromolecules derived from the intracellular and extracellular environment. An inherited single enzyme deficiency may prohibit adequate degradation of one or more of these compounds and result in a lysosomal storage disease (1). The phenotypic expression of these diseases is diverse, with early and late onset forms and variable extents of organ involvement. Our present study deals with the development of enzyme replacement therapy for one of these lysosomal storage disorders: glycogen storage disease type II (GSDII; Pompe's disease).

GSDII is an autosomal recessive disorder, characterized by generalized muscle weakness and wasting due to deficiency of the lysosomal enzyme acid α-glucosidase. Respiratory insufficiency is life threatening for affected children and adults with a slowly progressive course. Severely affected infants succumb by cardio-respiratory insufficiency in the first to second year of life (2). In the early 1960s, an attempt was made to compensate lysosomal enzyme deficiencies by intravenous administration of enzyme preparations from a variety of natural sources. The first attempt concerned treatment of GSDII with acid α-glucosidase extracted from *Aspergillus niger* (3). The outcome of clinical trials performed in several lysosomal storage diseases remained disappointing for a long time; the enzyme dosage was too low and the enzyme targeting inadequate (4,5). The first successful attempt at enzyme replacement therapy was published in 1990 for type I Gaucher disease, a glucocerebrosidase deficiency causing glycolipid storage in macrophages (6,7). The glucocerebrosidase used in this trial was purified on a large scale from human placentas and was modified in the carbohydrate side chain in

*To whom correspondence should be addressed. Tel: +31 10 408 7153; Fax: +31 10 436 2536; Email: Reuser@ikg.fgg.eur.nl

order to fit the mannose receptor on the cell surface of macrophages storing the glycolipid. A very similar recombinant form of human glucocerebrosidase, imiglucerase (Cerezyme; Genzyme, Cambridge, MA), presently is produced in genetically engineered Chinese hamster ovary K1 cells (CHO cells). The same cell type is employed for the production of a number of other recombinant human lysosomal enzymes that are overexpressed and purified from the culture medium (8–18).

A characteristic feature of these enzymes is that they have carbohydrate side chains with mannose 6-phosphate groups (M6P). This facilitates their binding and endocytosis by cells with cell surface mannose 6-phosphate receptors (M6P receptor, IGFII receptor). The cardiomyocytes and skeletal muscle cells affected in GSDII by lysosomal glycogen storage express the M6P receptor on their surface (19,20) and are thereby targets for M6P-containing forms of acid α-glucosidase (15,17,21–24). The feasibility of acid α-glucosidase production in CHO cells has been demonstrated and the therapeutic potential of this enzyme has been tested. However, it remains a major effort and a costly procedure to realize large-scale enzyme production in CHO cells. With this in mind, we have initiated an investigation into the possibility of lysosomal α-glucosidase production in the milk of transgenic mammals, and demonstrated that trace amounts of recombinant human acid α-glucosidase are secreted in the milk of transgenic mice carrying the human acid α-glucosidase cDNA linked to the bovine α_{S1}-casein promoter, but the yield was too low to characterize the recombinant enzyme in detail and to study its therapeutic effect (25,26).

In this study, we demonstrate the feasibility of large-scale production of recombinant acid α-glucosidase in the milk of transgenic mice with a transgene containing the intact α-glucosidase gene instead of the cDNA. The enzyme was purified from the milk and characterized with regard to its structural, biochemical and functional features. In all aspects, it resembles the recombinant enzyme produced in the medium of genetically engineered CHO cells. The therapeutic potential is demonstrated by feeding the enzyme to cultured fibroblasts of a GSDII patient and by intravenous administration to GSDII knock-out (KO) mice, that recently were developed in our laboratory for this and other purposes (27).

RESULTS

Generation of transgenic mice

To obtain high level expression of recombinant human acid α-glucosidase in the mammary gland of transgenic mice, we have fused the acid α-glucosidase gene in its genomic context to the bovine α_{S1}-casein promoter in a multi-step cloning procedure (as described in Materials and Methods). The transgene is depicted in Figure 1A and comprises 6.3 kb of the bovine α_{S1}-casein gene [promoter and 5'-untranslated region (5'-UTR)] and 28.5 kb of the human acid α-glucosidase gene including all exons, introns and the 3'-UTR. This construct was injected into the pronucleus of fertilized oocytes to generate transgenic mice. Nine founders were obtained and they were crossed with wild-type CBA/BrA × C57Bl/6 mice. One male founder appeared to be infertile, two others were germline chimeras. The remaining six founders transmitted the transgene in a Mendelian fashion. Litter sizes were normal and littermates were indistinguishable. The copy number of the transgene in the offspring ranged from two to 10 copies as judged from the intensity of the hybridization signal on Southern blots from tail DNA using human acid α-glucosidase cDNA as probe (Table 1).

Transgene expression

Northern and western blotting were performed to demonstrate transcription and translation of the transgene. Mice from different transgenic lines were tested. Figure 1B and C shows as a representative example the results obtained with mouse 4312 of line 2585 with an approximate copy number of 10. The northern blot (Fig. 1B) reveals very high expression of the transgene in the lactating mammary gland, and low expression in various other organs, such as brain, lungs, liver, salivary gland and intestine. Also, mammary gland-specific overexpression is shown by western blotting. Some other tissues contain human acid α-glucosidase at a much lower concentration (Fig. 1C).

Table 1. Characteristics of transgenic mouse lines

| Line number | Estimated copy number[a] | mRNA level[b] | Acid α-glucosidase concentration in milk samples | | | |
			Average[c]	Range	n	m
2470	10	++	248	11–1095	38	3
2471	2	+++	113	13–260	8	2
2474	2	+	31	4–99	9	2
2475	4	++	81	20–238	15	4
2477	4	+	72	5–215	8	3
2585	10	+++	514	24–2048	38	8
Non-transgenic	–	–	3	2–3	7	

[a]The copy number of the transgene was estimated by Southern blotting.
[b]The level of mRNA in the mammary gland of lactating females was estimated by northern blotting.
[c]The concentration of acid α-glucosidase in the milk (μg/ml) was calculated from the enzymatic activity measured with the artificial substrate 4-methylumbelliferyl-α-D-glucopyranoside.
n, number of milk samples (sample collection was started 7 days after birth); m, number of mice used for sample collection.
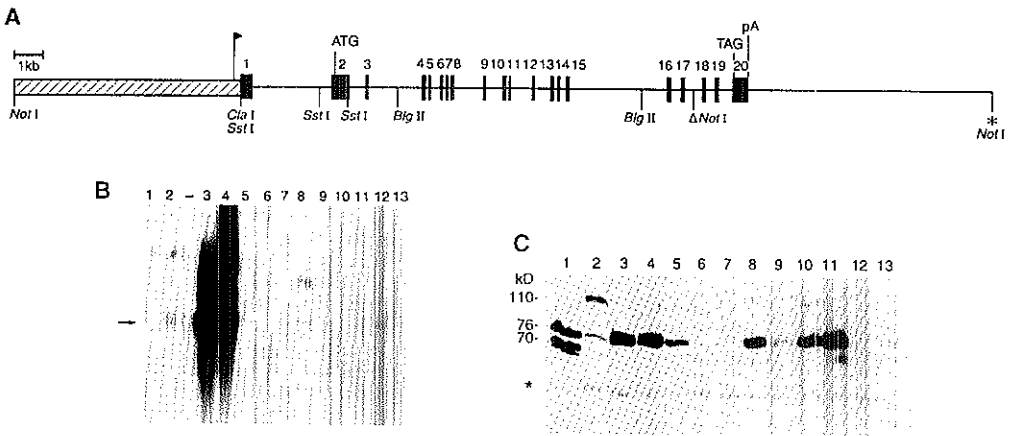
A

B

C

Figure 1. The acid α-glucosidase transgene and its expression. (A) Structure of the bovine α_{S1}-casein–human acid α-glucosidase transgene. Solid boxes, exons of the human acid α-glucosidase gene; line, introns and adjacent 3' sequences; striated box, bovine α_{S1}-casein promoter region; flag, transcription initiation site; ATG, translation initiation site; TAG, translation stop site; pA, polyadenylation site; Δ*Not*I, deleted *Not*I site; *, location of the *Bam*HI–*Bgl*II fusion site. The essential restriction sites *Cla*I, *Sst*I, *Bgl*II and *Not*I are indicated. (B) Northern blot analysis of transgene expression. Twenty micrograms of RNA extracted from various tissues of a lactating transgenic mouse (mouse 4312 of line 2585, with a copy number of 10) was loaded in each lane and transferred onto a Hybond-N filter. The filter was hybridized with a ³²P-labelled human specific acid α-glucosidase cDNA probe as described in Materials and Methods. Lane 1 contains 1 µg of bovine mammary gland RNA mixed with 19 µg of mammary gland RNA of a non-transgenic mouse. Lane 2 contains 20 µg of human fibroblast RNA. The other lanes contain RNA samples from the left mammary gland (lane 3), the right mammary gland (lane 4), brain (lane 5), lungs (lane 6), kidney (lane 7), liver (lane 8), salivary gland (lane 9), muscle (lane 10), heart (lane 11), intestine (lane 12), ovaries and uterus (lane 13). The arrow points to the human acid α-glucosidase messenger. Higher bands are probably caused by aberrant splicing. (C) Immunoblot analysis of human acid α-glucosidase expression in various tissues of mouse 4312 (line 2585). The acid α-glucosidase activity in tissue homogenates was measured with 4-methylumbelliferyl-α-D-glucopyranoside, and the human enzyme was immunoprecipitated selectively with mouse anti-human acid α-glucosidase antibodies from a sample containing 100 nmol of activity. The precipitate was applied to SDS–PAGE and subsequently blotted onto a nitrocellulose filter. Acid α-glucosidase was visualized with rabbit antibodies using chemiluminescence detection. Human placental acid α-glucosidase (0.1 µg, lane 1) and 5.5 µl of milk of the transgenic mouse (lane 2) were used as markers. The molecular masses of the 110 kDa precursor and the 76 and 70 kDa mature forms of acid α-glucosidase are given in kDa. Immunoglobulins are indicated with an asterisk. Lanes 3–13 show the same panel of tissues as described in (B).

High level expression of the transgene in the mammary gland

The six different transgenic mouse lines were compared by northern blot analysis to select those with the highest transgene expression in the mammary gland. RNA was extracted from the mammary gland of lactating F1 females at day 11 post-partum of their second litter. As a reference, we used mammary gland RNA from a lactating transgenic mouse producing human lactoferrin under control of the bovine α_{S1}-casein promoter and from a lactating non-transgenic cow. The results are shown in Figure 2. The blot was hybridized sequentially with a probe recognizing the 5'-UTR of the bovine α_{S1}-casein gene, identifying both the acid α-glucosidase and the reference mRNAs (Fig. 2A), with a human acid α-glucosidase cDNA probe exclusively recognizing the human acid α-glucosidase messenger (Fig. 2B), and a murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe specific for GAPDH mRNA as a control for gel loading (Fig. 2C). A phosphoimager was used to digitize the hybridization signals. The acid α-glucosidase mRNA level appeared to be lowest in mouse lines 2477 and 2474 and highest in lines 2585 and 2471 (Fig. 2A and B), but in the latter two lines is still significantly lower than the level of human lactoferrin mRNA in the human lactoferrin transgenic mice. There is no strict correlation between the copy number of the transgene and the acid α-glucosidase mRNA steady-state level in the mammary gland (Table 1).

The acid α-glucosidase mRNA expressed in the mammary gland of transgenic mice is translated as shown by western blotting (Fig. 1C, lanes 3 and 4) and immunocytochemistry (Fig. 3). High expression of endogenous β-casein is observed in all epithelial cells in the lactating mammary gland of transgenic (data not shown) and non-transgenic animals (Fig. 3A), while human acid α-glucosidase is not detectable in the latter mammary gland (Fig. 3B). A mosaic expression pattern of the acid α-glucosidase transgene is detected in the mammary gland of mouse 2785 (line 2474) with low mRNA expression (Fig. 3C), whereas intermediate mRNA expression in mouse 4316 (line 2470) leads to staining of all epithelial cells (Fig. 3D).

Secretion of recombinant acid α-glucosidase

The milk of founders, F1 and F2 females was assayed for acid α-glucosidase content by measuring the enzyme activity (Table 1 and Fig. 4) and by western blotting (Fig. 1C, lane 2). Figure 4 illustrates for mouse 3616 (line 2585) that the acid α-glucosidase concentration in the milk is low in the first few days after birth of the litter, but increases from day 7 and reaches an optimum at day 13–14 of the lactation period. This expression curve holds for all mouse lines that were obtained, with slight variations in time of onset and number of days to optimal production. The data are summarized
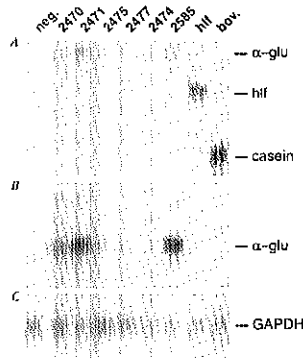
97

Figure 2. Northern blot analysis of human acid α-glucosidase expression in the mammary gland of different transgenic lines. Each lane contains 20 μg RNA. The blot was hybridized with (A) a bovine α_{S1}-casein oligo (18 bp of exon 1), recognizing the bovine α_{S1}-casein 5'-UTR, which is part of the acid α-glucosidase and human lactoferrin transgenes; (B) a human acid α-glucosidase probe; and (C) a GAPDH probe for comparing the quantities of mRNA loaded. The numbers above the lanes refer to the different transgenic mouse lines. neg., non-transgenic mouse (negative control); hlf, mouse transgenic for human lactoferrin. One microgram of bovine mammary gland RNA, mixed with 19 μg of RNA from a non-transgenic mouse, was loaded in the lane marked bov. The positions of human acid α-glucosidase (α-glu), human lactoferrin (hlf), bovine α_{S1}-casein (casein) and GAPDH transcripts are indicated.

in Table 1. The acid α-glucosidase concentration in the milk ranges from 4 μg/ml to 2 mg/ml. The acid α-glucosidase in the milk is predominantly of the 110 kDa precursor type. A minor amount represents 76 kDa mature enzyme (Fig. 1C, lane 2).

Characterization of the recombinant enzyme

The recombinant acid α-glucosidase was purified from transgenic mouse milk by a combination of concanavalin A–Sepharose and G200 Sephadex affinity chromatography as described in Materials and Methods. Two fractions were obtained: one (I) containing the 110 kDa precursor and the other (II) containing the 76 kDa mature form of acid α-glucosidase (Fig. 5). The biochemical and structural features of the recombinant enzyme purified from the milk were compared with those of

natural and recombinant forms of acid α-glucosidase obtained from human placenta and culture medium of genetically engineered CHO cells (Table 2). There are no major differences between the various precursor preparations. The various preparations of the mature enzyme also have very similar characteristics, with respect to pH optimum, K_m and V_{max} values and specific activity. The precursor from both mouse milk and CHO medium typically has a several-fold higher K_m for the natural substrate than do the mature forms from the respective sources.

The two fractions of acid α-glucosidase from murine milk were characterized further with regard to their N-terminal amino acid sequence. The precursor fraction I contains two molecular species in equimolar amounts. One starts at amino acid position 70 counted from the first methionine, the other seven residues further (Table 3). The same starting positions and an additional start at position 68 were found for precursor preparations from CHO cell medium. Mature acid α-glucosidases from mouse milk, human placenta and CHO cell medium all start at amino acid 123, although the latter two enzyme preparations also contain isoforms starting at position 122. With respect to the glycosylation pattern, the acid α-glucosidase species from the different fractions and sources are very similar. Removal of the carbohydrate chains with endoF leads to a comparable mass reduction on SDS–PAGE (Fig. 6).

Therapeutic potential of recombinant acid α-glucosidase; *in vitro* studies with GSDII fibroblasts

Precursor (I) and mature (II) enzyme from milk of transgenic mice was fed to cultured fibroblasts of a patient with GSDII to investigate its therapeutic potential. Fraction I enzyme was internalized in an M6P receptor-dependent manner. The acid α-glucosidase activity in the cells increased from 0.5 to 344 nmol of 4-methylumbelliferyl-α-D-glucopyranoside converted per mg protein/h by addition of enzyme over a period of 48 h. The effect obtained with acid α-glucosidase precursor preparations from human urine and CHO cell medium and with mature acid α-glucosidase isolated from bovine testes was much the same (15,21,28). No increase of activity was obtained in the presence of 5 mM M6P. In contrast to fraction I, fraction II enzyme was not internalized by the fibroblasts. Uptake of recombinant fraction I enzyme is documented further by the molecular mass reduction from 110 kDa to 95 and 76 kDa (Fig. 7A) and most of all by the disappearance of the lysosomal glycogen storage in cells of the patient, as demonstrated by electron microscopy (Fig. 7C).

