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GLYCOGENOSIS TYPE II

A STUDY ON CLINICAL HETEROGENEITY AND ENZYME REPLACEMENT THERAPY

GLYCOGENOSE TYPE II

EEN STUDIE MET BETREKKING TOT KLINISCHE HETEROGENITEIT EN ENZYMVERVANGINGSTHERAPIE

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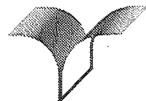

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Aan Pieter

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OBJECTIVES

Glycogenosis type II is a lysosomal storage disorder caused by deficiency of acid α-glucosidase and characterized by heart failure and skeletal muscle weakness.
The experimental work described in this thesis was performed to elucidate the cause of clinical heterogeneity in this disease and to test the feasibility of receptor mediated enzyme replacement therapy.

CHAPTER I

THE LYSOSOMAL SYSTEM AND RELATED DISORDERS

I.1 Lysosomes

The lysosomes as an entity were first recognized by De Duve as organelles containing acid phosphatase. At present it is known that they contain many different acid hydrolases (De Duve et al. 1955, 1983, Bainton 1981). Lysosomes have a variety of functions. They are part of the innate immune system and involved in the killing of multifarious microorganisms by leucocytes. They are the target in receptor mediated endocytosis (see I.6-I.8), and thus play an important role in the turnover of hormones and liberation of essential nutrients from their carrier proteins. Furthermore, they have a function in degradation of intracellular components, a mechanism defined as autophagy. In addition, lysosomes are involved in remodeling of tissues as cartilage and bone (Baron et al. 1985).

I.2 Lysosomal storage disorders

The concept of lysosomal storage diseases arose from the studies of Hers on type II glycogenosis (Hers 1963). He recognized that the accumulation of lysosomal glycogen was based on the deficiency of a single lysosomal enzyme, acid α-glucosidase. This pioneering discovery was followed by the recognition of various other lysosomal enzyme deficiencies involved in mucopolysaccharidoses (Bach et al. 1972, Neufeld et al. 1975, Cantz and Gehler 1976) and sphingolipidoses (Brady et al. 1966, Weinreb et al. 1968). At present about 30 distinct lysosomal storage disorders are known (Table 1, Galjaard 1980, Stanbury et al., 1983), whereas lysosomes are assumed to contain 60-70 different lysosomal enzymes (Barrett and Heath 1977). An explanation for this seeming discrepancy could be that some lysosomal storage disorders are not compatible with life, or on the contrary do not elicit symptoms. Further-

Table 1. The lysosomal storage disorders

DISEASE	DEFECTIVE ENZYME
Glycogenosis type II/ Pompe	acid α-glucosidase
Mucopolysaccharidoses	
MPS I/Hurler/Scheie	α-L-iduronidase
MPS II/Hunter	iduronate-sulfate sulfatase
MPS IIIA/Sanfilippo A	heparan-sulfate sulfatase
MPS IIIB/Sanfilippo B	N-acetyl-α-D-glucosaminidase
MPS IIIC/Sanfilippo C	acetyl Co A:α-glucosaminide-N-acetyltransferase
MPS IIID/Sanfilippo D	N-acetyl-α-D-glucosamine-6-sulphate sulfatase
MPS IVA/Morquio A	galactosamine-6-sulfate sulfatase
MPS IVB/Morquio B	β-galactosidase
MPS IVC/Morquio C	?
MPS VI/Maroteaux-Lamy	arylsulfatase B
MPS VII/Sly	β-glucuronidase
Mucolipidoses	
ML I/sialidosis	N-acetyl-α-neuraminidase
MLII/ I-Cell	N-acetyl-glucosaminyl phosphotransferase
MLIII/pseudo-Hurler polydystrophy	N-acetyl-glucosaminyl phosphotransferase
Mucolipidosis IV	?
Oligosaccharidoses	
Mannosidosis A	α-mannosidase
Mannosidosis B	β-mannosidase
Fucosidosis	α-fucosidase
Aspartylglucosaminuria	aspartylglucosaminidase

Table 1. continued

DISEASE	DEFECTIVE ENZYME
Sphingolipidoses	
GM1 gangliosidosis	β-galactosidase
GM2 gangliosidosis/Tay Sachs	hexosaminidase A
GM2 gangliosidosis/Sandhoff	hexosaminidase A and B
Galactosialidosis	protective protein
Ceramidetrihexoside lipidosis/Fabry	α-galactosidase
Metachromatic leukodystrophy	arylsulfatase A
Mucosulfatidosis	multiple sulfatase deficiency
Glucosylceramide lipidosis/ Gaucher	glucocerebrosidase
Lipogranulomatosis/ Farber	ceramidase
Sphingomyelin lipidosis/ Niemann-Pick A/B	sphingomyelinase
Galactosylceramide lipidosis/Krabbe	galactocerebrosidase
α-N-acetylgalacto-saminidase deficiency	α-N-acetylgalactosaminidase
Lipofuscinosis	?
Niemann-Pick C	?
Cholesteryl ester storage disease/Wolman	acid lipase
Free N-acetylneuraminic acid storage disease	defective sialic acid transport
Salla disease	
Cystinosis	defective cystine transport