Table 2. Characteristics of purified forms of acid α-glucosidase

Source	Mouse milk	CHO medium[a]	Mouse milk	CHO medium[a]	Placenta
Isoform (kDa)	110	110	76	76	76/70
Sp. act. (μmol 4-MU/mg/h[b])	369 ± 181 (6)[c]	276 ± 72 (3)[c]	261 ± 57 (3)[c]	351 ± 71 (5)[c]	331 ± 89 (7)[c]
pH optimum, 4-MU substrate	4.5–5.0	4.5–5.0	4.5–5.0	4.5–5.0	4.5–5.0
pH optimum, glycogen substrate	4.0–4.5	4.0–4.5	4.0–4.5	4.0–4.5	4.0–4.5
K_m for 4-MU (mM)	0.63	0.71	0.86	0.57	0.76
K_m for glycogen at pH 4.3 (mg/ml)	484.6	425.3	54.8	43	42.2
V_{max} for 4-MU (nmol/mg/h)	0.21	0.36	0.43	0.38	0.54
V_{max} for glycogen (nmol glucose formed/mg/h)	6.58	9.72	5.25	5.14	6.63

[a]Details about these recombinant forms of human acid α-glucosidase are described by Fuller *et al.* (15).
[b]4-MU, 4-methylumbelliferyl-α-D-glucopyranoside.
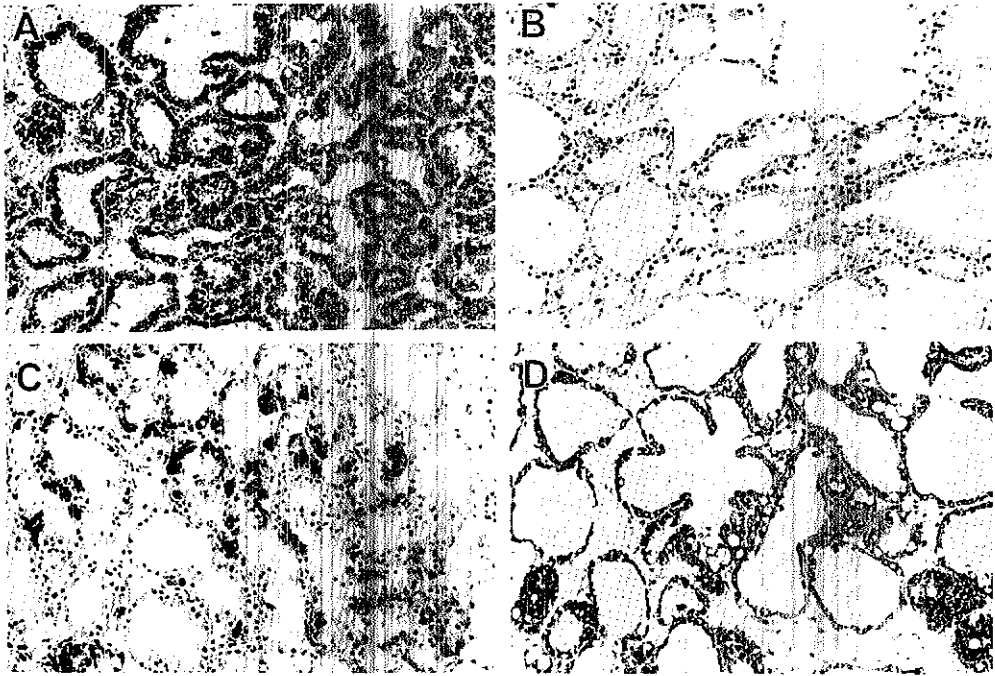[c]The number of different purifications is given in parentheses.

Figure 3. Immunocytochemistry of the lactating mammary gland. (A and B) Sections from a non-transgenic mouse incubated with an antibody against murine β-casein (A) and human acid α-glucosidase (B). (C) A section of a transgenic mouse with low acid α-glucosidase mRNA expression (mouse 2785, line 2474) and (D) with intermediate expression (mouse 4316, line 2470) both incubated with rabbit anti-human acid α-glucosidase antibodies. Immunoperoxidase conjugates were used for staining.

Table 3. N-terminal amino acid sequence of purified forms of acid α-glucosidase

Source	Isoform	N-terminus	
Mouse milk	precursor	77	AVPTQXDV...
		70	AHPGRPRA...
CHO medium	precursor	70	AHPGRPRA...
		77	AVPTQXDV...
		68	AQAHPGRP...
Human urine	precursor[a]	70	AHPGRPRA...
Mouse milk	mature	123	GQPWXFFP...
CHO medium	mature	122	MGQPWXFF...
		123	GQPWXFFP...
Placenta	mature	122	MGQPWXFF...
		123	GQPWXFFP...

[a]The N-terminal sequence of human urine acid α-glucosidase precursor was described by Wisselaar *et al.* (45).

Therapeutic potential of recombinant acid α-glucosidase; *in vivo* studies with GSDII KO mice

We were able to collect enough enzyme from the mouse milk to perform a pilot experiment on the *in vivo* efficacy of the recombinant enzyme in our recently developed KO mouse model of GSDII. The model mimics the severe infantile form of human GSDII in that the acid α-glucosidase activity is fully deficient, and both heart and skeletal muscles are involved. The GSDII KO mice were given a single 100 μg dose of enzyme intravenously and they were sacrificed 2 days later to measure the acid α-glucosidase activity in tissues. Affected and non-affected littermates received injections of phosphate-buffered saline (PBS) as placebo. The results are shown in Table 4. The increase in acid α-glucosidase activity resulting from enzyme administration is highest in liver and spleen, but is also significant in heart, skeletal muscle and other organs. Notably, the activity is not increased in brain and spinal cord of treated animals. Western blot analysis of heart and triceps brachii (Fig. 8A) shows that the injected 110 kDa milk precursor is converted in the tissues of treated animals to mature enzyme of 76 kDa. In this respect, the milk enzyme also behaves the same as the recombinant acid α-glucosidase produced by CHO cells (shown for quadriceps, Fig. 8B).
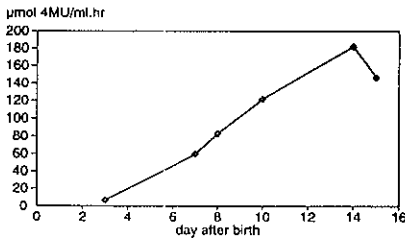
Figure 4. A representative curve of the acid α-glucosidase activity during lactation. The acid α-glucosidase activity in the milk of a transgenic mouse was measured with the artificial substrate 4-methylumbelliferyl-α-D-glucopyranoside during lactation (see Materials and Methods); 1 μmol of activity corresponds to 3.3 μg of enzyme.
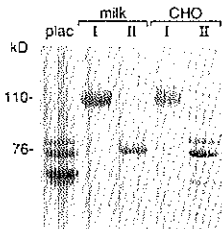
Figure 5. SDS-PAGE analysis of acid α-glucosidase purified from human placenta, milk of transgenic mice and medium of genetically engineered CHO cells. Fraction I contains the 110 kDa precursor and fraction II the 76 kDa mature form. The gel was stained with Coomassie brilliant blue. Plac., human placental enzyme.
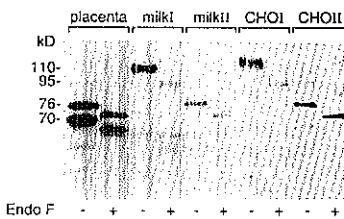
Figure 6. The glycosylation of acid α-glucosidase purified from human placenta, milk of transgenic mice and medium of genetically engineered CHO cells compared by endoF digestion. After endoF (+) or mock (−) treatment, the enzyme preparations were applied to SDS-PAGE and subsequently analysed by western blotting. Acid α-glucosidase was visualized with rabbit antibodies in combination with chemiluminescence staining. Fraction I contains the precursor and fraction II the mature enzyme.
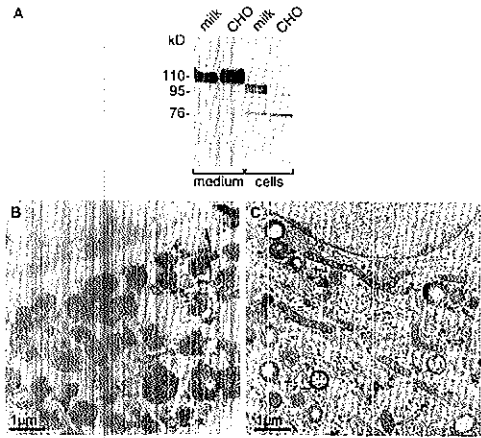
Figure 7. Uptake and processing of recombinant acid α-glucosidase by fibroblasts of a patient with GSDII, and clearance of lysosomal glycogen. (A) Western blot analysis of acid α-glucosidase from treated fibroblasts and culture medium 48 h after enzyme addition. Fraction I enzyme purified from the milk of transgenic mice is compared with fraction I enzyme purified from the medium of genetically engineered CHO cells. The enzyme is visualized with rabbit antibodies against human placental acid α-glucosidase and chemiluminescence staining. (B and C) Electron micrographs of untreated (B) and treated (C) cultured fibroblasts.

DISCUSSION

Receptor-mediated enzyme replacement therapy has proven its value for patients with type 1 Gaucher disease (6,7) and is under development for a number of other lysosomal storage diseases. The M6P receptor is foreseen as the enzyme target in most of these other diseases because of its role in lysosome-directed transport and its widespread tissue distribution (29). The requirement for phosphorylated mannose residues on the therapeutic enzyme limits the choice of enzyme source or biotechnological production system. The glycosylation and subsequent mannose 6-phosphorylation of lysosomal proteins is lacking or inadequate in bacteria, yeast, fungi, baculovirus-infected insect cells and plants (30). Due to the lack of natural sources, production of lysosomal proteins by genetically engineered CHO cells or in the milk of transgenic animals are two remaining practical options. The first production system has been explored broadly, and products have been tested *in vitro* and in animal models (10–12,15–17,24,31–36). The results are promising, but the predicted high production costs are a major concern. Potentially cheaper production in milk of transgenic animals is under development for typical milk and secretory proteins, but has hardly been explored for lysosomal enzymes (37). In our initial attempt to express and produce acid α-glucosidase in the mammary gland of mice, we used the human cDNA linked to the bovine α_{S1}-casein promoter (26). Although we succeeded in providing proof of the principle, the expression levels were low. By linking the entire genomic DNA sequences to the casein
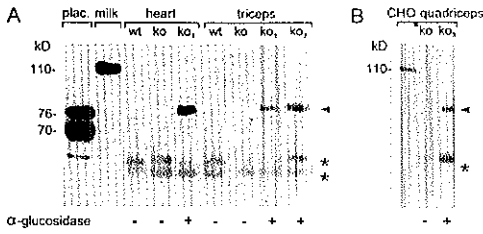
Figure 8. Uptake and processing of human recombinant acid α-glucosidase by heart and skeletal muscle tissue of GSDII mice. Mice were injected intravenously with 100 μg of the 110 kDa human precursor purified from milk of transgenic mice (A) and medium of transfected CHO cells (B). The mice were killed 2 days later with an overdose of anaesthetics and the circulation was perfused with PBS. Homogenates of heart muscle (2 mg of protein) and skeletal muscle (3.5 mg of protein) were analysed for the presence of recombinant human acid α-glucosidase by western blotting after immunoprecipitation as described in Figure 1C, except that rabbit anti-human acid α-glucosidase antibodies were used for precipitation and human specific mouse anti-acid α-glucosidase antibodies for detection on the blot. Enzyme preparations from milk, CHO cell medium and human placenta were loaded as reference samples. Wt, wild-type; KO, GSDII knockout mice that received PBS (–); KO₁, KO₂ and KO₃, GSDII KO mice that received enzyme (+). The arrowhead points to the 76 kDa human mature enzyme. Immunoglobulins are marked with an asterisk.

promoter we have increased the expression levels by 1000-fold. Up to 2 mg acid α-glucosidase/ml were measured in the milk of the highest producers. Other proteins also have an increased yield, when genomic instead of cDNA sequences are used for transgene construction. In general, the expression is highest when the gene locus is left intact and the vector system is designed to operate as an integration site-independent entity (30,38,39). Differences in expression levels of acid α-glucosidase in the six transgenic mouse lines described here point to integration site-dependent transgene expression. To achieve the most optimal production level of acid α-glucosidase, one can experiment by adding insulator elements at both ends of the acid α-glucosidase transgene, which may eliminate effects of the integration site, and remove the transcription/translation inhibitory elements in intron 1 of the gene (40).

The acid α-glucosidase production during lactation follows a curve similar to that of milk proteins such as α_{S1}-casein (41). The transgene expression is not fully mammary gland specific; other tissues also contain human acid α-glucosidase mRNA and protein, but in much lower concentrations than the mammary gland. This leaky expression is not unique, it has been described for other transgenes even for those encoding milk proteins (30). We have little concern that the (ectopic) transgene expression causes any harm to the animals, because acid α-glucosidase is an endogenous household enzyme that is naturally present in all cell types and body fluids. In addition, the overproduced acid α-glucosidase is thought to be secreted at the apical side of the epithelial cells along with the bulk of milk proteins. Ultimately, the transgenic mice look perfectly healthy. They give birth to healthy litters, with Mendelian distribution of the trait, and they have a normal average life span.

Table 4. Uptake of acid α-glucosidase by tissues of GSDII knockout mice

	Acid α-glucosidase activity (nmol 4-MU/mg protein/h)			
	Wild-type placebo	Knockout placebo	Knockout milk α-glucosidase[a]	Knockout CHO α-glucosidase[a]
Liver	49.4	0.8	209.7 (428)	286.6 (580)
Spleen	58.4	0.7	187.5 (321)	249.2 (426)
Kidney	31.3	1.9	6.7 (21)	10.9 (35)
Thymus	27.6	0.5	7.9 (29)	10.5 (38)
Lung	28.7	0.8	5.0 (17)	ND
Heart	21.9	0.5	7.7 (35)	27.8 (127)
Triceps	13.2	0.2	1.6 (12)	3.4 (26)
Quadriceps femoralis	10.3	0.3	3.0 (29)	3.6 (35)
Gastrocnemius	14.8	0.2	2.4 (16)	6.2 (42)
Tongue	17.6	0.3	7.8 (44)	18.6 (106)
Cerebellum	66.0	0.2	1.0 (1.5)	0.6 (0.9)
Cerebrum	51.2	0.2	0.8 (1.5)	0.7 (1.4)
Spinal cord	63.8	ND	1.0 (1.5)	ND

Enzyme activity was assayed, at pH 4.0, 2 days after intravenous injection of acid α-glucosidase or a physiological salt solution (placebo). The endogenous activities in the wild-type mice are the means of three individual mice. The activities in the GSDII knockout mice injected with either placebo or acid α-glucosidase are each from a single animal, except for heart and muscle injected with milk acid α-glucosidase where it concerns the mean activity in two animals.
[a]The figures within parentheses are the activities in the treated GSDII knockout mouse as a percentage of wild-type mice.
ND, not done.

More intriguing is the finding of a mosaic expression pattern of human recombinant acid α-glucosidase in the mammary gland of a relatively low producer (Fig. 3C). The underlying mechanism is unclear. One possible explanation is irreversible inactivation of the transgene, by methylation, rearrangement or excision processes during the developmental stages. Another explanation can be differential activation of milk protein genes in individual mammary gland cells. Similar mosaic expression patterns have been reported for endogenous and transgenically expressed milk proteins in sheep, cattle and mice (42–44). Mice with intermediate and high acid α-glucosidase expression in the milk produce human acid α-glucosidase in all the epithelial cells of the alveoli.

Obviously, the relatively high concentration of recombinant acid α-glucosidase in the milk facilitates extraction and purification. In essence, pure enzyme was obtained in just two purification steps. Of the two fractions obtained, fraction 1 has the same properties as the acid α-glucosidase precursor extractable from human urine and the medium of genetically engineered CHO cells. These properties concern the substrate specificity, the pH optimum, the molecular mass on reducing SDS–PAGE (before and after deglycosylation) and N-terminal amino acid sequence (15,21,45). Similarly, fraction II resembles, in terms of its properties, the 76 kDa mature acid α-glucosidase from human placenta and the 76 kDa fraction of CHO cell medium. Thus, it appears that the post-translational modifications of recombinant human acid α-glucosidase proceed in the mouse mammary gland in the same way as in human fibroblasts, muscle cells and CHO cells (45–47).

The therapeutic potential of the recombinant enzymes produced in milk and by CHO cells was compared *in vitro* and *in vivo*. Only the precursor but not the mature form of acid α-glucosidase from both sources is taken up by cultured fibroblasts of a patient with GSDII. Uptake is in both cases mediated by the M6P receptor and is followed by natural proteolytic conversion of the 110 kDa precursor to 76 kDa mature enzyme. The activity towards the natural substrate increases by this process. As a result, the lysosomal glycogen accumulation is diminished. In all these features, the recombinant enzymes are not different from the natural precursor isolated from human urine (21).

In vivo, the acid α-glucosidase produced in mouse milk gave results similar to those of the enzyme produced by CHO cells. Two days after treatment of GSDII KO mice, the enzyme activity in heart and skeletal muscle samples had increased from <3% to >12% of the wild-type activity. The same proteolytic maturation is observed *in vivo* as *in vitro*, suggesting that the enzyme has entered the cardiomyocytes and skeletal muscle fibres.

Altogether, the production of recombinant human acid α-glucosidase in the mammary gland of transgenic animals seems a good alternative to production by CHO cells because of lower intrinsic costs and similar therapeutic potential. Guided by these positive results, we have started large-scale production of recombinant acid α-glucosidase in the milk of transgenic rabbits.

MATERIALS AND METHODS

Construction of the expression cassette and generation of transgenic mice

The expression cassette (Fig. 1A) was constructed by fusion of the human acid α-glucosidase gene (48) to 6.3 kb of the bovine αS1-casein gene promoter (49). The human acid α-glucosidase gene was cloned by Hoefsloot *et al.* in 1990 (48) and is available

as three *Bgl*II fragments: a 5′ fragment of 10.5 kb with 5 kb upstream sequences plus exons 1–3, an 8.5 kb middle fragment containing exons 4–15, and a 14 kb 3′ fragment containing exons 16–20 plus 9 kb of 3′-flanking sequences. The three fragments were subcloned into the *Bgl*II site of plasmid pKUN1 derivatives with an adapted polylinker (50) and the plasmids were named p10.5αglu, p8.5αglu and p14αglu, respectively. The 5′-UTR was first removed from p10.5αglu by a partial digestion with *Sst*I followed by self-ligation. This resulted in plasmid p5′αgluSstI starting at the *Sst*I site in exon 1. Since the *Not*I site in intron 17 interfered with the cloning strategy, it was removed by digestion of p14αglu with *Not*I and re-ligation after blunting the sticky ends. This adapted 3′ fragment was excised from p14αglu with *Bgl*II and *Bam*HI, and inserted into the (remaining) 3′ *Bgl*II site of p5′αgluSstI, resulting in p5′3′αgluEx1. The orientation of the fragment was checked by restriction enzyme analysis. Subsequently, the middle fragment was excised from p8.5αglu with *Bgl*II and inserted in the de-phosphorylated *Bgl*II site between the 5′ and 3′ α-glucosidase gene fragments of p5′3′αgluEx1, resulting in pαgluEx1. The correct orientation of the fragment was confirmed with enzyme digestions. The acid α-glucosidase gene was excised from the plasmid with *Cla*I and *Not*I double digestion and ligated together with the 6.3 kb *Not*I–*Cla*I bovine αS1-casein promoter fragment into cosmid pWE15, on *Not*I digestion and dephosphorylation, resulting in cosmid c8αgluEx1. The expression cassette was excised from the vector by *Not*I digestion, isolated and microinjected into the pronuclei of fertilized mouse oocytes (CBA/BrA × C57Bl6) using standard procedures (51).