more, it is possible that the incidence of various lysosomal storage diseases is so low, that a proper diagnosis has not been made until now. For example, the occurrence of α-N-acetylgalactosaminidase deficiency was established for the first time in 1987 (Van Diggelen et al. 1987, 1988), whereas the existence of this disease had been postulated more than 10 years earlier (Callahan et al. 1973, Schram et al. 1978).
Apart from enzyme deficiencies, lysosomal storage diseases can result from defects in the transport of degradation products from the lysosomes to the cytoplasm. This is observed in cystinosis and Salla disease (Gahl et al. 1982, Renlund et al. 1986, Mancini et al. 1986).
Most lysosomal diseases have an incidence at birth from 1 in 25,000 to 1 in 100,000. All together these disorders present once in 5000 live births. This incidence is comparable to that of Duchenne muscular dystrophy, and is higher than the incidence of phenylketonuria (1 in 10,000), for which a routine screening program in newborns exists. In certain ethnic groups lysosomal diseases occur exceptionally frequently, like Tay Sachs disease and Gaucher type I among Ashkenazi Jews. Most lysosomal disorders are inherited in an autosomal recessive mode. Only Fabry and Hunter disease are transmitted as X-linked traits. In the latter conditions also (female) carriers may present clinical symptoms due to selective inactivation of the X-chromosome (see for review Stanbury et al. 1983, McKusick 1986).
As a consequence of the distinct action spectra of lysosomal enzymes, the clinical presentation of lysosomal storage disorders differs considerably (Galjaard 1980, Stanbury et al. 1983, Galjaard and Reuser 1984). For example, bone malformations are observed in all mucopolysaccharidoses, but they are most severe in the Morquio syndromes. In the latter disease patients show no mental retardation, whereas this is a prominent feature in most other lysosomal diseases. In Fabry disease and glycogenosis type II neither of the two characteristics are encountered.
Extensive variability of clinical symptoms within one lysosomal disorder is common, and has sometimes led to confusion about the nomenclature. For example Hurler and Scheie disease both caused by α-L-iduronidase deficiency were initially defined as MPS I and V respectively. Later they have been reclassified as MPS IH and IS under the assumption that they represent different mutations within the same gene. Also Wolman disease and cholesteryl ester storage disease, and mucolipidosis II and III were thought to have a distinct etiology. The opposite occurred for GM1 gangliosidosis and galac-

tosialidosis. In both diseases a deficiency of β-galactosidase was established. Thus, it was assumed that they were variants of one disorder, despite their clinically distinct presentation (Pinsky et al. 1974, Loonen et al. 1974, Galjaard et al. 1975). At present it is known that in the latter variant enhanced degradation and inactivation of β-galactosidase and neuraminidase (Wenger et al. 1978) is induced by deficiency of a protective protein needed for stabilization and/or activation of these enzymes (D'Azzo et al. 1982, Hoogeveen et al. 1983). Another interesting situation is encountered in the Sanfilippo syndromes A, B, C, and D. These variants are based on distinct lysosomal enzyme deficiencies, but represent a group of patients with similar clinical symptoms.

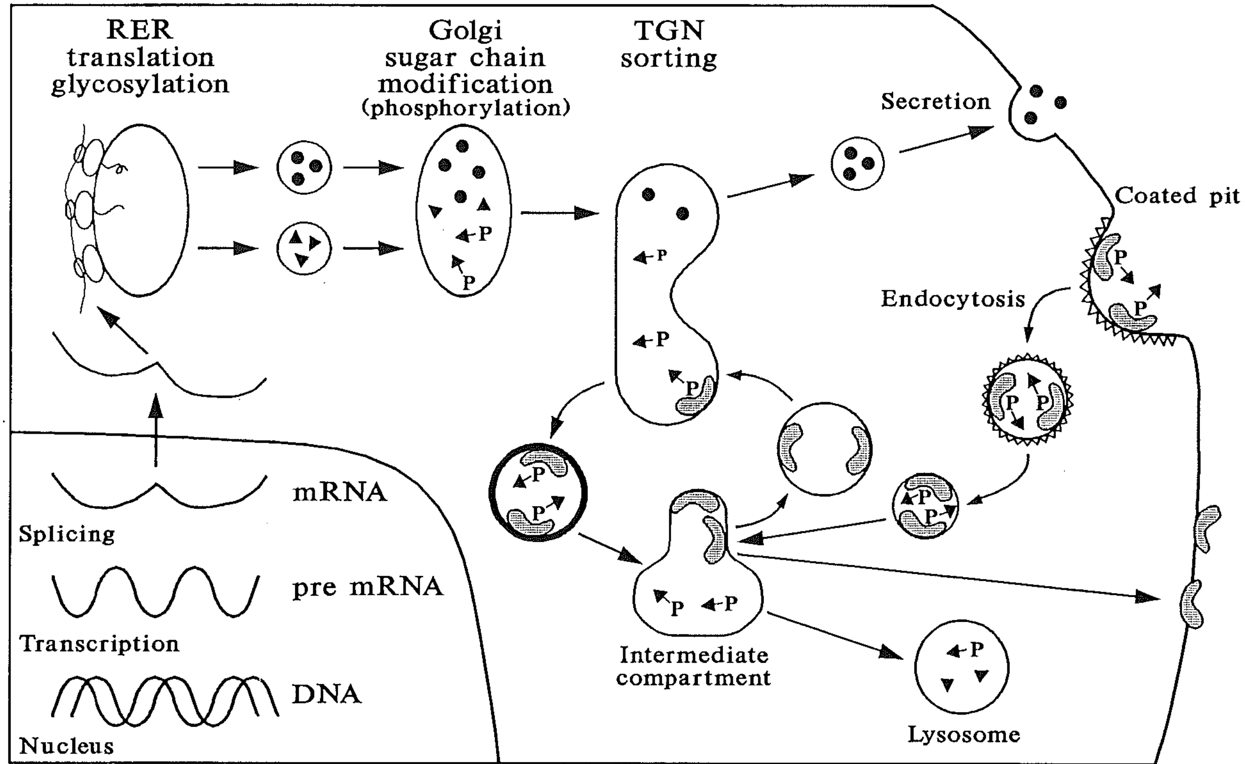
It has been demonstrated for some lysosomal storage disorders that the clinical diversity within one disease is primarily based on differences in residual activity of the deficient enzyme. A very clear example in this respect is glycogenosis type II (Mehler and DiMauro 1977, Shanske et al. 1986, Reuser et al. 1978, 1987, Publications I, II, III, V this thesis). Also in Tay-Sachs disease, Niemann-Pick disease, and Mucolipidosis II and III such a relation has been suggested (Inui et al. 1985, Gal et al. 1980, Ben-Yoseph et al. 1986). The cause of clinical heterogeneity in Gaucher's disease is still a matter of controversy, since the glucocerebrosidase activity is virtually the same in all phenotypes. However, it was demonstrated with immunoblotting and immuno-electron microscopy that in the relatively mild non-neuronopathic type 1 variant mature enzyme protein is present in the lysosomes of most patients (Ginns et al. 1982, Jonsson et al. 1987, Willemsen et al. 1987, 1988), whereas this is not the case in the acute (type 2) and subacute (type 3) neuronopathic forms. An explanation could be that the enzyme activity measured in the test tube does not reflect the in vivo situation. It was demonstrated that glucocerebrosidase activity in delipidated enzyme preparations derived from type I patients could be stimulated by the addition of activators (Glew et al. 1982). In vivo activator proteins are required to make lipids accessible to the lysosomal enzyme (Fürst et al. 1988). For instance, deficiency of an activator protein has been established in GM2-gangliosidosis. The patients show a clinical picture identical to that resulting from a deficiency of the corresponding lysosomal enzyme (see for review Sandhoff 1984). Lysosomal storage disorders with a defined enzyme deficiency can be diagnosed with reliability. However, rare pseudodeficiencies, such as the reported α-L-iduronidase and arylsulfatase A deficiencies in healthy individuals, may

complicate the matter (Whitley et al. 1987, Hohenschutz et al. 1988). Diagnostic material such as leucocytes, skin and muscle biopsies are easily available for postnatal diagnosis. Prenatal diagnosis can be performed on cultured amniotic fluid cells, obtained between 16-20 weeks of pregnancy (Galjaard 1980), or on chorionic villi, obtained in week 8-10 (Brambati et al. 1985, Galjaard and Kleijer 1985, Kleijer 1986). Development of the latter technique has been an important step forward. Heterozygote detection via enzyme diagnosis is often not reliable, since the enzyme activities measured are frequently within the control range.

I.3 Synthesis and transport of lysosomal enzymes

The realization of a lysosomal enzyme starts at the DNA level (Fig.1). Human DNA consists of 6×10^9 base pairs and contains an estimated 50,000-70,000 genes. Genes comprise several coding (exons) and non-coding regions (introns). Only 1-2% of the DNA represents coding sequences (McKusick and Ruddle 1977). Transcription of genes into mRNA is initiated from promoter sequences. Introns are spliced out and the resulting mature mRNA leaves the nucleus via the nuclear pores and becomes attached to the ribosomes. Translation of the messenger starts at the first AUG triplet coding for methionine and being part of a consensus sequence for translation initiation. In lysosomal, secretory and plasmamembrane glycoproteins the first methionine is followed by a few basic and a stretch of hydrophobic aminoacids, which form the signal sequence. This peptide is recognized by a signal recognition particle (SRP), which through binding to a docking protein, guides and couples the ribosomes to the membrane of the endoplasmic reticulum (RER), whereafter translation proceeds (Blobel 1980). The nascent protein enters the lumen of the RER. The signal sequence is detached from most lysosomal and secretory proteins, but may remain present as membrane anchor for plasma membrane proteins. Subsequently, Asn residues in the sequence (Asn-X-Ser/Thr) are glycosylated via en bloc transfer of an oligosaccharide precursor from dolichol pyrophosphate (Kornfeld and Kornfeld 1985). After limited oligosaccharide trimming the proteins leave the RER in smooth vesicles and enter the Golgi complex.
During transfer through the Golgi cisternae, the oligosaccharide chains of the secretory and plasmamembrane proteins are modified further to a sialic acid containing complex type of sugar chains (Fig.2). Most lysosomal enzymes