Southern blot hybridization of genomic DNA

DNA was extracted from tail biopsies, digested with *Eco*RI and subjected to agarose gel electrophoresis and Southern blotting to detect the transgene. An *Nsi*I–*Nco*I bovine αS1-casein fragment was used as probe. The copy number of the transgene was determined by Southern blot analysis on digestion with *Sac*I and hybridization with acid α-glucosidase cDNA PCR fragments as probes. The band intensities were compared with those of a serial dilution of plasmid DNA. A phosphoimager with Image Quant program (Molecular Dynamics) was used for quantitation.

Collection and storage of tissue and milk samples

Bovine mammary gland samples were obtained through the local slaughterhouse. Mouse milk was collected from day 7 after birth, unless stated otherwise, with a hand-made milking apparatus and stored at –20°C. Mice were killed by cervical dislocation. Tissue samples were quick-frozen in liquid nitrogen and stored at –20°C for subsequent isolation of RNA and biochemical analyses.

RNA isolation and northern blotting

Total RNA was isolated using the RNA-zol B method (Tel-Test). RNA (20 µg) was separated on a 1% agarose–formaldehyde gel, and transferred to Hybond-N membranes (Amersham). To detect the human acid α-glucosidase mRNA, three PCR fragments covering the human acid α-glucosidase cDNA were used as probe. An oligonucleotide of exon 1 of the bovine αS1-casein gene was used as a probe to detect the acid α-glucosidase and hIf mRNA levels in transgenic mice and the endogenous αS1-casein

mRNA level in cows. A GAPDH probe was used to quantify the total amount of mRNA in the different tissue samples.

Acid α-glucosidase activity and protein assay

Mouse tissues were homogenized in PBS using an ultra turrax (TP 19–10, 20.000 Upm; 170 W; Janke & Kunkel KG). After removal of the large debris at 10 000 g (twice for 15 min), the supernatant was stored at −20°C. The supernatants, crude milk and purified milk fractions were assayed for acid α-glucosidase activity with 4-methylumbelliferyl-α-D-glucopyranoside as substrate at pH 4.3 unless stated otherwise. The hydrolysis of glycogen was measured as described (52). The protein content of the samples was determined using the bicinchoninic acid (BCA) protein assay (Pierce).

SDS–PAGE and western blotting

Acid α-glucosidase was immunoprecipitated from tissue homogenates or cell lysates with mouse or rabbit antibodies raised against human placental acid α-glucosidase complexed to protein A–Sepharose 4B as described (26). The complex was washed, boiled in sample buffer and applied to 8% SDS–PAGE. Acid α-glucosidase was blotted onto nitrocellulose filters and visualized with rabbit or mouse anti-human placental acid α-glucosidase antibodies using the ECL detection kit (Amersham).

Immunocytochemistry

Mammary gland tissue was taken from transgenic and non-transgenic mice at day 11 of the second lactation period. The tissue was embedded in tissue tek (Sakura Fine tek Europe BV, Zoeterwoude, The Netherlands) and frozen in liquid nitrogen. Cryostat sections (6 µm) were incubated with rabbit anti-human placental acid α-glucosidase antiserum in combination with swine anti-rabbit IgG antibodies conjugated to horseradish peroxidase (Dako) and stained with diaminobenzidine (Dako). The sections were counterstained with Gill's haematoxylin.

Acid α-glucosidase purification and characterization

Recombinant human acid α-glucosidase was extracted from milk. The extraction and purification procedure consisted of three steps. Milk fat and casein were removed by centrifugation at 1000 g for 45 min. The whey fraction was applied to concanavalin A–Sepharose 4B. Bound proteins were eluted with 100 mM methyl α-D-mannopyranoside (Sigma) and, after concentration, purified to homogeneity on a Sephadex G200 column (46). The acid α-glucosidase-containing fractions were pooled in such a way that fraction I contained the 110 kDa acid α-glucosidase precursor and fraction II the 76 kDa mature enzyme. These fractions were characterized further. The pH optimum of the recombinant acid α-glucosidase was determined in a buffer system of sodium acetate (0.1 M) and sodium phosphate (0.1 M). The K_m and V_{max} were determined at pH 4.3. N-terminal amino acid sequence analysis was performed as described (15). Samples for endoF digestion (*N*-glycosidase F; Boehringer Mannheim) were brought to pH 7.5 (100 mM sodium phosphate, 10 mM EDTA and 0.1% SDS). After boiling for 5 min, cooling and addition of NP-40 in a final concentration of 0.5% (BDH), 0.2 U of endoF was added and the samples were incubated for 16 h at 37°C, according to the manufacturer's recommendations. The

samples were analysed by SDS–PAGE followed by western blotting as described above.

Addition of recombinant acid α-glucosidase to cultured fibroblasts

Confluent fibroblasts in 35 mm diameter tissue culture dishes were incubated with acid α-glucosidase equivalent to 500 nmol of 4-methylumbelliferyl-α-D-glucopyranoside converted/h/1.5 ml medium. Endocytosis via the M6P receptor pathway was assessed by competing with 5 mM M6P. Media and cells were harvested 48 h later. Cell lysates were prepared by four cycles of freeze–thawing of cell pellets in 150 µl of PBS. Both cell lysates and media were assayed for acid α-glucosidase activity and analysed by western blotting.

Enzyme administration

Fraction I (110 kDa acid α-glucosidase) was sterilized through microfiltration and administered intravenously via the tail vein to 8- and 16-week-old GSDII KO mice in a single dosage of 100 µg per 130 µl PBS. Control animals received 130 µl PBS. Two days after enzyme administration, the mice were anaesthetized, and the circulation was perfused with PBS. Tissues were collected and processed as described above.

REFERENCES

1. Hers, H.G. and Van Hoof, F. (1973) *Lysosomes and Storage Diseases.* Academic Press, New York.
2. Hirschhorn, R. (1995) Glycogen storage disease type II: acid α-glucosidase (acid maltase) deficiency. In Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. (eds), *The Metabolic and Molecular Bases of Inherited Disease,* Vol. 1. McGraw-Hill, pp. 2443–2464.
3. Baudhuin, P., Hers, H.G. and Loeb, H. (1964) An electron microscopic and biochemical study of type II glycogenosis. *Lab. Invest.,* 13, 1139–1152.
4. Desnick, R.J. (1980) *Enzyme Therapy in Genetic Diseases: 2.* Alan R. Liss, New York.
5. Tager, J.M., Hooghwinkel, G.J.M. and Daems, W.T. (1974) *Enzyme Therapy in Lysosomal Storage Diseases.* North Holland Publishing, Amsterdam.
6. Barton, N.W., Furbish, F.S., Murray, G.J., Garfield, M. and Brady, R.O. (1990) Therapeutic response to intravenous infusions of glucocerebrosidase in a patient with Gaucher disease. *Proc. Natl Acad. Sci. USA,* 87, 1913–1916.
7. Barton, N.W., Brady, R.O., Dambrosia, J.M., Di Bisceglie, A.M., Doppelt, S.H., Hill, S.C., Mankin, H.J., Murray, G.J., Parker, R.I., Argoff, C.E., Grewal, R.P., Yu, K.T. *et al.* (1991) Replacement therapy for inherited enzyme deficiency. Macrophage-targeted glucocerebrosidase for Gaucher's disease. *N. Engl. J. Med.,* 324, 1464–1470.
8. Scott, H.S., Anson, D.S., Orsborn, A.M., Nelson, P.V., Clements, P.R., Morris, C.P. and Hopwood, J.J. (1991) Human α-L-iduronidase: cDNA isolation and expression. *Proc. Natl Acad. Sci. USA,* 88, 9695–9699.
9. Anson, D.S., Taylor, J.A., Bielicki, J., Harper, G.S., Peters, C., Gibson, G.J. and Hopwood, J.J. (1992) Correction of human mucopolysaccharidosis type-VI fibroblasts with recombinant *N*-acetylgalactosamine-4-sulphatase. *Biochem. J.,* 284, 789–794.

10. Bielicki, J., Hopwood, J.J., Wilson, P.J. and Anson, D.S. (1993) Recombinant human iduronate-2-sulphatase: correction of mucopolysaccharidosis-type II fibroblasts and characterization of the purified enzyme. *Biochem. J.*, 289, 241–246.

11. Bielicki, J., Fuller, M., Guo, X.H., Morris, C.P., Hopwood, J.J. and Anson, D.S. (1995) Expression, purification and characterisation of recombinant human *N*-acetylgalactosamine-6-sulphatase. *Biochem. J.*, 311, 333–339.

12. Bielicki, J., Hopwood, J.J., Melville, E.L. and Anson, D.S. (1998) Recombinant human sulphamidase: expression, amplification, purification and characterization. *Biochem. J.*, 329, 145–150.

13. Unger, E.G., Durrant, J., Anson, D.S. and Hopwood, J.J. (1994) Recombinant α-L-iduronidase: characterization of the purified enzyme and correction of mucopolysaccharidosis type I fibroblasts. *Biochem. J.*, 304, 43–49.

14. Kakkis, E.D., Matynia, A., Jonas, A.J. and Neufeld, E.F. (1994) Overexpression of the human lysosomal enzyme α-L-iduronidase in Chinese hamster ovary cells. *Protein Express. Purif.*, 5, 225–232.

15. Fuller, M., Van der Ploeg, A., Reuser, A.J.J., Anson, D.S. and Hopwood, J.J. (1995) Isolation and characterisation of a recombinant, precursor form of lysosomal acid alpha-glucosidase. *Eur. J. Biochem.*, 234, 903–909.

16. Weber, B., Blanch, L., Clements, P.R., Scott, H.S. and Hopwood, J.J. (1996) Cloning and expression of the gene involved in Sanfilippo B syndrome (mucopolysaccharidosis III B). *Hum. Mol. Genet.*, 5, 771–777.

17. Van Hove, J.L.K., Yang, H.W., Wu, J.Y., Brady, R.O. and Chen, Y.T. (1996) High-level production of recombinant human lysosomal acid α-glucosidase in Chinese hamster ovary cells which targets to heart muscle and corrects glycogen accumulation in fibroblasts from patients with Pompe disease. *Proc. Natl Acad. Sci. USA*, 93, 65–70.

18. Litjens, T., Bielicki, J., Anson, D.S., Friderici, K., Jones, M.Z. and Hopwood, J.J. (1997) Expression, purification and characterization of recombinant caprine *N*-acetylglucosamine-6-sulphatase. *Biochem. J.*, 327, 89–94.

19. Salminen, A. and Marjomaki, V. (1985) Phosphomannosyl receptors of lysosomal enzymes in cardiac and skeletal muscles of young and old mice. *Comp. Biochem. Physiol.*, 82, 259–262.

20. Taylor, J.E., Scott, C.D. and Baxter, R.C. (1985) Comparison of receptors for insulin-like growth factor II from various rat tissues. *J. Endocrinol.*, 115, 35–41.

21. Oude Elferink, R.P.J., Brouwer-Kelder, E.M., Surya, I., Strijland, A., Kroos, M., Reuser, A.J.J. and Tager, J.M. (1984) Isolation and characterization of a precursor form of lysosomal α-glucosidase from human urine. *Eur. J. Biochem.*, 139, 489–495.

22. Van der Ploeg, A.T., Kroos, M., van Dongen, J.M., Visser, W.J., Bolhuis, P.A., Loonen, M.C. and Reuser, A.J.J. (1987) Breakdown of lysosomal glycogen in cultured fibroblasts from glycogenosis type II patients after uptake of acid alpha-glucosidase. *J. Neurol. Sci.*, 79, 327–336.

23. Van der Ploeg, A.T., Loonen, M.C.B., Bolhuis, P.A., Busch, H.M.F., Reuser, A.J.J. and Galjaard. H. (1988) Receptor-mediated uptake of acid α-glucosidase corrects lysosomal glycogen storage in cultured skeletal muscle. *Pediatr. Res.*, 24, 90–94.

24. Kikuchi, T., Yang, H.W., Pennybacker, M., Ichihara, N., Mizutani, M., Van Hove, J.L.K. and Chen, Y.T. (1998) Clinical and metabolic correction of Pompe disease by enzyme therapy in acid maltase-deficient quail. *J. Clin. Invest.*, 101, 827–833.

25. Lee, S.H. and De Boer, H.A. (1994) Production of biomedical proteins in the milk of transgenic dairy cows: the state of the art. *J. Control. Release*, 29, 213–221.

26. Bijvoet, A.G.A., Kroos, M.A., Pieper, F.R., de Boer, H.A., Reuser, A.J.J., van der Ploeg, A.T. and Verbeet, M.P. (1996) Expression of cDNA-encoded human acid α-glucosidase in milk of transgenic mice. *Biochim. Biophys. Acta*, 1308, 93–96.

27. Bijvoet, A.G.A., Van de Kamp, E.H.M., Kroos, M.A., Ding, J.H., Yang, B.Z., Visser, P., Bakker, C.E., Verbeet, M.P., Oostra, B., Reuser, A.J.J. and Van der Ploeg, A.T. (1998) Generalized glycogen storage and cardiomegaly in a knockout mouse model of Pompe disease. *Hum. Mol. Genet.*, 7, 53–62.

28. Reuser, A.J., Kroos, M.A., Ponne, N.J., Wolterman, R.A., Loonen, M.C., Busch, H.F., Visser, W.J. and Bolhuis, P.A. (1984) Uptake and stability of human and bovine acid alpha-glucosidase in cultured fibroblasts and skeletal muscle cells from glycogenosis type II patients. *Exp. Cell Res.*, 155, 178–189.

29. Kaplan, A., Achord, D.T. and Sly, W.S. (1977) Phosphohexosyl components of a lysosomal enzyme are recognized by pinocytosis receptors on human fibroblasts. *Proc. Natl Acad. Sci. USA*, 74, 2026–2030.

30. Houdebine, L.M. (1994) Production of pharmaceutical proteins from transgenic animals. *J. Biotechnol.*, 34, 269–287.

31. Sands, M.S., Vogler, C., Kyle, J.W., Grubb, J.H., Levy, B., Galvin, N., Sly, W.S. and Birkenmeier, E.H. (1994) Enzyme replacement therapy for murine mucopolysaccharidosis type VII. *J. Clin. Invest.*, 93, 2324–2331.

32. Shull, R.M., Kakkis, E.D., McEntee, M.F., Kania, S.A., Jonas, A.J. and Neufeld, E.F. (1994) Enzyme replacement in a canine model of Hurler syndrome. *Proc. Natl Acad. Sci. USA*, 91, 12937–12941.

33. Enomaa, N., Danos, O., Peltonen, L. and Jalanko, A. (1995) Correction of deficient enzyme activity in a lysosomal storage disease, aspartylglucosaminuria, by enzyme replacement and retroviral gene transfer. *Hum. Gene Ther.*, 6, 723–731.

34. Kakkis, E.D., McEntee, M.F., Schmidtchen, A., Neufeld, E.F., Ward, D.A., Gompf, R.E., Kania, S., Bedollia, C., Chien, S.L. and Shull, R.M. (1996) Long-term and high-dose trials of enzyme replacement therapy in the canine model of mucopolysaccharidosis I. *Biochem. Mol. Med.*, 58, 156–167.

35. Crawley, A.C., Brooks, D.A., Muller, V.J., Petersen, B.A., Isaac, E.L., Bielicki, J., King, B.M., Boulter, C.D., Moore, A.J., Fazzalari, N.L., Anson, D.S., Byers, S. and Hopwood, J.J. (1996) Enzyme replacement therapy in a feline model of Maroteaux--Lamy syndrome. *J. Clin. Invest.*, 97, 1864–1873.

36. Ioannou, Y.A., Zeidner, K.M., Friedman, B. and Desnick, R.J. (1996) Fabry disease: enzyme replacement therapy in α-galactosidase A deficient mice. *Am. J. Hum. Genet.*, 59, A15.

37. Wall, R.J., Martin, B.M., Stubblefield, B., Eliason, W., Pursel, V.G., Henninghausen, L., Ginns, E.I. and Sidransky, E. (1994) The production of human glucocerebrosidase in the milk of lactating mammals. *Am. J. Hum. Genet.*, 55, A179.

38. Brinster, R.L., Allen, J.M., Behringer, R.R., Gelinas, R.E. and Palmiter, R.D. (1988) Introns increase transcriptional efficiency in transgenic mice. *Proc. Natl Acad. Sci. USA*, 85, 836–840.

39. Palmiter, R.D., Norstedt, G., Gelinas, R.E., Hammer, R.E. and Brinster, R.L. (1991) Heterologous introns can enhance expression of transgenes in mice. *Proc. Natl Acad. Sci. USA*, 88, 478–482.

40. Raben, N., Nichols, R.C., Martiniuk, F. and Plotz, P.H. (1996) A model of mRNA splicing in adult lysosomal storage disease (glycogenosis type II). *Hum. Mol. Genet.*, 5, 995–1000.

41. Rijnkels, M., Kooiman, P.M., Platenburg, G.J., Van Dixhoorn, M., Nuijens, J.H., De Boer, H.A. and Pieper, F.R. (1995) High-level expression of the bovine αs1-casein in milk of transgenic mice. *Transgenic Res.*, 6, 1–10.

42. Molenaar, A.J., Davis, S.R. and Wilkins, R.J. (1992) Expression of α-lactalbumin, αs1-casein, and lactoferrin genes is heterogeneous in sheep and cattle mammary tissue. *J. Histochem. Cytochem.*, 40, 611–618.

43. Faerman, A., Barash, I., Puzis, R., Nathan, M., Hurwitz, D.R. and Shani, M. (1995) Dramatic heterogeneity of transgene expression in the mammary gland of lactating mice: a model system to study the synthetic activity of mammary epithelial cells. *J. Histochem. Cytochem.*, 43, 461–470.

44. Dobie, K.W., Lee, M., Fantes, J.A., Graham, E., Clark, A.J., Springbett, A., Lathe, R. and McClenaghan, M. (1996) Variegated transgene expression in mouse mammary gland is determined by the transgene integration locus. *Proc. Natl Acad. Sci. USA*, 93, 6659–6664.

45. Wisselaar, H.A., Kroos, M.A., Hermans, M.M.P., van Beeumen, J. and Reuser, A.J.J. (1993) Structural and functional changes of lysosomal acid α-glucosidase during intracellular transport and maturation. *J. Biol. Chem.*, 268, 2223–2231.