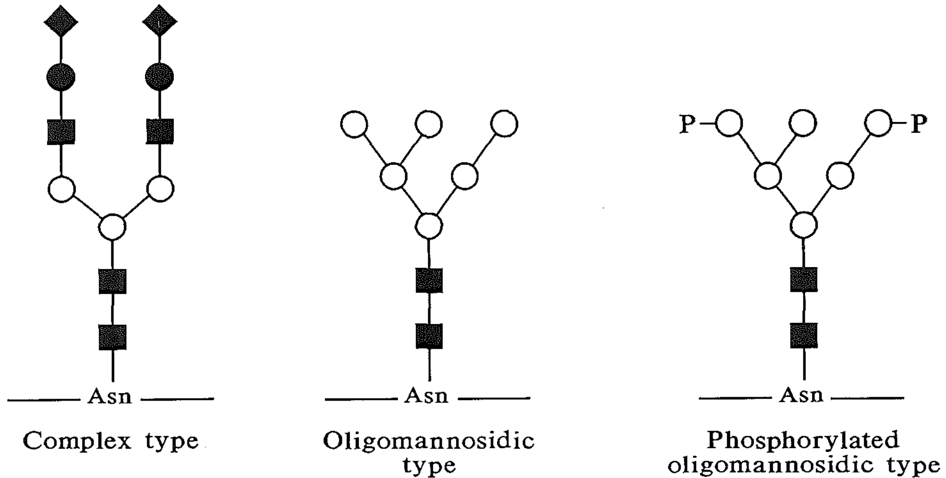
Fig 1. Synthesis and transport of lysosomal and secretory proteins



= Mannose 6-phosphate receptor ; ● = Secretory protein ; ▶ = Lysosomal enzyme

undergo different carbohydrate modifications. A subset of high-mannose type oligosaccharide chains are phosphorylated at the 6-carbon position of mannose by the concerted action of two enzymes, probably localized in the cis-Golgi (Reitman and Kornfeld 1981, Waheed et al. 1981). First, N-acetylglucosaminyl-phosphotransferase transfers N-acetylglucosamine-1-phosphate to selected mannose residues. Then, N-acetylglucosamine-1-phosphodiester α-N-acetylglucosaminidase removes the N-acetylglucosamine to expose the recognition marker (see for review Kornfeld 1986, 1987, Von Figura and Hasilik 1986).
Acquisition of the phosphomannosyl recognition marker is essential for trans-

Figure 2. Examples of Asparagine-linked oligosaccharides



○ =Mannose , ● =Galactose , ◆ =Sialic acid , P =Phosphate
■ =N-acetylglucosamine

port of most lysosomal enzymes to the lysosomes by means of mannose 6-phosphate (M6P) receptors. It is uncertain where the lysosomal enzymes and the M6P receptor meet en route to the lysosomes. Brown and Farquhar (1984) suggested that ligand and receptor associate in the cis-Golgi complex and leave the Golgi via smooth vesicles. Geuze et al. (1985), and Willemsen et al. (1988) found ligand and receptor in all Golgi cisternae with high concentration in the Trans Golgi Network (TGN). A recent report of Duncan and

Kornfeld (1988) suggests that the ligand first encounters the M6P receptor in the TGN, where the actual sorting of glycoproteins is thought to occur. Secretory and plasmamembrane proteins are transported to the outer cellular surface via smooth vesicles (Griffiths and Simons 1986). Lysosomal enzymes travel via coated vesicles to a prelysosomal acidic compartment where receptor and ligand dissociate (Brown et al. 1986, Griffiths et al. 1988). It is proposed that in this compartment the endocytotic and the endogenous pathway intermingle. The lysosomal enzymes are segregated in lysosomes and the M6P receptor cycles back to the Golgi complex.
In I-cell disease the phosphomannosyl recognition marker is not acquired due to deficiency of the phosphotransferase. Nevertheless some membrane associated lysosomal enzymes like glucocerebrosidase and acid phosphatase are transported to the lysosomes (Van Dongen et al. 1985). For acid phosphatase it was demonstrated recently that the enzyme actually arrives in the lysosome as a transmembrane protein (Pohlmann et al. 1988, Waheed et al. 1988). For glucocerebrosidase it has been demonstrated that complex oligosaccharide formation is required for efficient routing to the lysosomes. The enzyme does not undergo oligosaccharide phosphorylation (Aerts et al. 1986, 1988).

I.4 The cation independent mannose 6-phosphate receptor

Two M6P receptors have been identified. A larger cation independent and a smaller cation dependent receptor (See Pfeffer 1988 for review). Most is presently known about the cation independent receptor. This receptor was first recognized for its function in endocytosis of phosphorylated lysosomal enzymes (Kaplan et al. 1977, Sando and Neufeld 1977). Purification of the receptor from bovine liver by Sahagian and coworkers revealed a 215 kD protein as determined by SDS polyacrylamide gel electrophoresis (Sahagian et al. 1981). Recent cloning of the cDNA coding for the receptor and determination of the amino acid sequence showed that the molecular mass of the nascent receptor is approximately 270 kD, and after glycosylation 300 kD (Lobel et al. 1987, 1988, Oshima et al. 1988). The M6P receptor is predominantly localized intracellularly (90%), and only transiently exposed at the plasma membrane (10%) (Creek and Sly, 1984). Evidence exists that there is exchange between these different pools of receptors, since antiserum bound to cell surface receptors blocks eventually both receptor pools (Von