46. Reuser, A.J.J., Kroos, M., Oude Elferink, R.P.J. and Tager, J.M. (1985) Defects in synthesis, phosphorylation, and maturation of acid α-glucosidase in glycogenosis type II. *J. Biol. Chem.*, 260, 8336–8341.

47. Willemsen, R., Van der Ploeg, A.T., Busch, H.F.M., Zondervan, P.E., Van Noorden, C.J.F. and Reuser, A.J.J. (1993) Synthesis and *in situ* localization of lysosomal α-glucosidase in muscle of an unusual variant of glycogen storage disease type II. *Ultrastruct. Pathol.*, 17, 515–527.

48. Hoefsloot, L.H., Hoogeveen-Westerveld, M., Reuser, A.J.J. and Oostra, B.A. (1990) Characterization of the human lysosomal alpha-glucosidase gene. *Biochem. J.*, 272, 493–497.

49. Platenburg, G.J., Kootwijk, E.P.A., Kooiman, P.M., Woloshuk, S.L., Nuijens, J.H., Krimpenfort, P.J.A., Pieper, F.R., de Boer, H.A. and Strijker, R. (1994) Expression of human lactoferrin in milk of transgenic mice. *Transgenic Res.*, 3, 99–108.

50. Konings, R.N., Luiten, R.G. and Peeters, B.P. (1986) Mike, a chimeric filamentous phage designed for the separate production of either DNA strand of pKUN vector plasmids by F+ cells. *Gene*, 46, 269–276.

51. Hogan, B., Beddington, R., Costantini, F. and Lacy, E. (1994) *Manipulating the Mouse Embryo: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

52. Reuser, A.J.J., Koster, J.F., Hoogeveen, A. and Galjaard, H. (1978) Biochemical, immunological, and cell genetic studies in glycogenosis type II. *Am. J. Hum. Genet.*, 30, 132–143.

Chapter 7

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

(Submitted)

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

Agnes G.A. Bijvoet, Hans van Hirtum, Marian A. Kroos, Esther H.M. van de Kamp, Onard Schoneveld, Pim Visser, Just P.J. Brakenhoff, Miranda Weggeman, Emile J. van Corven, Ans T. Van der Ploeg, and Arnold J.J. Reuser.

Glycogen storage disease type II (GSDII) is a member of the family of inheritable lysosomal storage diseases. The underlying deficiency of acid α-glucosidase leads to glycogen storage in heart-, skeletal- and smooth muscle in different degrees of severity. The clinical spectrum covers infants, children and adults, who all have a progressive loss of skeletal muscle function[1]. Affected infants have generalized glycogen storage and cardiomegaly as conspicuous additional symptom. There is currently no treatment for this fatal disease[2], which is similarly manifested in naturally occurring animal models and in knockout mice[3-5]. For testing the feasibility of enzyme replacement therapy (ERT), we have started large-scale production of recombinant human acid α-glucosidase (rhGAA) in the milk of transgenic rabbits and now demonstrate the therapeutic effect of this enzyme in our knockout mouse model. Full correction of acid α-glucosidase deficiency was obtained in all tissues except brain after intravenous enzyme administration. Prolonged treatment of mice resulted in degradation of lysosomal glycogen in heart-, skeletal-, and smooth muscle and improved tissue morphology, despite an advanced state of disease at the start of treatment. Hereby, we have demonstrated the feasibility of pharmaceutical protein production in milk of transgenic rabbits and obtained a solid basis for an imminent start of a Phase II clinical trial in patients with GSDII, after the recent completion of a Phase I study in healthy volunteers.

Soon after the discovery of acid α-glucosidase deficiency in GSDII[6], it was speculated that lysosomal storage diseases could be treated by supplementing the missing enzyme. However, after more than 10 years of intensive investigations and unsuccessful clinical try-outs, the idea of enzyme replacement therapy (ERT) was virtually abandoned. In retrospect, the disappointing results can be largely ascribed to administration of low enzyme doses from unfavorable sources and specificity with respect to immune-tolerance and cellular targeting. At present, ERT for lysosomal storage diseases is again vividly pursued, mainly stimulated by the clinical application of receptor mediated ERT for Gaucher disease and promising outcome of exploratory studies for other lysosomal diseases including GSDII[7-14]. Cell culture systems and transgenic animal technology were investigated for large-scale production of acid α-glucosidase as medicine for GSDII[10,13,15,16]. Based on qualitatively and quantitatively favorable results with production of rhGAA in milk of transgenic mice, we have chosen for transgenic rabbits to realize in limited time span the industrial enzyme production. For targeting α-glucosidase expression to the milk of rabbits, the same bovine αS1-casein/human acid α-glucosidase gene-expression cassette was used as previously tested in mice[13]. Transgenic rabbits of the selected

strain produce up to 8 gram rhGAA per liter milk. The purified rhGAA has an apparent molecular mass of 110 kD and thereby compares to precursor acid α-glucosidase from human urine[17] (Fig. 1a).

A series of experiments was performed to assess the therapeutic efficacy of the purified rhGAA in acid α-glucosidase deficient knockout (KO) mice. A single enzyme dose (17 mg/kg) resulted, two days after administration, in normalization of acid α-glucosidase activity in all tissues except brain. In another initial test, one C57/Bl6 and two FVB KO mice received, with a six day interval, four rhGAA injections of 40-68 mg/kg each (three untreated KO mice served as negative control and 3 normal mice were included to assess normal acid α-glucosidase activity levels). The acid α-glucosidase activity in liver of treated mice increased to 20-60 times that of normal mice. The levels in heart and skeletal muscle increased to 3 times normal. Not the administered 110 kD precursor, but the 76 kD mature form of acid α-glucosidase was recovered from the tissues (Fig. 1b). This provides evidence for uptake by lysosomes in which the 76 kD species is formed as natural result of post-translational modification. Localization of the enzyme in lysosomes was further demonstrated in hepatocytes by visualization with immuno-electron microscopy (Fig. 1c). Uptake of enzyme by liver and heart was accompanied by reduction of the glycogen content, but no such effect was seen in skeletal muscle (data not shown).

Fig. 1 *rhGAA: SDS-PAGE analysis of purified enzyme, conversion to mature enzyme in recipient mouse tissues. lysosomal localization and acid α-glucosidase activity and glycogen content of mouse tissues after 25 weeks of treatment with rhGAA. a, Purified rhGAA (32 μgram), stained with Coomassie brilliant blue, appears on a non-reduced 4-12 % gradient gel as a single molecular species of 110 kD, known as the acid α-glucosidase precursor (the 220 kD doublet results from aggregation). b, Conversion of the 110 kD precursor to mature 76 kD 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 immuno-precipitation 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 immuno-electron microscopy in 60 nm lowicryl sections, using the same rabbit antibodies as described under b. N = nucleus, C = bile canaliculus.*
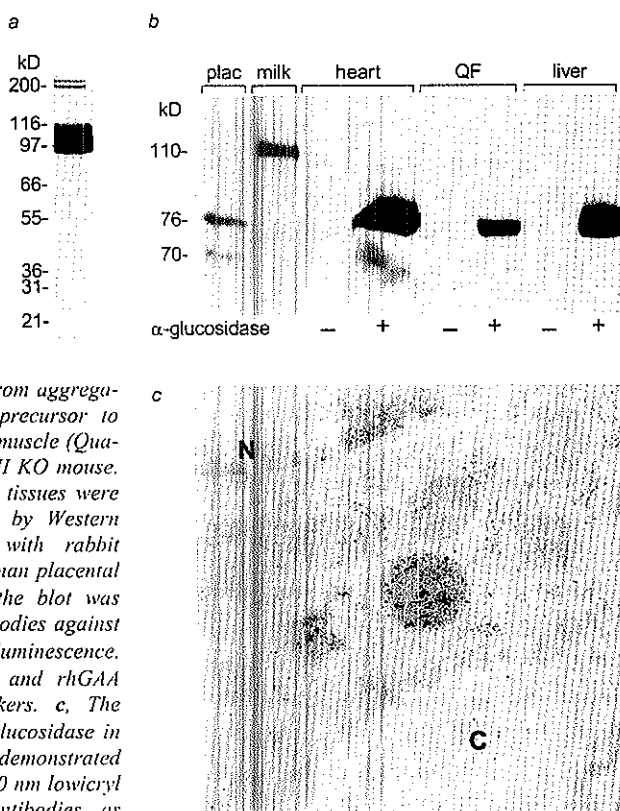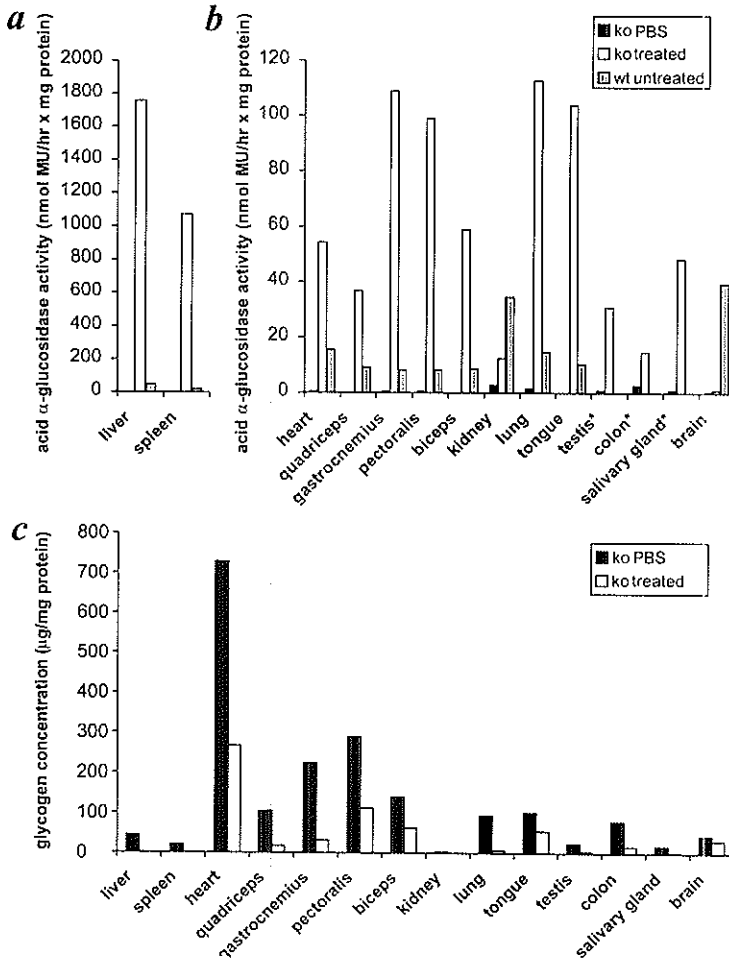
Fig. 2. a, b, The acid α-glucosidase activity was determined with the artificial substrate 4-methylum-belliferyl-α-D-glucopyranoside (MU) in liver and spleen (a) and other tissues (b). c, The glycogen content was measured as described in materials and methods. Wild type levels are between 0 and 2 μg/mg protein (not shown). Data represent the average of two mice in each group. *: Wild type levels of testis, colon and salivary gland were not determined.

Encouraged by these results we decided to subject six months old mice with advanced tissue pathology to prolonged treatment (14 to 25 weeks). The protocol included 16 (12 FVB and 4 C57/Bl6) 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. All treated FVB mice had developed an antibody titer (approximately one fifth of injected enzyme was immuno-precipitable per calculated total serum volume). No titer was measured in serum of the C57/Bl6 mice. Three enzyme- and three placebo-treated FVB
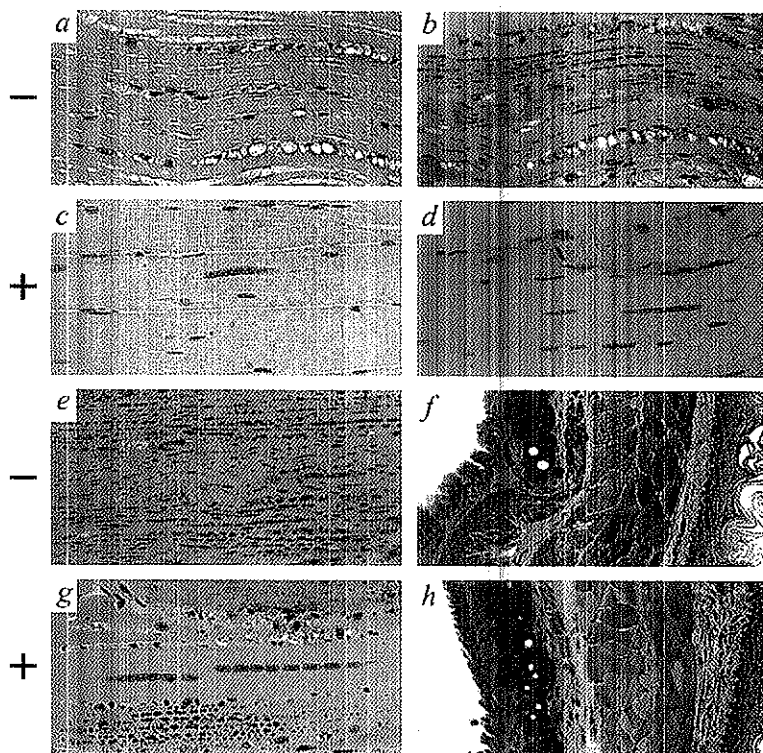
Fig. 3 *Correction of glycogen storage and improvement skeletal muscle morphology of KO mice after prolonged treatment with rhGAA. Panels a, b, c, d, gastrocnemius, e, g, pectoralis, and f, h, esophagus and trachea. Sections a and c were stained with haematoxylin azofloxin, all others were stained with periodic acid-Schiff (PAS) to demonstrate glycogen storage. Sections a, b, e, f are from placebo treated mice (-), and sections c, d, g, h, from rhGAA treated mice (+).*

mice were killed at this stage to evaluate the therapeutic effect of enzyme administration. The acid α-glucosidase activity in liver had increased (120 times normal), and the liver glycogen content had normalized (2.5 µg/mg protein in treated versus 24 µg/mg in untreated mice). Only one of the three treated mice showed increased acid α-glucosidase activity in heart and skeletal muscle, but this was not accompanied by a lowering of the glycogen content in these tissues. The disappointing results could relate to the antibody titers.

To overcome the immunologic response of FVB mice and to secure successful completion of the experiment, the dose was raised four fold for both FVB as well as C57/Bl6 mice that did not have an antibody titer. Analysis of the three FVB mice after the 4th high dose injection (18 injections in total) revealed that acid α-glucosidase activity had increased up to sixty times normal in liver, four times normal in heart, and five times normal in skeletal muscle. The glycogen store in liver was depleted. The average reduction of the glycogen content was 39 % for heart (288 µg/mg treated versus 470 µg/mg untreated) and 10 % for skeletal muscle (125 µg/mg treated versus 140 µg/mg untreated).
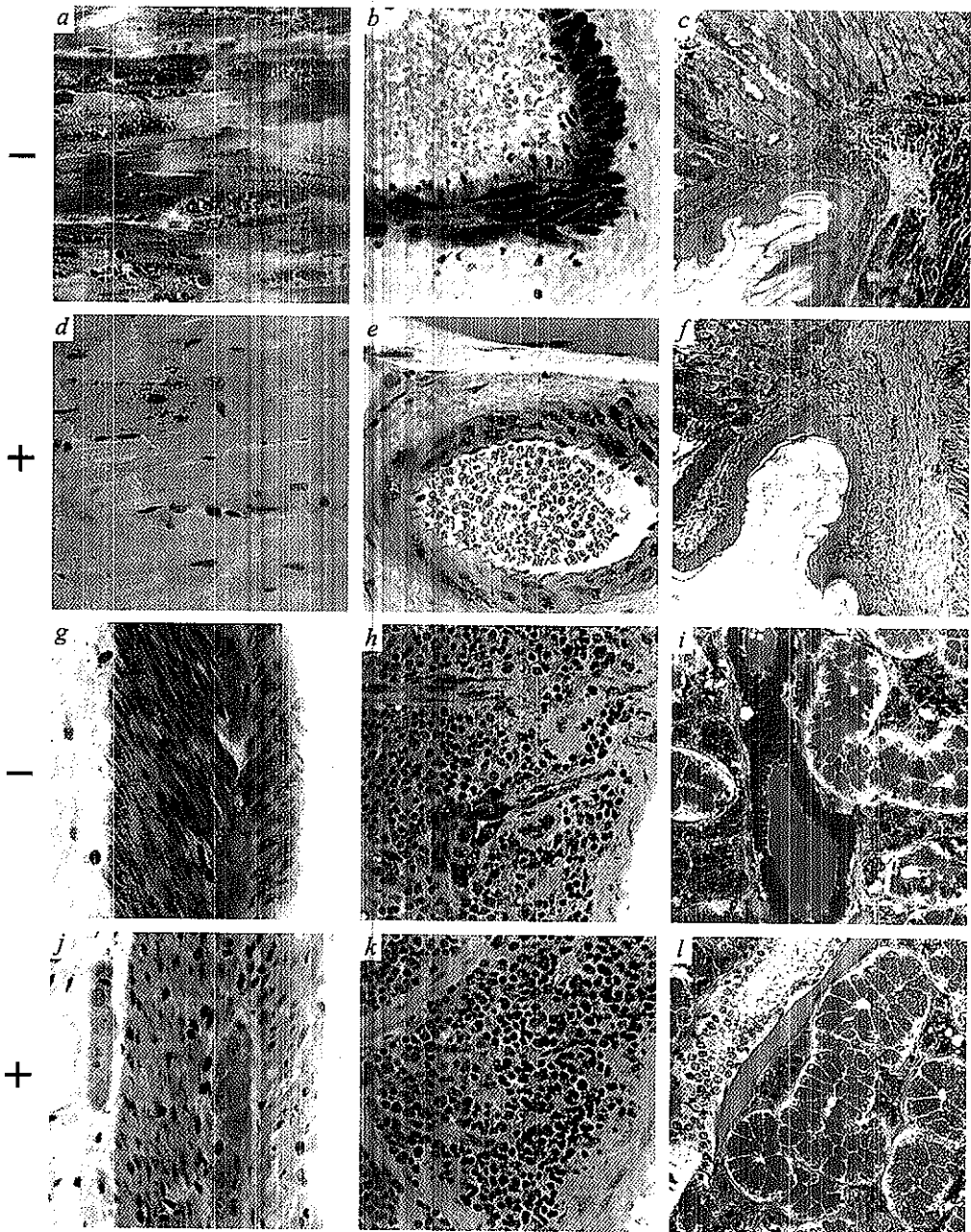
Fig. 4 *Correction of glycogen storage and improved morphology of heart, smooth muscle and salivary glands of KO mice after prolonged treatment with rhGAA. Panels a, d, heart, b, e, muscular artery, c, f, stomach, g, j, colon, h, k, spleen and i, l, salivary gland. The sections were stained with PAS. Sections a-c, g-i are from placebo treated mice (-), and sections d-f, j-l from rhGAA treated mice (+).*

Two C57/Bl6 KO mice continued to receive weekly doses of 68 mg/kg till week 25, despite the detection of a low antibody titer after the 20th injection (approximately 5 % of injected enzyme was immuno-precipitable 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, except for kidney (36 % of normal) and brain (2 % of normal) (Fig. 2*a* and *b*). Importantly, the glycogen content of these tissues, except brain, had either reversed to normal or was significantly reduced when compared with the values measured in placebo-treated mice (Fig. 2*c*). Comparison of tissue sections of treated and untreated animals provided further and convincing evidence for the effectiveness of enzyme replacement therapy. This is illustrated in Fig. 3 (skeletal muscle), Fig. 4 (heart, smooth muscle and epithelial cells of ducts of salivary gland) and Fig. 5 (electron microscopy of heart muscle). Clearance of glycogen containing vacuoles was observed in almost all muscle fibers of the gastrocnemius (Fig. 3*a,b* compared with *c,d*), the quadriceps femoralis (not shown) and the longitudinal and circular skeletal muscle layers around the esophagus (Fig. 3*f, 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. 3*c, d* and *g*). The pectoralis major showed partial correction with intra and inter-fiber segmental variation (Fig. 3*g* compared with *e*). The correction of cardiomyocytes was impressive in some areas of the heart (Fig. 4*a* and *d* and Fig. 5), but cells staining with periodic acid-Schiff (PAS) were still present in other areas. Also, smooth muscle cells of arteries and veins, a prominent site of glycogen storage in GSDII, had lost most of lysosomal glycogen, but were not in all instances fully corrected (Fig. 4*b* and *e*). Smooth muscle of the bladder showed little response as judged by the intensity and spreading of PAS staining, but degradation of lysosomal glycogen became evident using electron microscopy (not shown). In contrast, smooth muscle of the stomach and digestive tract responded very well to enzyme administration with dramatic reduction of PAS staining (Fig. 4*c, f, g* and *j*). A summary of the corrective effect of enzyme infusion is given in Table 1.