Figura et al. 1984). The bulk of receptors is present in the TGN (Geuze et al. 1984, 1985, Willemsen et al. 1988, Griffiths et al. 1988), where it has a function in the sorting of lysosomal enzymes. In lower amount the receptor is present throughout the Golgi complex, in endosomes, intermediate compartment, coated vesicles and on the plasma membrane. M6P receptors are not present in lysosomes. The receptor shows optimal binding of lysosomal enzymes at pH 6-7. Dissociation of receptor ligand complex occurs below pH 5.5. (Von Figura and Hasilik 1986, Tong et al. 1988). The M6P receptor is a transmembrane protein. The extracellular domain comprises 90% of the molecule, the residual part consists of a transmembrane region and a C-terminal cytoplasmic part (Sahagian and Steer 1985). Recently, Kornfeld and coworkers have shown that this cytoplasmic domain has a specific functional importance. Removal of the outer half of the cytoplasmic portion abolishes sorting capacities of the receptor, whereas complete removal of the cytoplasmic part leads to loss of the endocytotic capacity (Kornfeld, personal communication). Surprising was the finding of Morgan et al. (1987) that the cDNA sequence of the human insulin-like growth factor (IGF) II receptor showed a high degree of homology with the bovine cation independent M6P receptor (Lobel et al. 1987). Comparison of the full length cDNA of the human M6P receptor with the IGF II receptor showed that the two proteins are identical (Oshima et al. 1988). The extracellular part of the receptor is composed of 15 cysteine rich repeat units of 145 aminoacids. Despite the high degree of homology between these units, the receptor contains only 2 binding places for M6P. Evidence exists that IGF II and M6P bind at distinct places, since both proteins are bound in a non competitive manner (Morgan et al. 1987, Tong et al. 1988, Waheed et al. 1988, Kiess et al. 1988). This is not unexpected since the IGF II is not a glycoprotein, and thus does not expose the M6P recognition marker (Pfeffer 1988). One of the repeats contains an additional 43-residue segment that is similar to the type II repeat of fibronectin. It has been proposed that this area has a function in IGF II binding (Lobel et al. 1988, Pfeffer 1988). The exact function of IGF II is unknown, but the protein may have a function in early neonatal development (Morgan et al. 1987, Brown et al. 1986). IGF II is present in rather high concentrations in plasma and it has been speculated that receptor mediated endocytosis is a way of IGF II turnover (Pfeffer 1988, Oshima et al. 1988).

I.5 The cation dependent mannose 6-phosphate receptor

The cation dependent receptor is a 46 kD protein that requires divalent cations for ligand binding. The receptor was first recognized by Hoflack and Kornfeld (1985) in cell types deficient in M6P/IGF II receptors, and seems to have a function in sorting of lysosomal enzymes similar to the larger receptor (Hoflack et al. 1987). Many cell types contain both receptors, and it is puzzling why cell types expose two receptors with similar functions. Both receptors are present in bovine liver in equal amounts (Hoflack and Kornfeld 1985), whereas the 46 kD receptor is the predominant phosphomannosyl receptor present in bovine testis (Distler and Jourdian 1987). No cell line has yet been identified that lacks the 46 kD receptor. Although expressed at the cell surface, the 46 kD receptor is not able to mediate endocytosis of phosphorylated lysosomal enzymes (Stein et al. 1987). The pH optimum of the receptor is between 6 and 6.4 (Hoflack et al. 1987). Indeed, above this pH affinity for ligands decreases significantly, but is not completely abolished. Dissociation of receptor and ligand occurs below pH 5.3. The receptor functions as a dimer, and has the capacity to bind 2 mannose 6-phosphate containing ligands. The affinity is highest for ligands exposing 2 phosphomonoesters, as demonstrated for the larger receptor. Cloning of the cDNA coding for the receptor has revealed only sparse homology with the larger receptor (Pohlmann et al. 1987, Dahms et al. 1987). The cytoplasmic and transmembrane parts are completely distinct. In the extracellular part, however, one 145 amino acid long cysteine rich domain is found similar to the repeat units that make up the M6P/IGF II receptor. This has led to the hypothesis that both receptors are evolutionary related (Lobel et al. 1988).

I.6 Mannose 6-phosphate receptor mediated endocytosis

Since the M6P/IGF II receptor was first recognized for its function in endocytosis of phosphorylated lysosomal enzymes, it was proposed that the receptor had a function in recapture of these proteins after secretion (Creek and Sly, 1984). The receptor has been demonstrated on various cell types (See Table 2). In fibroblasts, about 0.5-1% of the plasmamembrane proteins are M6P/IGF II receptors (Von Figura and Hasilik 1986). The receptors are often found clustered in membrane areas that contain clathrin (Willingham

et al. 1981, Geuze et al. 1985, Mellman et al. 1987). These areas are called coated pits because of their fuzzy appearance in electron microscopy. Once the phosphorylated enzymes are bound to the receptor, the coated pit invaginates and pinches off from the plasma membrane and becomes a coated vesicle. Shortly after loosing contact with the plasmamembrane, the clathrin coat is disassembled, and a smooth vesicle remains. The further route of the vesicles has not been fully elucidated. It has been proposed that the smooth vesicles fuse with the endosomal compartment and that the receptor-ligand complex moves further to a prelysosomal compartment. This latter compartment has been defined as multivesicular endosome, or intermediate compartment, and is the possible location where the endogenous and endocytosed lysosomal enzymes meet (Brown et al. 1986, Griffiths et al. 1988). From this compartment lysosomal enzymes are channeled to lysosomes, while the M6P receptor cycles back to the Golgi complex or the plasma membrane. An interesting question remains how differential transport to these locations is organized. Modification of the cytoplasmic domain of the receptor by site-directed mutagenesis of the cloned receptor cDNA can possibly give an answer.