Based on these results, speculations can be made as to the prospects of enzyme replacement therapy in humans using rhGAA from rabbit milk. It is reassuring that this recombinant enzyme is well tolerated over a relatively long period. The intravenously infused enzyme reaches and corrects the tissues most directly involved in symptomatology, which are heart-, skeletal- and smooth muscle, despite 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]. Although neurologic symptoms have not been documented in this disease, the inability to correct glycogen accumulation in the CNS is a point of concern, when ERT would extend the life of affected infants. Mental and motor developmental milestones need to be monitored cautiously. Another potential complication of ERT is the formation of neutralizing antibodies, as suggested by the different results obtained with FVB and C57/Bl6 mice. Antibody formation is expected to be less of a risk in juvenile and adult GSDII patients, who have residual enzyme activity, than in infants without catalytically active enzyme.

In conclusion, large-scale production of rhGAA was accomplished in the milk of transgenic rabbits, and the enzyme is of therapeutic quality when tested in KO mice. While these studies were in progress a Phase I clinical trial, assessing the safety, tolerance and pharmacokinetics of

rhGAA in healthy volunteers, was completed. These results together set the stage to conduct a Phase II clinical trial on the safety and potential efficacy of this recombinant enzyme in patients with GSDII.
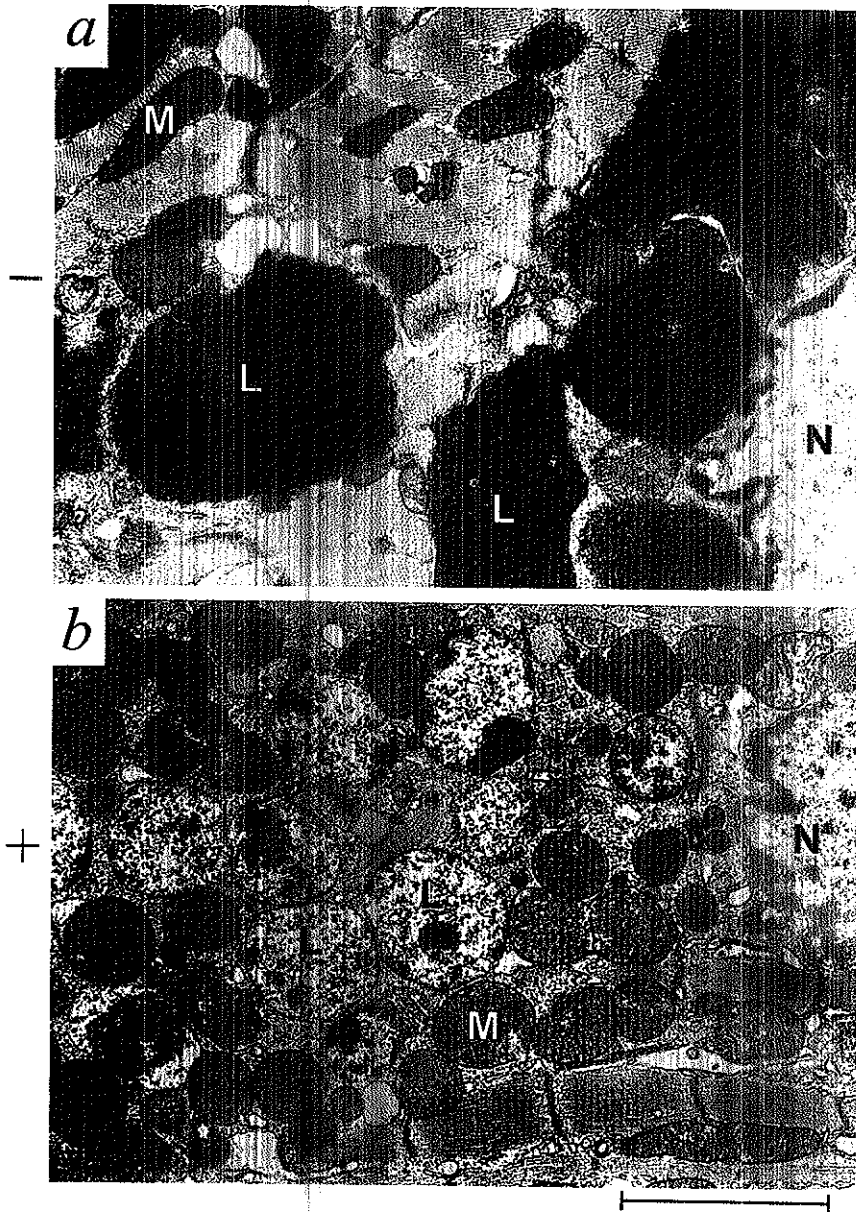

*Fig. 5 Electron microscopy showing degradation of lysosomal glycogen in heart muscle of KO mice after prolonged treatment with rhGAA. **a**, placebo treated (-), **b**, treated with rhGAA (+). N: nucleus, L: lysosome, M: mitochondria . (bar = 2 μm)*

Table 1. Improvement of tissue morphology after prolonged enzyme treatment, as judged by light-(LM) and electron microscopy (EM)[a]

Skeletal muscle	LM	EM	Other tissues	LM	EM
quadriceps	+++		liver	+++	+++
gastrocnemius	++++		spleen	++++	
pectoralis	+	+	kidney	++++	
triceps	+		salivary gland	++++	
diaphragm	+	++	epidydimus	+++	
tongue	+		plexi of Meissner		
esophagus	+++		and Auerbach	+++	
Heart and smooth muscle			cartilage	-	
heart	++	++	adipose tissue	+++	
vessels[a]	++ /-	+++/+	brain	-	
lung	++				
stomach	++++				
ileum	+++				
jejunum	++++				
duodenum	+++				
colon	+++				
rectum	++++				
bladder	-	+			

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

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[4]. The mice were backcrossed for two generations in either FVB or C57/Bl6 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[13]. 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 according to Van Corven et al. (manuscript in preparation). 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), 2 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 immuno-precipitated from tissue homogenates with rabbit antibodies raised against human placental acid α-glucosidase complexed to protein A-Sepharose 4B as described[16]. The complex was washed, boiled in sample buffer and applied to 8 % SDS-PAGE. Acid α-glucosidase was blotted onto nitro-cellulose filters and visualized with mouse anti human placental acid α-glucosidase antibodies using the ECL detection kit (Amersham).

Treatment protocol. rhGAA from rabbit milk was brought to a final concentration of 2.5 mg/ml (low dose) and 10 mg/ml (high dose) in phosphate buffered saline (PBS), filter sterilized, and injected in 200 µl volume in the tail vein. Single dose injections of enzyme were given to three FVB female KO mice of three months old. Three other KO females received PBS as placebo. Two groups of mice were used for short term treatment: three, 3 months old, KO females with FVB background in the first group, and two, 8 months, old KO littermates with C57/Bl6 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 with a six-day interval. An intra peritoneal injection of 0.05 mg/kg clemastinum (Tavegil®; Sandoz Pharma AG, Bazel, Swiss) was given thirty minutes before the last enzyme infusion because an anaphylactic reaction occurred shortly after the third injection. Mice were fasted 16 hrs before killing to deplete non-lysosomal glycogen. Tissues were collected after perfusion with PBS.

For prolonged treatment, 12 KO females with FVB background and 4 KO males with C57/Bl6 background, all 6 months of age, were included. Weekly injections were given. Eight mice (6 females and 2 males) received enzyme, the other eight (6 females and 2 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), and the last 11 again high dose (68 mg/kg). Intraperitoneal injections with clemastinum were given from the third week on, 30 minutes before enzyme/placebo administration, to prevent anaphylactic shock. Blood samples were taken in week 13, 16, 18 and 21. Six FVB mice were killed on day two after the 13th injection and six more after the 18th injection. The four C57/Bl6 mice were killed two days after the 25th injection. Mice were fasted 16 hrs 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 (4MU) as substrate, at pH 4.3 as described[4]. 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-amylase and α-amyloglucosidase. The amount of liberated glucose was determined with the glucose-oxidase method[18].

Histology. For light microscopy, tissues were fixed with 4 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) and embedded in glycol methacrylate (GMA) according to standard procedures. Sections of 4 µm were stained with periodic acid-Schiff (PAS) reagent and haematoxylin 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[19] and embedded in epon. For immuno-electron microscopy tissues were fixed with 1 % acroleine and 0.4 % glutaraldehyde in 0.1 M cacodylate buffer, and embedded in Lowicryl. Ultrathin sections were immuno-stained 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[20].

References

1. Engel, A.G., Gomez, M.R., Seybold, M.E. & Lambert, E.H. The spectrum and diagnosis of acid maltase deficiency. *Neurology* 23, 95-106 (1973).
2. Hirschhorn, R. Glycogen storage disease type II: acid α-glucosidase (acid maltase) deficiency. In *The metabolic and molecular bases of inherited disease* 7th edn Vol. 1 (eds Scriver, C.R., Beaudet, A.L., Sly, W.S. & Valle, D.) 2443-2464 (McGraw-Hill, Inc., 1995).
3. Walvoort, H.C. Glycogen storage diseases in animals and their potential value as models of human disease. *J. Inherit .Metab. Dis.* 6, 3-16 (1983).
4. Bijvoet, A.G.A. *et al.* Generalized glycogen storage and cardiomegaly in a knockout mouse model of Pompe disease. *Hum. Mol. Genet.* 7, 53-62 (1998).
5. Raben, N. *et al.* Targeted disruption of the acid α-glucosidase gene in mice causes an illness with critical features of both infantile and adult human glycogen storage disease type II. *J. Biol. Chem.* 273, 19086-19092 (1998).
6. Hers, H.G. α-glucosidase deficiency in generalized glycogen storage disease (Pompe's disease). *Biochem. J.* 86, 11-16 (1963).
7. Barton, N.W., Furbish, F.S., Murray, G.J., Garfield, M. & Brady, R.O. Therapeutic response to intravenous infusions of glucocerebrosidase in a patient with Gaucher disease. *Proc. Natl. Acad. Sci. USA* 87, 1913-1916 (1990).
8. Van der Ploeg, A.T., Kroos, M.A., Willemsen, R., Brons, N.H.C. & Reuser, A.J.J. Intravenous administration of phosphorylated acid a-glucosidase leads to uptake of enzyme in heart and skeletal muscle of mice. *J. Clin. Invest.* 87, 513-518 (1991).
9. Kakkis, E.D. *et al.* Long-term and high-dose trials of enzyme replacement therapy in the canine model of mucopolysaccharidosis I. *Biochem. Mol. Med.* 58, 156-167 (1996).
10. Van Hove, J.L.K.. Yang, H.W., Wu, J.Y., Brady, R.O. & Chen, Y.T. High-level production of recombinant human lysosomal acid a-glucosidase in Chinese hamster ovary cells which targets to heart muscle and corrects glycogen accumulation in fibroblasts from patients with Pompe disease. *Proc. Natl. Acad. Sci. USA* 93, 65-70 (1996).
11. Sands, M.S. *et al.* Murine mucopolysaccharidosis type VII: Long term therapeutic effects of enzyme replacement and enzyme replacement followed by bone marrow transplantation. *J. Clin. Invest.* 99, 1596-1605 (1997).
12. Crawley, A.C. *et al.* Enzyme replacement therapy from birth in a feline model of mucopolysaccharidosis type VI. *J. Clin. Invest.* 99, 651-662 (1997).
13. Bijvoet, A.G.A. *et al.* Recombinant human acid α-glucosidase: high level production in mouse milk, biochemical characteristics, correction of enzyme deficiency in GSDII KO mice. *Hum. Mol. Genet.* 7, 1815-1824 (1998).
14. Kikuchi, T. *et al.* Clinical and metabolic correction of Pompe disease by enzyme therapy in acid maltase-deficient quail. *J. Clin. Invest.* 101, 827-833 (1998).
15. Fuller, M., Van der Ploeg, A., Reuser, A.J.J., Anson, D.S. & Hopwood, J.J. Isolation and characterisation of a recombinant, precursor form of lysosomal acid alpha-glucosidase. *Eur. J. Biochem.* 234, 903-909 (1995).
16. Bijvoet, A.G.A. *et al.* Expression of cDNA-encoded human acid α-glucosidase in milk of transgenic mice. *Biochim. Biophys. Acta* 1308, 93-96 (1996).
17. Oude Elferink, R.P.J. *et al.* Isolation and characterization of a precursor form of lysosomal α-glucosidase from human urine. *Eur. J. Biochem.* 139, 489-495 (1984).
18. Van der Ploeg, A.T. *et al.* Breakdown of lysosomal glycogen in cultured fibroblasts from glycogenosis type II patients after uptake of acid alpha-glucosidase. *J. Neurol. Sci.* 79, 327-336 (1987).
19. De Bruijn, W.C. Glycogen, its chemistry and morphologic appearance in the electron microscope. 1. A modified OsO_4 fixative which selectively contrasts glycogen. *J. Ultrastruct. Res.* 42, 29-50 (1973).
20. De Jonge, A.J.R., De Smit, S., Kroos, M.A. & Reuser, A.J.J. Cotransfer of syntenic human genes into mouse cells using isolated metaphase chromosomes or cellular DNA. *Hum. Genet.* 69, 32-38 (1985).

Chapter 8

Discussion and future prospects

Discussion and future prospects

Mouse models for human glycogen storage disease type II: genetic background and phenotypic diversity

Despite the availability of naturally occurring animal models of GSDII, it was decided for practical reasons to generate a knockout mouse model of the disease by targeted disruption of the murine acid α-glucosidase gene in embryonic stem cells (Chapter 3). A neo gene cassette was inserted in exon 13 via homologous recombination. The knockout allele is backcrossed into both the FVB and C57Bl/6 background. The mice discussed in Chapters 3, 4, and 7 were backcrossed for one to three generations and have a mixed 129-C57Bl/6 or 129-FVB background. The knockout mice with a C57Bl/6 background seem to be more severely affected than the ones with FVB background.

Meanwhile, three other mouse models have been reported. Two were generated by the insertion of a neo cassette in exon 6 (ex6 $^{neo}/_{neo}$) and exon 14 (ex14 $^{neo}/_{neo}$), respectively, and have a mixed 129-C57Bl/6 background (Raben *et al.* 1998a,b). Another model with an in-frame deletion of exon 6 ($\Delta6/\Delta6$) was obtained via Cre-lox mediated recombination on a 129-FVB background (Raben *et al.* 1998b). The four mouse models are the same in that they lack normal acid α-glucosidase mRNA, protein, and enzymatic activity, and have progressive glycogen storage in heart and skeletal muscle. They develop overt clinical symptoms between 7-12 months, but a detailed comparison of these four mouse models is not possible because the mice were evaluated by different methods. We have described the pathological features of our mice in detail (Chapter 4), but the pathological description of the others is as yet limited.

At 4 months of age, our knockout mice (ex13 $^{neo}/_{neo}$) are less active than their normal littermates when measured by the spontaneous use of a running wheel. The three other mouse models were alternatively tested in an open field test. Both the ex6 $^{neo}/_{neo}$ and ex14 $^{neo}/_{neo}$ mice have markedly reduced motor activity as early as 3-4 weeks after birth, while the $\Delta6/\Delta6$ mice perform as good as their unaffected heterozygous littermates at this age (Raben *et al.* 1998a). The differences may largely result from differences in genetic background as suggested by the observation that heterozygous ex6 $^{neo}/+$ and ex14 $^{neo}/+$ mice (with 129-C57Bl/6 background) perform significantly worse than animals heterozygous for the exon 6 deletion ($\Delta6/+$ with 129-FVB background).

A model depicting the molecular basis of phenotypic diversity in lysosomal storage disease is presented in Fig. 1 with GSDII as an example. The disease phenotype is primarily dictated by the types of mutation in the acid α-glucosidase alleles determining the level of residual activity, but is modulated by a large number of other genes. In case of GSDII, the "modifier genes" may influence the biosynthesis and intracellular transport of the mutant acid α-glucosidase and the cytoplasmic load or lysosomal delivery of glycogen in the muscle fibers.

The impact of genetic background and modifier genes on phenotypic expression is illustrated in several naturally occurring and artificially made knockout mouse models (Baribault *et al.* 1994; Erickson 1996; Rozmahel *et al.* 1996; Wilson 1996; Casal and Wolfe
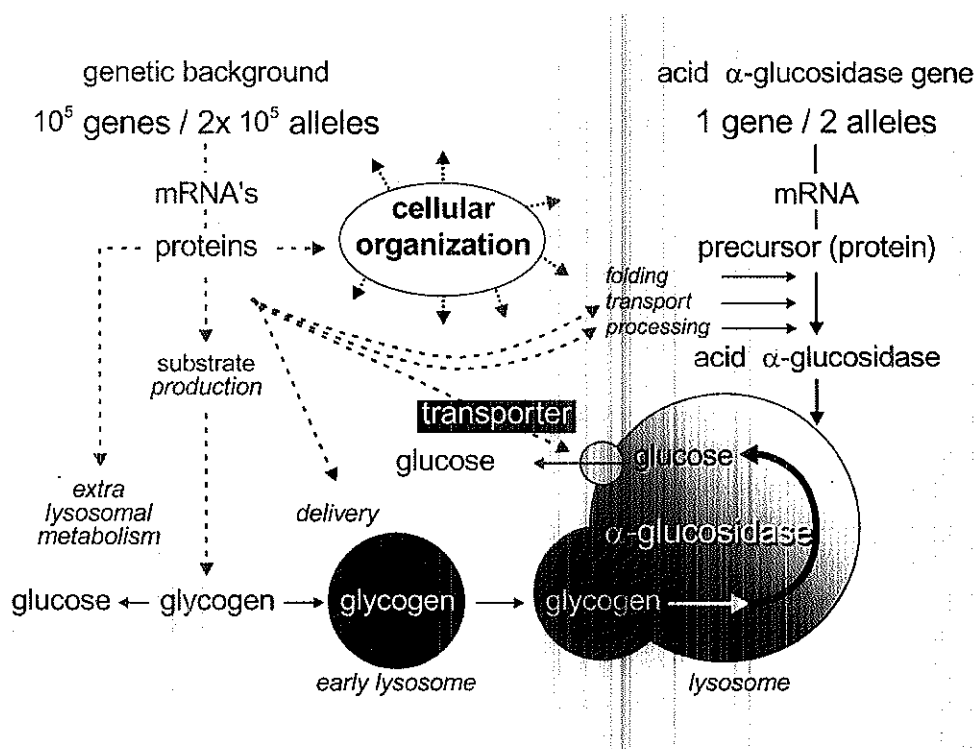
Figure 1. A model proposing the molecular basis of clinical diversity. The right side of the figure shows schematically the biosynthesis of acid α-glucosidase (dictated by one gene c.q. two alleles) and the proteins involved (solid lines). Once delivered to lysosomes, acid α-glucosidase degrades glycogen to glucose. The left side of the figure illustrates how the genetic background (2x 10⁵ alleles) influences the lysosomal degradation of glycogen by regulating extralysosomal glycogen metabolism and delivery to lysosomes (dashed lines). The depicted processes depend on the cellular organisation which varies in different cell types.

1998). A salient example of phenotypically diverse expression of one mutation on different backgrounds is that of epidermal growth factor. The phenotype of knockout mice ranges from lethality around implantation to three weeks post partum survival in different genetic backgrounds (Sibilia and Wagner 1995; Threadgill *et al.* 1995).

Genetic background, or modifier genes, also may play a role in the clinical heterogeneity in GSDII. The same IVS-1 splice site mutation is found in early-onset juvenile and very late-onset adult forms of the disease. To evaluate the impact of genetic background factors, two more GSDII knockout mouse strains were made that develop clinical symptoms earlier (Raben *et al.* 1998a). These strains were obtained by crossing the ex6 $^{neo}/_{neo}$ mice with transgenic mice overexpressing glycogen synthetase (GS) or GlutI glucose transporter. Both ex6 $^{neo}/_{neo}$/GS and ex6 $^{neo}/_{neo}$/GlutI mice show overt clinical symptoms already at 3-5 months, indicating that the elevated cytoplasmic glycogen level accelerates the course of GSDII as the model in Fig. 1 predicts. These mice may be additionally informative in studying the pathological process and the effect of therapeutic interventions.

Two approaches can be followed to demonstrate that the genetic background, indeed, has a significant impact on the clinical course of the GSDII knockout mice. The first is to compare the four knockout constructs on an identical genetic background. The easiest option is to introduce them all in strain 129, because pure 129 mice can be obtained relatively easy by blastocyst injection of the 129 embryonic stem cells and subsequent intracrossing of the mosaic and heterozygous offspring in strain 129. The $\Delta6/\Delta6$ mice can only be obtained by backcrossing, because transgenic Cre mice, needed to create the knockout, have an FVB-background. The second approach is to breed one and the same knockout allele into various backgrounds and compare the different lines. At present, we already have almost pure strains (nine times backcrossed) in both the C57Bl/6 and the FVB background for our ex13 $^{neo}/_{neo}$ knockout mouse model. The clinical, biochemical and pathological parameters of the various strains have to be compared under identical conditions and with the same methods. Besides the tests previously used (observation at smooth surface, open field and running wheel) other tests are under development for evaluation of the mice such as: echocardiography and ECG, measurement of muscle strength, and morphometry to determine muscle fiber size.

Comparison of GSDII in humans and mice

Based on the completeness of the acid α-glucosidase deficiency and the tissue pathology (cardiac and neuronal involvement), the knockout mouse models of GSDII parallel the human infantile form of the disease. However, when the species are compared by relative age, all the animal models of GSDII resemble the adult-onset human disease, because there are no clinical signs before adulthood.

The tissue pathology and clinical symptoms of the various animal models of GSDII may clarify in part the disease process in humans. Frequent vomiting is a prominent clinical symptom in dogs with GSDII and is probable related to weakness of the muscles in the esophagus (Walvoort *et al.* 1984). In our knockout mice, excessive glycogen storage is found in the upper digestive tract; in the tongue, the masseter, and the esophagus. If this would similarly occur in humans it could in part explain the feeding difficulties reported in both the infantile and the late-onset forms of the disease. Furthermore, the animal studies (including the ones on our knockout mice) emphasize the involvement of smooth muscles in the pathological process. This phenomenon is generally underestimated in the human disease. The pathological changes in the smooth muscle layer of blood vessels, observed in cattle, sheep, quail and in our mice, may find their parallel in dilatation and aneurysms of the basilar artery reported for some adult patients (Makos *et al.* 1985; Miyamoto *et al.* 1985; Braunsdorf 1987; Matsuoka *et al.* 1988; Kretzschmar *et al.* 1990). The deficient bladder function in late-onset GSDII may relate to storage in the smooth muscle cells of the bladder, as illustrated in our knockout mice.

In addition, the knockout mouse model can be informative about a possible neurologic component in the disease process. Glycogen storage is evidently present in the peripheral and central nervous system of affected mice, as it is in other animal models and patients with the infantile form of GSDII, but a clear neurologic component has not been documented.

In conclusion, knockout mice have been valuable for studies on the pathological process, and are valuable for evaluating the influence of modifier genes on this process. In addition, they are particularly useful for the development of therapeutic strategies.

Recombinant human acid α-glucosidase produced in milk of transgenic animals and its therapeutic effect in a mouse model

The M6P receptor mediated uptake of acid α-glucosidase by cultured cells of patients with GSDII and the correction of lysosomal glycogen storage were promising signs to attempt treatment of GSDII by enzyme replacement therapy (Van der Ploeg *et al.* 1988a,b, 1990, 1991). The large amounts of enzyme necessary for pre-clinical studies and clinical application of enzyme replacement therapy can only be obtained via biotechnological production methods. The choice of production system depends on several factors. The recombinant human acid α-glucosidase should be glycosylated and phosphorylated to have the right high-uptake properties. Bacteria, yeast, and insect cells are excluded as production system, because they miss the post-translational machinery to modify mammalian proteins correctly (Martiniuk *et al.* 1992; Houdebine 1994; Wu *et al.* 1996; Colman 1998). However, future refinements of these systems by co-expression of mammalian glycosyltransferases and phosphotransferases may change the situation.

Investigations on the production of recombinant eukaryotic proteins by transgenic plants and in the urine of transgenic animals have started only recently. At the beginning of the experimental work described in this thesis, only two options were available for the production of recombinant human acid α-glucosidase. It could be done in genetically engineered, cultured mammalian cells, or in the milk of transgenic animals. Both systems have shown to be successful (Fuller *et al.* 1995; Bijvoet *et al.* 1996, 1998; Van Hove *et al.* 1996). The short period needed to develop lab-scale production of enzyme for *in vitro* and *in vivo* pre-clinical testing is an advantage of the CHO cell production system. Several proteins including lysosomal enzymes are successfully produced by genetically engineered CHO cells (see Chapter 1), and some are already approved for therapeutic application and are safe and effective. However, the production levels in CHO cell culture systems are relatively low and large-scale production is expensive. For example, glucocerebrosidase (so far the only therapeutic lysosomal enzyme on the market) produced in CHO cells for the treatment of Gaucher disease costs about $80,000-312,000 per patient of 50 kg per year (Grabowski *et al.* 1998).

In contrast, the production of therapeutic proteins in milk of transgenic animals is expected to be very efficient with high yield and probably with low costs (Houdebine 1994; Colman 1998). No recombinant human protein from milk is as yet on the market, but clinical trials have started with recombinant human α_1-antitrypsine, human antithrombin III, human lactoferrin and human acid α-glucosidase from milk of transgenic sheep, goat, cattle and rabbits, respectively (see Table 1).

The choice of animal species to be used for protein production in milk is largely determined by the amount of protein needed for clinical application and the time needed for development. Cows produce the largest milk volume, but it takes 32 months before the first milk can be

Table 1. Production of potentially therapeutic proteins in the milk of transgenic livestock

Product	Indication	Function	Phase[a]	Ref
Acid α-glucosidase	GSDII	glycogen degradation	PII	Chapter 7
Protein C	acute heart infarction	anti-coagulation	pc	(1)
Cl-esterase inhibitor	hereditary angioedema acute myocardial infarction	counteracts swelling	pc	(2)
Tissue plasminogen activator	heart attack or stroke	dissolves blood clots	pc	(3)
Factor VIII	hemophilia A	blood clotting	pc	(4)
Protein C	acute heart infarction	anti-coagulation	pc	(1, 5)
α1-antitrypsin	cystic fibrosis congenital emphysema	inhibits elastase	PII	(6)
Factor IX	hemophilia B	blood clotting	pc	(7)
Protein C	acute heart infarction	anti-coagulation	pc	(1)
Fibrinogen	severe tissue damage	blood clot formation	pc	(1)
Factor VIII	hemophilia A	blood clotting	pc	(8)
Antithrombine III	bypass surgery	anti-coagulation	PIII	(4)
Malaria vaccine MSP-1	malaria	vaccination	pc	(9)
CD-4-MAB[b] and other MAB's	cancer and other diseases	drug targeting (antigen recognition)	pc	(9)
Tissue plasminogen activator	heart attack or stroke	dissolves blood clots	pc	(10)
Lactoferrin	infectious arthritis gastrointestinal tract infections	prevents and inhibits infection	PI	(11)
Factor VIII	hemophilia A	blood clotting	pc	(2)
Cl-esterase inhibitor	hereditary angioedema acute myocardial infarction	counteracts swelling	pc	(2)
Fibrinogen	heavy tissue damage	blood clot formation	pc	(2)
Serum albumin	trauma patients	plasma expansion	pc	(9, 12)
Collagen type I	tissue and bone trauma	tissue and bone repair	pc	(2)
Bile salt stimulated lipase	pre-term infants	lipid degradation	pc	(12)

[a]*phase of research: pc: pre-clinical research; PI: Phase I clinical trial; PII: Phase II clinical trial; PIII: Phase III clinical trial.* [b]*MAB: monoclonal antibody.*

(1) Colman 1998; (2) De Ron and Van Beynum 1999; (3) Riego et al. 1993; (4) Drohan 1997; (5) Velander et al. 1992; (6) Carver et al. 1993; (7) Clark et al. 1989; (8) Halter et al. 1993; (9) Genzyme Transgenics Corporation internet homepage: http://www.genzyme.com; (10) Ebert et al. 1991; (11) Krimpenfort et al. 1991; (12) PPL therapeutics internet homepage: http://www.ppl.therapeutics.co.uk

Table 2. Species specific lactation volumes and generation times

	Mouse	Rabbit	Pig	Sheep	Goat	Cow
Volume per lactation (liters)	0.0008	1	150	400	500	10,000
Gestation time (months)	0.7	1	4	5	5	9
Time to sexual maturity (months)	1.8	5	7	8	8	15
Time from oocyte injection to first milk (months)	3.25	7	15	18 (9)[a]	18 (9)[a]	33 (12)[a]
Time from oocyte injection to production (months)	8.3	19	37	44 (31)[b]	44 (31)[b]	81 (57)[b]

[a] *first milk by premature induction of lactation.*
[b] *first production by use of nuclear transfer techniques.*

collected and about 78 months are required for the start of large scale production (see Table 2 and Fig. 2). For pigs, sheep, goat and rabbit the time scales are shorter, but also the milk production levels are lower (Table 2). Mice are usually chosen for proof of principle studies, because milk can be obtained within four months after transgene injection, and enough protein can be collected for enzyme characterization and for the first pilot studies on clinical effectiveness in a laboratory setting. However, studies in transgenic mice are not necessarily valid for the transgene function in other species. For instance, both α_1-antitrypsin and protein C are produced in higher quantity and better quality in larger animals such as sheep and pigs than in mice (Wright *et al.* 1991; Velander *et al.* 1992). In addition, the choice of production species depends on species specific processing effects, which may affect the quality of the recombinant human protein. For instance, recombinant protein C, produced in the milk of transgenic sheep, has the most optimal properties in terms of γ-carboxylation needed for bioactivity when compared to protein C produced in the milk of transgenic mice, rabbits, and pigs (Colman 1998).

We have demonstrated the feasibility of lysosomal enzyme production in the milk of transgenic mice (Chapters 5 and 6). Recombinant human acid α-glucosidase is produced in the milk of this species at levels up to 1.5 µg/ml for the cDNA transgene construct and 2 mg/ml for the genomic construct, respectively. The acid α-glucosidase obtained from the milk has a molecular mass of 110 kD and resembles the natural human acid α-glucosidase precursor from human urine and the recombinant precursor secreted by genetically engineered CHO cells in many aspects. Importantly, the enzyme contains the properties necessary for uptake by cultured cells and target tissues of GSDII knockout mice. Based on these encouraging results a project was started to develop, in collaboration with Pharming B.V. Leiden, a system to produce recombinant human enzyme for long term treatment of GSDII knockout mice and eventually humans. Rabbits were chosen as producers, because of their relatively short generation time and sufficient milk volume. They were generated using the same hybrid gene construct and technology that was used to make the transgenic mice. Production levels of 3 gram per liter were obtained, which is concentration wise at least 30 times higher than in culture medium of

A

Male founder

birth
F1 females
(males)

birth F2
evaluation
F1 milk
breed F1 males

breed
production
herd

birth
founder
F0 male

breed
founder

breed
F1 female(s)
(males)

birth F2
production
herd

milk
from
production
herd

Start

oocyte injection

birth
founder
F0 female

breed
founder

breed
F1 male
(females)

breed
production
herd

Female founder

birth F1
male (females)
evaluation
founder milk

birth F2
production
herd

milk
from
production
herd

B

birth
identical
founders
F0 females

birth F1
male & females
evaluation
milk

milk
from
production
herd

Start

breed
founders

breed
production
herd

nuclear
trans-
plantation

Gestation period

Time to sexual maturity

Figure 2. Stages in the generation of a milking herd of transgenic animals using conventional breeding methods (A) and using nuclear transfer techniques (B). See table 2 for accurate (gestational and time to sexual maturity) periods per species. (Adapted and modified from Colman 1996; Wall et al. 1997; De Ron and Van Beynum 1999).

genetically modified CHO cells. The recombinant enzyme produced by transgenic rabbits was used for long term treatment of the knockout mice and resulted in correction of the acid α-glucosidase deficiency and degradation of the lysosomal glycogen in virtually all tissues except the CNS. The advanced state of tissue pathology at the start of treatment was at least partially reversed in all tissues, except brain. This assuring result has meanwhile led to Phase I and Phase II studies of enzyme replacement therapy in humans with the recombinant human enzyme from rabbit milk.

Enzyme replacement therapy in humans

The preclinical studies described in this thesis have given useful information for a phase II clinical trial in patients. The recombinant human acid α-glucosidase produced in rabbit milk was well tolerated by the mice over a period of six months with weekly injections. However, several animals showed a hypersensitive reaction after the third injection. A similar hypersensitivity may be encountered in patients with the infantile form of GSDII who do not produce any endogenous acid α-glucosidase, but may also occur in late-onset patients when the enzyme preparation would contain trace amounts of foreign proteins. Interestingly, the knockout mice with FVB background developed a clear antibody response during the repeated enzyme infusions, but the C57Bl/6 mice gave hardly any response (Chapter 7). Of note, about 15 % of the Gaucher patients receiving enzyme replacement therapy develop antibodies to the administered glucocerebrosidase but 90 % of them became antibody negative (tolerized) after 28 months of continued therapy (Grabowski et al. 1998). The literature reports that immune responses in animal models can be treated by the induction of immunological tolerance, the removal of reactive antibodies prior to the administration of an effective treatment dose or treatment with antihistamines (Brooks et al. 1998).

The immunogenicity of the recombinant human acid α-glucosidase may vary between mice, rabbits and humans, because of potential cross-species differences in post-translational modification. and the responses in humans may vary per patient depending on the type of acid α-glucosidase defect and the recipient's genetic background. This holds for recombinant enzyme produced by any production system. For instance, antibody formation to recombinant CHO-proteins has been reported in studies on animal models for other lysosomal diseases (Shull *et al.* 1994; Crawley *et al.* 1996; Sands *et al.* 1997; Brooks *et al.* 1998). Circulating antibodies to the administered enzyme can potentially result in enzyme inactivation (neutralization), degradation and mistargeting. However, although about 15 % of the Gaucher patients receiving enzyme replacement therapy develop antibodies, only 3 patients are reported to have antibodies with a neutralizing effect (Grabowski *et al.* 1995; Ponce *et al.* 1997).