I.7 Other glycoprotein receptors

Other glycoprotein receptors which have been identified are the mannose receptor on macrophages and the fucose and asialoglycoprotein receptor on hepatocytes (Table 2). Endocytosis via these receptors involves the same mechanism as described for the M6P/IGF II receptor. These receptors, however, do not move to the Golgi complex, and have no function in transport of endogenous proteins.

Most soluble lysosomal enzymes purified from human tissues have mannose rich sugar chains and are therefore recognized by the mannose receptor. In plasma many glycoproteins have complex type of sugar chains with sialic acid as the terminal sugar, and are very slowly cleared from the circulation. Upon removal of the outermost sialic acid, a penultimate galactose is exposed which is recognized by the asialoglycoprotein receptor, and could thus play a role in the clearance of circulating glycoproteins. The biological function of the fucose receptor is not fully understood.

TABLE 2

Glycoprotein receptors involved in endocytosis

Receptor	cell type	Reference
galactose/ N-acetylgalactosamine	hepatocyte	(1)
fucose	hepatocyte	(2,3)
mannose/ N-acetylglucosamine	macrophages	(4,5)
mannose 6-phosphate/ IGF II	fibroblasts	(6)
	hepatocytes	(7)
	macrophages	(8)
	skeletal muscle	(9)
	smooth muscle	(10)

(1) Ashwell and Morell 1974, (2) Prieels et al. 1978, (3) Furbish et al. 1980, (4) Stahl et al. 1976, (5) Achord et al. 1978, (6) Kaplan et al. 1977, (7) Ullrich et al. 1979 (8) Shepherd and Stahl 1984, (9) Reuser et al. 1984, (10) Hasilik et al. 1981.

I.8 Receptor mediated endocytosis in general

Receptor mediated endocytosis is a mechanism which serves many purposes (Stahl and Schwartz 1986, Brown et al. 1983). It can for instance be used for the delivery of certain nutrients; cholesterol associated to LDL via the LDL receptor, vitamin B12 associated to transcobalamin II via the transcobalamin II receptor, and iron associated to transferrin via the transferrin receptor. Furthermore, certain hormones are degraded in the lysosomes after accomplishing their task. Examples are insulin, epidermal growth factor, chorionic gonadotropin. Probably, the lysosome is also the final destination of IGFII. In addition, certain viruses use receptors for cell entry (Zeichhardt et al. 1985).

CHAPTER II

THERAPEUTICAL APPROACHES IN LYSOSOMAL STORAGE DISORDERS

II.A ENZYME REPLACEMENT THERAPY

A.1 Introduction

In the early seventies it was generally assumed that the development of a therapy for lysosomal storage disorders was only a matter of time. This optimistic view was based on two important findings. Firstly, most lysosomal storage disorders appeared to be due to the deficiency of a single lysosomal enzyme (I.2). Secondly, the lysosomal apparatus was demonstrated to be accessible to exogenous macromolecules (De Duve 1964). Knowledge about specific receptor mediated endocytosis had not yet been obtained. The feasibility of a therapy for lysosomal storage disorders was further sustained by findings of Fratantoni et al. (1968), who demonstrated that cultured fibroblasts derived from Hunter and Hurler patients were able to correct each other mutually by exchange of enzyme. In addition, Jacques et al. (1969) established that artificial storage of sucrose in rat liver could be prevented by prior injection of invertase. Various attempts at in vitro correction of lysosomal diseases followed, using partially purified enzymes, and apparently with success (Neufeld and Cantz 1973, Porter et al. 1971, Dawson et al. 1973, Brot et al. 1974, Neufeld 1980).

A.2 Applications

The first attempt at enzyme replacement therapy for lysosomal storage diseases was performed for glycogenosis type II (Baudhuin et al. 1964). For this purpose, a crude extract of Aspergillus Niger was obtained, containing α-glucosidase activity towards glycogen at lysosomal pH. Intravenous injection of the enzyme preparation in mice had resulted in a significant increase

of acid α-glucosidase activity in muscle and liver immediately after administration, but enzyme activity declined rapidly over the following hours. A considerable decrease of hepatic glycogen stores was measured in healthy animals, suggesting an effect on the cytoplasmic glycogen pool. Intramuscular and intravenous administration of the extract to various patients with infantile glycogenosis type II had limited success (Baudhuin et al. 1964, Lauer et al. 1968, Tyrell al. 1976).

Hug et al. (1967, 1968) treated a 5 months old infant for 116 days with the enzyme preparation. After the first 18 days of treatment a pronounced increase in liver α-glucosidase activity was recorded, and complete clearance of glycogen storage vacuoles in the liver was observed by electron microscopy. A slight clinical improvement and amelioration of ECG abnormalities was reported. However, the positive effects could not be maintained. At 116 days of treatment the child developed an immune nephritis due to antibody formation against the heterologous Aspergillus Niger preparation, and the therapeutical efforts were stopped. The patient died 7 days later of cardiorespiratory failure. At postmortem examination, the pathology of liver and muscle was completely identical to that in severe classical Pompe disease. Thus, this type of treatment did not have a favourable effect on the course of the disease.

Also the few attempts at enzyme supplementation with small amounts of homologous enzyme purified from human placenta were unsuccessful (Hers and Van Hoof 1973, De Barsy and Van Hoof 1974). As in the former studies with Aspergillus Niger α-glucosidase, enzyme uptake was reported in liver, but no effect on glycogen stores was obtained. The authors concluded that uptake of the enzyme in the liver does not lead to reversal of glycogen storage.

Similar therapeutical interventions were performed for other lysosomal storage disorders (see for review Tager et al. 1974, Desnick 1980). For this purpose, various enzymes from human sources were purified. Noteworthy are the results obtained for Fabry disease (Brady et al. 1973, Desnick et al. 1979). Intravenous administration of α-galactosidase to patients resulted in a transient but significant decrease (58%) of accumulated plasma ceramidetrihexoside levels. This was assumed to result from redistribution of storage compounds to tissues cleared of storage products by endocytosed exogenous enzyme. This is of particular interest, since the clinical symptoms in Fabry disease result directly from storage in endothelial cells. The latter cells derive their storage compounds from blood plasma.

One of the problems encountered in enzyme replacement therapy is the predominant uptake of infused enzymes by the liver. Since in Gaucher disease type I the major storage occurs in macrophages, including the Kupffer cells of the liver, the latter disorder seemed the candidate of choice for treatment by enzyme replacement therapy. Extensive studies were performed (Brady et al. 1974, 1984, 1985, Beutler et al. 1980). Brady and coworkers treated about 13 Gaucher patients repeatedly with large doses of human placental glucocerebrosidase. Some patients received treatment for more than 5 years. The initial results were very promising, since a significant decrease in liver-, plasma- and erythrocyte associated glucocerebroside was observed. However, the long term clinical effects were inconclusive, except that none of the patients required splenectomy because of thrombocytopenia. Importantly, no antibody formation was detectable, despite frequent and long-term exposure to exogenous enzyme. Probably, the presence of endogenous mutant enzyme protein prevents an antigenic reaction.

A.3 Remarks

With the present knowledge about receptor mediated endocytosis, and the composition of the carbohydrate chains of lysosomal enzymes, the limited success of enzyme therapy can largely be explained (See section I.6, I.7). Various lysosomal enzymes purified from human placenta are rapidly cleared from the circulation by mannose/N-acetylglucosamine receptors of macrophages (Stahl et al. 1976, Achord et al. 1977, 1978, Steer et al. 1979). Sugar analysis of e.g. human placental acid α-glucosidase revealed that the enzyme contains predominantly oligomannosidic types of sugar chains (Mutsaers et al. 1987), which are not phosphorylated (Reuser et al. 1984). This might explain why in the clinical trials of De Barsy and Van Hoof (1974), uptake of placental enzyme in liver did not result in breakdown of glycogen. Enzyme was probably taken up by Kupffer cells, whereas glycogen storage is most prominent in hepatocytes.
Structural analysis of the carbohydrate chains of glucocerebrosidase purified from human placenta revealed that the enzyme predominantly contained complex type of sugar chains (Furbish et al. 1984). Sialic acid (neuraminic acid) is the terminal sugar followed by galactose and N-acetylglucosaminyl residues, respectively. Glycoproteins terminating with sialic acid are slowly cleared from the circulation, since they are not recognized by a specific cell

surface receptor (Furbish et al. 1978). Indeed, desialylation of the native enzyme decreased the plasma half life from 21 to 1.2 min, and this enzyme was recovered almost exclusively in hepatocytes. When subsequently the galactose residues were removed, the modified glucocerebrosidase was cleared with a half life of 1.5 min, and uptake of enzyme was found mainly in the Kupffer cells of the liver, mediated by the N-acetylglucosamine/mannose receptors. Uptake of enzyme by macrophages in vitro was even further enhanced when additional mannose residues components were coupled to the native enzyme (Doebber et al. 1982). The authors conclude that the native human placental glucocerebrosidase used in clinical trials is probably taken up by inefficient fluid phase endocytosis, which could explain the poor clinical results (Brady et al. 1974, 1984, 1985).

A.4 Stabilization of enzymes

Various attempts have been made to reduce the rapid clearance of infused enzymes from the circulation (see for review Desnick 1980). Enzymes were packaged in liposomes and erythrocyte ghosts (Gregoriadis 1976, Tyrell et al. 1976, Beutler et al. 1980). Success was limited and in addition liposomes appeared to stimulate the antigenic response. Stabilization of enzymes by complexation with other macromolecules appeared more successful e.g. human placental acid α-glucosidase covalently linked to albumin appeared highly resistant to bioinactivation (Poznansky and Bhardwaj 1980). Furthermore, polyvinylpyrrolidone-β-hexosaminidase complexes appeared to circulate significantly longer than β-hexosaminidase alone (Von Specht et al. 1979).
With respect to enzyme stabilization, promising results were obtained in severe combined immunodeficiency disease based on adenosine deaminase (ADA) deficiency (Hershfield et al. 1987). In this disease T cell function is abolished due to accumulation of toxic phosphorylated metabolites. The T-cell entrapped storage products are in equilibrium with substrates in plasma. Addition of polyethylene glycol (PEG) to bovine adenosine deaminase prolonged the plasmahalf life of the enzyme in patients from a few minutes to 48-72 hours, diminished the immunogenicity, and initiated restoration of T-cell function. Possibly enzymes coupled to PEG can have wider applicability e.g in Fabry disease (See section II.A.2).