Another important issue in the phase II trial is the monitoring of the long-term effect of the glycogen storage in the central nervous system. Little information as to this effect is available. Neither infants with GSDII nor knockout mice show apparent signs of functional CNS impairment. However, the appearance of neurological symptoms can not be excluded, when enzyme replacement therapy is effective and prolongs the patient's life. Therefore, it is essential to monitor the mental and motoric milestones of infants under treatment.

Concluding remarks

The work described in this thesis has contributed to the understanding of glycogen storage disease type II and will help to elucidate the molecular and pathological processes in patients with this disease. Furthermore, it has stimulated and steered the development of enzyme replacement therapy for this and other lysosomal diseases by the presentation of a new method for the large-scale production of lysosomal enzymes. The potential efficacy of enzyme replacement therapy for GSDII has been demonstrated in a mouse model of this disease. This

animal model has additional value for dose finding, for fundamental studies on enzyme replacement therapy, and for testing of alternative therapeutic approaches in future.

References

Baribault, H., Penner, J., Iozzo, R.V. and Wilson-Heiner, M. (1994) Colorectal hyperplasia and inflammation in keratin 8-deficient FVB/N mice. *Genes Dev* 8: 2964-2973.

Bijvoet, A.G.A., Kroos, M.A., Pieper, F.R., de Boer, H.A., Reuser, A.J.J., van der Ploeg, A.T. and Verbeet, M.P. (1996) Expression of cDNA-encoded human acid α-glucosidase in milk of transgenic mice. *Biochim Biophys Acta* 1308: 93-96.

Bijvoet, A.G.A., Kroos, M.A., Pieper, F.R., Van der Vliet, M., De Boer, H.A., Van der Ploeg, A.T., Verbeet, M.P. and Reuser, A.J.J. (1998) Recombinant human acid α-glucosidase: high level production in mouse milk, biochemical characteristics, correction of enzyme deficiency in GSDII KO mice. *Hum Mol Genet* 7: 1815-1824.

Braunsdorf, W.E. (1987) Fusiform aneurysm of basilar artery and ectatic internal carotid arteries associated with glycogenosis type 2 (Pompe's disease). *Neurosurg* 21: 748-749.

Brooks, D.A., Hopwood, J.J. and King, B.M. (1998) Immune response to enzyme replacement therapy: clinical signs of hypersensitivity reactions and altered enzyme distribution in a high titre rat model. *Biochim Biophys Acta* 1407: 163-172.

Carver, A.S., Dalrymple, M.A., Wright, G., Cottom, D.S., Reeves, D.B., Gibson, Y.H., Keenan, J.L., Barrass, J.D., Scott, A.R., Colman, A. and et, a. (1993) Transgenic livestock as bioreactors: stable expression of human α-1-antitrypsin by a flock of sheep. *Biotechnology* 11: 1263-1270.

Casal, M.L. and Wolfe, J.H. (1998) Variant clinical course of mucopolysaccharidosis type VII in two groups of mice carrying the same mutation. *Lab Invest* 78: 1575-1581.

Clark, A.J., Bessos, H., J.O., B., Brown, P., Harris, S., Lathe, R., McClenaghan, M., Prowse, C., Simons, J.P., Whitelaw, C.B.A. and Wilmut, I. (1989) Expression of human anti-hemophilic factor IX in the milk of transgenic sheep. *Bio/Technology* 7: 487-492.

Colman, A. (1996) Production of proteins in the milk of transgenic livestock: problems, solutions, and successes. *Am J Clin Nutr* 63: 639S-645S.

Colman, A. (1998) Production of therapeutic proteins in the milk of transgenic livestock. *Biochem Soc Symp* 63: 141-147.

Cramer, C.L., Weissenborn, D.L., Oishi, K.K., Grabau, E.A., Bennett, S., Ponce, E., Grabowski, G.A. and Radin, D.N. (1996) Bioproduction of human enzymes in transgenic tobacco. *Ann N Y Acad Sci* 792: 62-71.

Crawley, A.C., Brooks, D.A., Muller, V.J., Petersen, B.A., Isaac, E.L., Bielicki, J., King, B.M., Boulter, C.D., Moore, A.J., Fazzalari, N.L., Anson, D.S., Byers, S. and Hopwood, J.J. (1996) Enzyme replacement therapy in a feline model of Maroteaux-Lamy syndrome. *J Clin Invest* 97: 1864-1873.

De Ron, A. and Van Beynum, G. (1999) Reactor op vier pootjes. *Natuur en Techniek* 67: 81-87.

Drohan, W.N. (1997) The past, present and future of transgenic bioreactors. *Thromb Haemost* 78: 543-547.

Ebert, K.M., Selgrath, J.P., DiTullio, P., Denman, J., Smith, T.E., Memon, M.A., Schindler, J.E., Monastersky, G.M., Vitale, J.A. and Gordon, K. (1991) Transgenic production of a variant of human tissue-type plasminogen activator in goat milk: generation of transgenic goats and analysis of expression. *Biotechnology* 9: 835-838.

Erickson, R.P. (1996) Mouse models of human genetic disease: which mouse is more like a man? *BioEssays* 18: 993-998.

Fuller, M., Van der Ploeg, A., Reuser, A.J.J., Anson, D.S. and Hopwood, J.J. (1995) Isolation and characterisation of a recombinant, precursor form of lysosomal acid α-glucosidase. *Eur J Biochem* 234: 903-909.

Grabowski, G.A., N.W., B., Pastores, G., Dambrosia, J.M., Banerjee, T.K., McKee, M.A., Parker, C., Schiffman, R., Hill, S.C. and Brady, R.O. (1995) Enzyme therapy in type 1 Gaucher disease: Comparative efficacy of mannose-terminated glucocerebrosidase from natural and recombinant sources. *Ann Intern Med* 122: 33-39.

Grabowski, G.A., Leslie, N. and Wenstrup, R. (1998). Enzyme therapy for Gaucher disease: the first five years. *Blood Rev* 12: 115-133.

Halter, R., Carnwath, J., Espanion, G., Herrmann, D., Lemme, L., Niemann, H. and Paul, D. (1993) Strategies to express factor VIII gene constructs in the ovine mammary gland. *Theriogenology* **39**: 137-149.

Hirschhorn, R. (1995). Glycogen storage disease type II: acid α-glucosidase (acid maltase) deficiency. *In: The metabolic and molecular bases of inherited disease.* Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D., (Eds) McGraw-Hill, Inc. **1**: 2443-2464.

Houdebine, L.M. (1994) Production of pharmaceutical proteins from transgenic animals. *J Biotechnol* **34**: 269-287.

Kretzschmar, H.A., Wagner, H., Hubner, G., Danek, A., Witt, T.N. and Mehraein, P. (1990) Aneurysms and vacuolar degeneration of cerebral arteries in late-onset acid maltase deficiency. *J Neurol Sci* **98**: 169-183.

Krimpenfort, P., Rademakers, A., Eyestone, W., van der Schans, A., van den Broek, S., Kooiman, P., Kootwijk, E., Platenburg, G., Pieper, F., Strijker, R. and et al. (1991) Generation of transgenic dairy cattle using 'in vitro' embryo production. *Biotechnology (N Y)* **9**: 844-847.

Makos, M.M., McComb, R.D., Adickes, E.D. and Bennett, D.R. (1985) Acid maltase deficiency and basilar artery aneurysms: a report of a sibship. *Neurology* **35 suppl 1**: 193-194.

Martiniuk, F., Tzall, S. and Chen, A. (1992) Recombinant human acid α-glucosidase generated in bacteria: antigenic, but enzymatically inactive. *Dna Cell Biol* **11**: 701-706.

Matsuoka, Y., Senda, Y., Hirayama, M., Matsui, T. and Takahashi, A. (1988) Late-onset acid maltase deficiency associated with intracranial aneurysm. *J Neurol* **235**: 371-373.

Miyamoto, Y., Etoh, Y., Joh, R., Noda, K., Ohya, I. and Morimatsu, M. (1985) Adult-onset acid maltase deficiency in siblings. *Acta Pathol Jpn* **35**: 1533-1542.

O'Sullivan, B.M., Healy, P.J., Fraser, I.R., Nieper, R.E., Whittle, R.J. and Sewell, C.A. (1981) Generalized glycogenosis in Brahman cattle. *Aust Vet J* **57**: 227-229.

Ponce, E., Moskovitz, J. and Grabowski, G. (1997) Enzyme therapy in Gaucher disease type I: effect of neutralizing antibodies to acid β-glucosidase. *Blood* **90**: 43-48.

Raben, N., Nagaraju, K., Lee, E., Ward, J., Byrne, B., Kessler, P., Sauer, B. and Plotz, P. (1998a) Modulation of disease severity in knockout mice with targeted disruption of the acid α-glucosidase gene. *Am J Hum Genet* **64 suppl**: A381.

Raben, N., Nagaralu, K., Lee, E., Kessler, P., Byrne, B., Lee, L., LaMarca, M., King, C., Ward, J., Sauer, B. and Plotz, P. (1998b) Targeted disruption of the acid α-glucosidase gene in mice causes an illness with critical features of both infantile and adult human glycogen storage disease type II. *J Biol Chem* **273**: 19086-19092.

Riego, E., Limonta, J., Aguilar, A., Perez, A., De Armas, R., Solano, R., Ramos, B., Castro, F.O. and De la Fuente, J. (1993) Production of transgenic mice and rabbits that carry and express the human tissue plasminogen activation cDNA under the control of a bovine α-S1 casein promoter. *Theriogenology* **39**: 1173-1185.

Rozmahel, R., Wilschanski, M., Matin, A., Plyte, S., Oliver, M., Auerbach, W., Moore, A., Forstner, J., Durie, P., Nadeau, J., Bear, C. and Tsui, L.C. (1996) Modulation of disease severity in cystic fibrosis transmembrane conductance regulator deficient mice by a secondary genetic factor. *Nat Genet* **12**: 280-287.

Sands, M.S., Vogler, C., Torrey, A., Levy, B., Gwynn, B., Grubb, J., Sly, W.S. and Birkenmeier, E.H. (1997) Murine mucopolysaccharidosis type VII: long term therapeutic effects of enzyme replacement and enzyme replacement followed by bone marrow transplantation. *J Clin Invest* **99**: 1596-1605.

Shull, R.M., Kakkis, E.D., McEntee, M.F., Kania, S.A., Jonas, A.J. and Neufeld, E.F. (1994) Enzyme replacement in a canine model of Hurler syndrome. *Proc Natl Acad Sci USA* **91**: 12937-12941.

Sibilia, M. and Wagner, E.F. (1995) Strain-dependent epithelial defects in mice lacking the EFG receptor. *Science* **269**: 234-238.

Threadgill, D.W., Dlugosz, A.A., Hansen, L.A., Tennenbaum, T., Lichti, U., Yee, D., LaMantia, C., Mourton, T., Herrup, K., Harris, R.C. and et al. (1995) Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. *Science* **269**: 230-234.

Van der Ploeg, A.T., Bolhuis, P.A., Wolterman, R.A., Visser, J.W., Loonen, M.C.B., Busch, H.F.M. and Reuser, A.J.J. (1988a). Prospect for enzyme therapy in glycogenosis II variants: a study on cultured muscle cells. *J Neurol* **235**: 392-396.

Van der Ploeg, A.T., Loonen, M.C.B., Bolhuis, P.A., Busch, H.M.F., Reuser, A.J.J. and Galjaard, H. (1988b). Receptor-mediated uptake of acid α-glucosidase corrects lysosomal glycogen storage in cultured skeletal muscle. *Pediatr Res* **24**: 90-94.

Van der Ploeg, A.T., Van der Kraaij, A.M.M., Willemsen, R., Kroos, M.A., Loonen, M.C.B., Koster, J.F. and Reuser, A.J.J. (1990). Rat heart perfusion as model system for enzyme replacement therapy in glycogenosis type II. *Pediatr Res* **28**: 344-347.

Van der Ploeg, A.T., Kroos, M.A., Willemsen, R., Brons, N.H.C. and Reuser, A.J.J. (1991). Intravenous administration of phosphorylated acid α-glucosidase leads to uptake of enzyme in heart and skeletal muscle of mice. *J Clin Invest* **87**: 513-518.

Van Hove, J.L.K., Yang, H.W., Wu, J.Y., Brady, R.O. and Chen, Y.T. (1996) High-level production of recombinant human lysosomal acid α-glucosidase in Chinese hamster ovary cells which targets to heart muscle and corrects glycogen accumulation in fibroblasts from patients with Pompe disease. *Proc Natl Acad Sci USA* **93**: 65-70.

Velander, W.H., Johnson, J.L., Page, R.L., Russell, C.G., Subramaniam, A., Wilkins, T.D., Gwazdauskas, F.C., Pittius, C. and Drohan, W.N. (1992) High-level expression of a heterologous protein in the milk of transgenic swine using the cDNA encoding human protein C. *Proc Natl Acad Sci USA* **89**: 12003-12007.

Wall, R.J., Kerr, D.E. and Bondioli, K.R. (1997) Trangenic diary cattle: genetic engineering on a large scale. *J Dairy Sci* **80**: 2213-2224.

Walvoort, H.C., Van Nes, J.J., Stokhof, A.A. and Wolvekamp, W.T.C. (1984) Canine glycogen storage disease type II (GSDII): a clinical study of four affected Lapland dogs. *J Am Anim Hosp Assoc* **20**: 279-286.

Wilson, J.M. (1996) Animal models of human diseases for gene therapy. *J Clin Invest* **97**: 1138-1141.

Wright, G., Carver, A., Cottom, D., Reeves, D., Scott. A., Simons, P., Wilmut, I., Garner, I. and Colman, A. (1991) High level expression of active human α-1-antitrypsin in the milk of transgenic sheep. *Biotechnology* **9**: 830-834.

Wu, J.Y., Van Hove, J.L.K., Huang, Y.S. and Chen, Y.T. (1996) Expression of catalytically active human multifunctional glycogen-debranching enzyme and lysosomal acid α-glucosidase in insect cells. *Biochem Mol Biol Int* **39**: 755-764.

Summary

Samenvatting

Summary

Glycogen storage disease type II (Pompe's disease) is a fatal lysosomal storage disorder with an autosomal recessive mode of inheritance. Skeletal muscle weakness is the most conspicuous clinical symptom. An additional cardiac involvement is seen in the most severe infantile variant of this disease. Affected infants succumb in the first to second year of life. Patients with late-onset disease become wheelchair bound and often artificial ventilation dependent. The muscle weakness is caused by loss of muscle fibers as a result of the lysosomal glycogen storage, which in turn is caused by the deficiency of the lysosomal enzyme acid α-glucosidase.

Lysosomes are acidic, membrane bound, cell-organelles that are involved in the degradation of a wide variety of intra and extracellular compounds. Over 40 different lysosomal enzymes are involved in this process, acting often in concert and sometimes with the aid of activator proteins. Each enzyme acts specifically on one or more substrates. Lysosomal storage diseases are characterized by the inherited deficiency of one of the lysosomal proteins resulting in the intralysosomal accumulation of certain substrates. Due to the different nature of the storage products in each of the lysosomal storage diseases, there is a substantial variation in tissue involvement and related symptomatology, despite the fact that lysosomes are present in almost every cell type. For most lysosomal storage diseases, there is at present no cure or adequate therapeutic intervention.

Background information on the lysosomal system and characteristics of lysosomal storage diseases are given in Chapter 1. This chapter also gives an overview of the currently existing mouse models of lysosomal storage diseases and describes the various approaches towards therapy. There are in principle two therapeutic options: the first is to prevent storage by substrate deprivation, and the second is to remove the storage products by supplementing the missing enzyme. The latter can be effectuated through direct administration of the enzyme (enzyme replacement therapy), through transplantation or implantation of cells producing and secreting the enzyme (like bone marrow transplantation) or through introduction of the gene in question into somatic cells (gene therapy). Examples of the different options are discussed. The last part of Chapter 1 deals with the application of biotechnology for the industrial production of therapeutic proteins with an emphasis on lysosomal enzyme production.

Chapter 2 provides information on glycogen storage disease type II (GSDII), a member of the lysosomal storage disease family. It was the first of these diseases in which the enzyme deficiency became identified. The disease is caused by mutations in the gene coding for acid α-glucosidase, and the resulting enzyme deficiency leads in different degrees of severity to lysosomal glycogen storage and a spectrum of clinical phenotypes. The severe infantile form of GSDII presents shortly after birth with cardiac enlargement and generalized muscle weakness, and is fatal within the first two years of life. There is no cardiac involvement in late onset forms of the disease, but skeletal muscle function is severely impaired with major risk of respiratory failure. Patients may benefit from respiratory support and possibly from certain dietary regimens, but effective therapy is as yet not available.

Several natural animal models of GSDII have been described, but only cattle and quail are available for therapeutic study. However, these models are not ideal because of the physical size

(cattle) and the evolutionary distance (quail) to humans. Therefore, a knockout mouse model of GSDII was made by disruption of exon 13 of the mouse acid α-glucosidase gene in embryonic stem cells through homologous recombination followed by implantation of these modified cells into blastocysts. Chapter 3 describes the methodology, documents the acid α-glucosidase deficiency in the homozygous knockout mice and illustrates the lysosomal glycogen storage in liver, heart and skeletal muscle. By biochemical and histological criteria, the knockout mice appear to mimic the human infantile form of the disease. With respect to the clinical course, there seems to be a difference, because affected infants have an average life span of one year whereas the knockout mice reach adulthood. However, the absolute time of the disease process (from gestation to death) of affected mice and the other animal models of GSDII is similar to that of affected infants. A more detailed investigation into the clinical symptoms and the distribution of lysosomal glycogen storage in the visceral organs, skeletal and smooth muscle of the knockout mouse model is described in Chapter 4.

Chapters 5 and 6 report on the production of recombinant human acid α-glucosidase in the milk of transgenic mice. The aim of these studies was to investigate the feasibility of large-scale production of therapeutic lysosomal protein in farm animals. Transgenic mice were generated by oocyte injection with hybrid constructs consisting of the human acid α-glucosidase cDNA or genomic sequences under control of the bovine α_{S1}-casein gene promoter. The transgenic female mice express human acid α-glucosidase in the mammary gland during lactation and secrete the enzyme in their milk. The maximal production level was 1.5 µg/ml using the cDNA construct (Chapter 5), but with the genomic construct a production up to 2.0 mg/ml (Chapter 6). The purified recombinant enzyme resembles in all aspects the natural acid α-glucosidase precursor from human urine and the recombinant precursor secreted by genetically engineered Chinese hamster ovary cells. The recombinant acid α-glucosidase was tested for its *in vitro* and *in vivo* effect. The enzyme is taken up by cultured fibroblasts of patients with GSDII in a mannose 6-phosphate receptor dependent manner and corrects both the acid α-glucosidase deficiency as well as the lysosomal glycogen storage. A single dose of enzyme, administered intravenously to GSDII knockout mice, corrects the acid α-glucosidase deficiency in all tissues except brain. Based on these promising results, the genomic construct was used for the industrial production of recombinant human acid α-glucosidase in the milk of transgenic rabbits. The potential therapeutic effect of this latter enzyme was demonstrated by treating GSDII knockout mice over a six months period. The experiments described in Chapter 7 demonstrate the degradation of lysosomal glycogen in heart, skeletal and smooth muscle and the reversal of tissue pathology. Chapter 8 discusses the work of this thesis in broader perspective.

The work reported in this thesis demonstrates the feasibility of recombinant human acid α-glucosidase production in the milk of transgenic rabbits and the potential value of this enzyme for treatment of patients with GSDII. It has laid a solid basis for the start of a phase II clinical trial of enzyme replacement therapy in humans, which has meanwhile begun in the department of Pediatrics of the Sophia Children's Hospital Rotterdam.

Samenvatting

Glycogeen stapelingsziekte type II (GSDII; Pompe's disease) is een erfelijke lysosomale stapelingsziekte met een fataal verloop. Algehele spierzwakte is het meest in het oog springend klinische symptoom. Wanneer de ziekte zich, in de meest ernstige vorm, reeds kort na geboorte manifesteert is ook de hartspier betrokken en is de levensverwachting van de patiënt van de patiënt gemiddeld niet meer dan een jaar. Patiënten met een mildere vorm van de ziekte krijgen pas op latere leeftijd symptomen. Zij worden op den duur rolstoelafhankelijk en hebben ademhalingsondersteuning nodig. De spierzwakte wordt veroorzaakt door het verlies van spiervezels ten gevolge van de lysosomale glycogeenstapeling. Het basale defect bij deze ziekte is een tekort aan het lysosomale enzym α-glucosidase.

Lysosomen zijn celorganellen, waarin een grote variatie van intra- en extracellulaire producten wordt afgebroken. Bij deze afbraak zijn meer dan 40 verschillende lysosomale enzymen en soms ook activator-eiwitten betrokken. Elk enzym werkt specifiek op één of meerdere substraten. Lysosomale stapelingsziekten worden veroorzaakt door de erfelijke deficiëntie van één van de lysosomale eiwitten, waardoor karakteristieke producten in het lysosoom accumuleren. De weefsel-betrokkenheid en de gerelateerde symptomatologie wordt bepaald door de eigenschappen van de verschillende stapelingsproducten. Tot op heden is er slechts voor een enkele lysosomale stapelingsziekte een therapeutische behandeling mogelijk.

Hoofdstuk 1 geeft achtergrond informatie over het lysosomale systeem en de lysosomale stapelingsziekten. In dit hoofdstuk wordt ook een overzicht gegeven van de bestaande muismodellen voor lysosomale ziekten en wordt besproken welke vormen van therapie theoretisch mogelijk zijn. In principe zijn er twee therapeutische benaderingen: de eerste is het voorkomen van stapeling door substraat deprivatie, de tweede is het verwijderen van de stapelingsproducten door toevoeging van het missende enzym. Dit laatste zou kunnen door rechtstreekse toediening van het enzym (enzym vervangingstherapie), door transplantatie of implantatie van cellen die het enzym produceren en uitscheiden (bijv. beenmerg transplantatie) of door gen therapie, waarbij het gen, nodig voor enzym productie, wordt geïntroduceerd in somatische cellen. Voorbeelden van de verschillende opties worden besproken. Het laatste deel van hoofdstuk 1 gaat over de toepassing van biotechnologie voor de industriële productie van therapeutische eiwitten met de nadruk op lysosomale eiwit productie.

Hoofdstuk 2 is toegespitst op glycogeen stapelingsziekte type II. De ziekte wordt veroorzaakt door mutaties in het gen coderend voor lysosomaal α-glucosidase en kent een spectrum van klinische fenotypen. De meest ernstig aangedane patiënten vertonen kort na geboorte algehele spierzwakte en hartvergroting. Zij hebben geen restactiviteit van lysosomaal α-glucosidase en overlijden meestal binnen de eerste twee levensjaren ten gevolge van cardiorespiratoire insufficiëntie. Bij de mildere variant van de ziekte is restactiviteit van het enzym aanwezig en is er geen betrokkenheid van het hart. De skeletspieren, inclusief de ademhalingsspieren, zijn ernstig aangedaan. De patiënten hebben baat bij ademhalings-

ondersteuning en mogelijk bij bepaalde diëten, maar een effectieve therapie is op dit moment nog niet beschikbaar.

Hoewel de ziekte bij verschillende diersoorten van nature voorkomt, waren alleen koeien en kwartels beschikbaar voor studies naar therapeutische mogelijkheden. Deze diermodellen zijn echter niet ideaal; koeien vanwege hun grootte en kwartels vanwege de evolutionaire afstand tot de mens. Muizen hebben voor dit soort studies de voorkeur. In hoofdstuk 3 wordt beschreven hoe (via knockout technologie) een muizenlijn werd verkregen met een volledige deficiëntie van lysosomaal α-glucosidase en glycogeenstapeling in onder andere lever, hart en skeletspieren. Op grond van biochemische en histologische criteria is het verkregen muismodel vergelijkbaar met de meest ernstige variant van GSDII. Bij vergelijking van het klinische proces lijken er verschillen te bestaan, omdat patiënten met de ernstige variant van de ziekte een gemiddelde levensverwachting hebben van een jaar, terwijl "knockout" muizen volwassen worden en zich zelfs kunnen voortplanten. Als echter de absolute lengte van het ziekteproces in beschouwing wordt genomen (van ééncellig embryo tot het moment van eerste symptomen en de leeftijd van overlijden) dan is het verloop van de ziekte in het muismodel en de andere diermodellen gelijk aan dat in de meest ernstige variant van GSDII. Hoofdstuk 4 geeft een gedetailleerde beschrijving van de klinische symptomen van de muizen en de betrokkenheid van de diverse organen wat betreft de mate van glycogeenstapeling.

De productie van recombinant menselijk α-glucosidase in de melk van transgene muizen wordt beschreven in de hoofdstukken 5 en 6. Het betreft een studie die het principe van lysosomale enzym productie in de melk van transgene zoogdieren aantoont. De transgene muizen werden gemaakt door de genetische informatie, nodig voor de aanmaak van humaan α-glucosidase, te injecteren in bevruchte eicellen. In praktijk werden twee "hybride" gen-constructen gebruikt. Bij de één werd uitgegaan van het cDNA, bij de ander van het genomische DNA van het menselijke lysosomale α-glucosidase. In beide gevallen werd het α-glucosidase gen onder de controle van de promoter van een melk-specifiek eiwit (koeien α_{S1}-caseine) geplaatst. Het menselijke α-glucosidase gen komt tot expressie tijdens de lactatie en het enzym wordt uitgescheiden in de melk. Het maximale productie-niveau was 1.5 μg/ml bij gebruik van het cDNA construct (hoofdstuk 5), terwijl met het genomische construct een productie-niveau van 2.0 mg/ml werd bereikt.

Het gezuiverde recombinante enzym lijkt in alle opzichten op de precursor van α-glucosidase die in menselijke urine voorkomt en op de recombinante precursor die uitgescheiden wordt door genetisch gemodificeerde, menselijk α-glucosidase producerende, Chinese hamster ovarium (CHO) cellen. Het enzym gezuiverd uit de melk wordt via de mannose 6-fosfaat receptor opgenomen door gekweekte fibroblasten van patiënten met GSDII en het corrigeert zowel de lysosomale α-glucosidase deficiëntie als de glycogeenstapeling. Eenmalige intraveneuze toediening van het recombinante enzym aan knockout muizen resulteert in de correctie van de enzym-deficiëntie in alle weefsels behalve de hersenen.

Op grond van deze veelbelovende resultaten is een begin gemaakt met de industriële productie van het recombinante, menselijke α-glucosidase in de melk van transgene konijnen.

Hiervoor wordt het genomische gen-construct gebruikt dat in de muizen werd getest. De potentiële therapeutische werkzaamheid van het enzym gezuiverd uit konijnenmelk wordt beschreven in hoofdstuk 7. Het lysosomale glycogeen in hart, skelet- en gladde spieren van de behandelde knockout muizen neemt af en de weefselschade is verminderd na wekelijkse toediening van het enzym gedurende zes maanden. In hoofdstuk 8 wordt het werk beschreven in dit proefschrift in een breder kader bediscussieerd.

Het in dit proefschrift beschreven werk demonstreert de mogelijkheid van productie van recombinant menselijk α-glucosidase in de melk van transgene dieren en de potentiële waarde van dit enzym voor de behandeling van patiënten met GSDII. Dit werk heeft de basis gelegd voor de start van een klinische trial (fase II), waarin de veiligheid en het effect van enzym vervangingstherapie bestudeerd wordt in patiënten. Deze trial is ondertussen begonnen op de afdeling Kindergeneeskunde van het Sophia Kinderziekenhuis te Rotterdam.

List of abbreviations

4MU	4-methylumbelliferyl-α-D-glucopyranoside
Arg	arginine
Asn	asparagine
Asp	aspartic acid
BMT	bone marrow transplantation
CD-M6P	cation dependent mannose 6-phosphate
(c)DNA	(complementary) Deoxyribonucleic Acid
CHO	Chinese hamster ovary
CI-M6P	cation independent mannose 6-phosphate
CNS	central nervous system
ER	endoplasmic reticulum
GlcNAc	N-acetylglucosamine
Glu	glutamic acid
GSDII	glycogen storage disease type II
IGFII	insulin like growth factor II
kb	kilo base
kD	kilo Dalton
KO/ko	knockout
LCR	locus control region
M6P	mannose 6-phosphate
MPS	mucopolysaccharidosis
(m)RNA	(messenger) Ribonucleic Acid
Pro	proline
rhGAA	recombinant human acid α-glucosidase
Ser	serine
SRP	signal recognition particle
TGN	trans golgi network
Thr	threonine
WT/wt	wild type

Curriculum Vitae

12 oktober 1966: Agnes Geertruida Antoinette Bijvoet geboren te Haarlem

1985   : Eindexamen gymnasium β, Triniteitslyceum te Haarlem.

1985 – 1991   : Scheikunde aan de Universiteit van Amsterdam. Specialisatie biochemie. Eén studiejaar is besteed aan extra algemeen chemische vakken.

April 1992   : Aanvang tweejarig onderzoeksproject op de afdeling Medische Biotechnologie, Universiteit van Leiden, bij Prof. Dr. H.A. De Boer, Dr. A.J.J. Reuser en Dr. M.Ph. Verbeet.

Augustus 1994 : Aanvang onderzoeksproject op de afdeling Klinische Genetica, Erasmus Universiteit Rotterdam, bij Prof. Dr. H. Galjaard, Mevr. Dr. A.T. van der Ploeg en Dr. A.J.J. Reuser.

Cursussen: Stralingshygiëne, deskundigheidsniveau 5b.
Strategies for purification, sequencing and structural analysis of proteins.
Engels: The Oxford examination in English as a foreign language, higher level.
Proefdierkunde (artikel 9), inclusief 100 studiebelastingsuren zoölogie en anatomie.

Vervolgens ging het werk in Rotterdam verder. Het DNA-lab rechts om de hoek, waar ik ben begonnen: Cathy, Carola , Pietro (Piet, m'n Italiaans is niet zo goed), Jeltje, Peter, Ester, Guido, Ingeborg, Marijke, Patrizia, Herma, Marcel en Robbert Jan), het eiwitlab, waar ik na de luchtverversing ben beland: Frans, André, Elly, Joris, Leontine, Filippo (Flip, m'n Italiaans blijft slecht), Mieke, Mark, Mirjam, Jeroen, Grazia; het stofwisselingslab: Jan, Rembert, Wistaria, Eric, Robin en Diny (bedankt voor de urine dunne laag plaatjes, jammer dat muizen toch net anders zijn), het biochemie-lab: Otto, Wim, Victor, Gerard, Patrick, Joke, Marijke en Jacqueline (bedankt voor de antwoorden op acute vragen, de onder handbereik zijnde literatuur, de hulp bij het stikstof tappen, glycogeen bepalingen en het opwerken van bloedjes), het DNA lab links om de hoek, Post en Pre nataal (Huub en Hannie bedankt voor de hulp bij de chromosoom bepalingen van de ES cellen), het secretariaat: Cilesta, Jeanette, Jacqueline, Jolanda, Hermine en Rita (bedankt voor de hulp bij het schrijven van brieven, faxen, telefoneren en de post op de juiste plek (in de zak = naar buiten, toch ?) deponeren), kortom alle bewoners en ex-bewoners (waaronder: E*, Henk, Manou, Aart, Coleta, Nicolle, Marjon, Elke, Erwin, Leon, Anita, Cecile en Karin) van de 24ste en ook 7de en 22ste etage: heel erg bedankt voor jullie inbreng, belangstelling en gezelligheid.

Natuurlijk ben ik ook diegenen dankbaar die juist buiten het lab hun inbreng hebben gehad. Familie, vrienden (waarvan een aantal reeds bovengenoemd is), Dorus en Doortje Rijkers, Anima Vitae, en alle anderen, bedankt voor jullie interesse, het begrip voor mijn afwezigheid en de ontspannende en gezellige uurtjes. Lieve Scott, Peter, Martijn, sinds kort ook Freek en Shannon en binnenkort "mini-Loeff", ik ben blij en dankbaar dat ik jullie mag leren kennen.
Om met Scott te spreken: "Wat gaat ie nòù doen?" Ik? Ik ga een ijsje eten. Maar pas na die mensen te hebben genoemd die me het meest nabij zijn.
Lieve mams, heel erg bedankt voor je betrokkenheid en alle steun. Het is aan jou en Marcel te danken dat het in huis geen enorme zooi geworden is en dat het geheel nu dan echt af is. Marcel, lief, bedankt voor alle steun, rust en ruimte die je me gegeven hebt om datgene te doen wat gedaan moest worden. Bij jou ben ik thuis.

Stellingen behorende bij het proefschrift:

Therapy for glycogen storage disease type II

Acid α-glucosidase production in milk
and enzyme replacement therapy in a mouse model

1. De productie van recombinant menselijke lysosomale eiwitten die *in vitro* en *in vivo* opgenomen worden is mogelijk in de melk van transgene zoogdieren.
   dit proefschrift

2. Op basis van de resultaten behaald na herhaald toedienen van recombinant menselijk zure α-glucosidase in GSDII knockout muizen is een klinische trial naar de effecten van enzym vervangingstherapie op patiënten met GSDII gerechtvaardigd.
   dit proefschrift

3. De mate van lysosomale stapeling van een bepaald substraat bij de totale afwezigheid van het betreffende enzym wordt niet bepaald door het stadium van ontwikkeling van het individu maar door de absolute leeftijd van de weefsels.
   de vele knockout muis modellen voor lysosomale stapelingsziekten
   Walvoort, J Inher Metab Dis 1983; 6:3-16.
   dit proefschrift

4. De genetische achtergrond is mede bepalend voor het fenotype ten gevolge van één mutatie in één bepaald gen.
   Raben et al. Hum Mol Genet 1996; 5:995-1000.
   Casal et al. Lab Invest 1998; 78:1575-1581.
   dit proefschrift

5. Bij het bestuderen van de mogelijkheid van substraat deprivatie als behandeling voor lysosomale stapelingsziekten dient rekening te worden gehouden met de mogelijkheid dat meerdere substraten stapelen.
   Liu et al. J Clin Invest 1999; 103: 497-505.

6. Het screenen op creatine kinase activiteit bij pasgeborenen kan leiden tot een vroege diagnose van Duchenne spierdystrofie en opent daarmee de mogelijkheid van tijdige erfelijkheidsvoorlichting en eventuele prenatale diagnostiek voor de betrokken families.
   Buschby et al. Lancet, 1999; 353:557-558.
   Parsons et al. 1996; Arch Dis Child; 74:550-553.

7. Wanneer intracytoplasmatische sperma injectie (ICSI) aangeboden wordt aan paren met fertiliteitsproblemen is naast goede voorlichting over de mogelijke effecten van het omzeilen van een natuurlijke biologische barrière bij de bevruchting op de ontwikkeling van de foetus en de ontwikkeling van het kind op latere leeftijd, ook genetisch onderzoek van man en vrouw van essentieel belang gezien de frequentie van genetische afwijkingen bij fertiliteitsproblemen.

> *Hewitson et al. Nature Med 1999; 5:431-433.*
> *Schlegel, J Androl 1999; 20:18-22/Lamb, J Androl 1999; 20:23-33.*
> *Thielemans et al. Eur J Obstet Gyn Reprod Biol 1998; 81:217-225.*
> *Tuerlings et al. Eur J Hum Genet 1998; 6:194-200.*

8. De term "identieke tweelingen" zou niet gebruikt moeten worden indien "monozygote tweelingen" bedoeld worden, gezien het voorkomen van verschillen in onder andere pigmentatie en haarkleur bij monozygote tweelingen.

> *Gringras, Lancet, 1999; 353:562.*
> *St Clair et al. Am J Med Genet 1998; 77:412-414.*
> *Machin, Am J Med Genet 1996; 61:216-228.*

9. De geldigheid van de derde wet van Newton (actiekracht = - reactiekracht) is niet beperkt tot de mechanica.

10. De combinatie promotieonderzoek in een laboratorium en ouderschap komt relatief vaker voor bij artsen en mannen.

Rotterdam 16 juni 1999   Agnes Bijvoet