A.5 The blood brain barrier

In many lysosomal storage disorders involvement of the central nervous system (CNS) is prominent. Since the CNS is carefully protected by the blood brain barrier (BBB) from endogenous and exogenous agents, the brain was soon considered the most arduous target in enzyme replacement therapy. To circumvent the BBB, intrathecal, intracerebral and intracisternal injections of purified lysosomal enzymes were applied, but without effect (Greene et al. 1969, Von Specht et al. 1979). As an alternative approach, attempts were undertaken to disrupt the BBB by infusion of hyperosmolar solutions (Rapoport et al. 1971). Barranger et al. (1979) reported that intra-arterial infusion of solutions of arabinose or mannitol immediately followed by administration of α-mannosidase or β-hexosaminidase resulted in uptake of enzyme in rat brain. Uptake of β-hexosaminidase was about 0.5% of the infused dose. The enzyme appeared to be predominantly localized in lysosomes (Neuwelt et al. 1983), but the uptake efficiency was considered too low to warrant a clinical trial.
Rattazzi et al. (1980) demonstrated that hexosaminidase A could be delivered to brains of cats with GM2-gangliosidosis by means of hyperbaric disruption of the BBB. Uptake was even further enhanced when mannans were infused together with the enzyme to block the mannose receptors of the liver. Degradation of GM2-ganglioside was, unfortunately, not observed due to minimal uptake of enzyme by the neurones (Rattazzi 1983). This is sustained by in vitro experiments showing that the endocytotic activity of neuronal cells is rather low, and many times exceeded by the uptake capacity of astrocytes and other non-neuronal cell types present in the CNS (Rattazzi et al. 1987). Noteworthy in this context is the recent development of a complex of hexosaminidase A and tetanus toxin fragment C (TTC). TTC has retained the neuron-binding and internalization properties of the holotoxin, but is non-toxic. In vitro, the complex is efficiently endocytosed by neuronal cells. Further results have to be awaited (Rattazzi et al 1987).

A.6 Further attempts at therapy

In attempts at enzyme replacement therapy it has always been a major problem to obtain a sufficient amount of purified enzyme. Transfusions with fresh

frozen plasma and leucocytes were applied, as an alternative method for enzyme supplementation, but with limited effect (Di Ferrante et al. 1971, Dekaban et al. 1972, Knudsen et al. 1971). Organ transplantations were performed to create a continuous source of "exogenous" enzyme. Increase of enzyme activity in organs of the acceptor was, however, not obtained (Groth et al. 1971, 1979, Van den Bergh et al. 1976). On the other hand, transplantations performed to replace severely impaired organs per se can still be worthwhile. Kidney transplantations have significantly prolonged the life span in nephropathic cystinosis (Fivush et al. 1987). Furthermore, it has been reported that treatment of these patients with cysteamine has an effect on cystine storage in vivo, and can help in maintaining renal function (Gahl et al. 1987).
Transplantations of fibroblasts and skin initially appeared to have some positive effects (Dean and Muir 1975), but this was not confirmed by later investigations (Dean et al. 1982, Adinolfi et al. 1986). Scaggiante and coworkers (1987) reported significant beneficial clinical effects and reversal of hepatosplenomegaly after transplantation of amniotic membranes in patients with Niemann-Pick type B. Amnion transplantations in various patients with other lysosomal storage disorders showed transient (Tylki- Szymanska et al. 1985) or no beneficial responses (Yeager et al. 1985). Also amnion implantations into cattle with glycogenosis type II provoked no positive effects (Howell et al. 1987).

II.B BONE MARROW TRANSPLANTATION

B.1 Introduction

Bone marrow transplantation has proven to be an efficacious therapy for various hematologic disorders. Since normal bone marrow continuously produces enzyme competent circulating blood cells, it has been proposed that bone marrow transplantations might also be effective in the treatment of lysosomal storage disorders. Several mechanisms for correction of enzyme deficiency have been suggested.

- Enzyme transfer from cell to cell. Olsen and coworkers have demonstrated that lymphocytes and macrophages are able to transfer various lysosomal enzymes like β-glucuronidase, β-hexosaminidase, α-mannosi-

dase and α-L-iduronidase to enzyme deficient fibroblasts by means of cell-cell contact (Olsen et al. 1981, 1983, Abraham et al. 1985, Dean et al. 1988). Transfer appeared independent of the mannose 6-phosphate receptor. Deficiency of glucocerebrosidase, a membrane bound lysosomal enzyme, could not be corrected in this model system. In these experiments donor cells were applied to fibroblasts in a 100 to 1 ratio.

- Replacement of enzyme deficient cell types. Macrophages, derived from bone marrow, home in various tissues e.g. liver (Kupffer cells), spleen, lung, peritoneum and lymphoid system. In addition, chondroclasts, osteoclasts, and Langerhans cells in the skin are derived from monocytes. Also microglia in the brain are probably macrophages (Oemichen 1982, Perry and Gordon 1988).
- Capture of secreted lysosomal enzymes. Stimulated macrophages secrete lysosomal enzymes, but secretion is minimal under normal conditions (Imort et al. 1983, Dean et al. 1988).

B.2 Applications

Bone marrow transplantation as a treatment for lysosomal storage disorders was first performed in Hurler disease (Hobbs et al. 1981). At present more than 30 Hurler patients have received a bone marrow transplant (Table 3). They are the largest group of patients transplanted for a lysosomal storage disease. In all surviving patients reversal of hepatosplenomegaly was observed, and in most cases improvement of corneal clouding and an increase of joint mobility (Krivit et al. 1988, Hobbs 1987, 1988). Overall, no amelioration of skeletal malformations was observed. This is consistent with the fact that storage of glycosaminoglycans in chondrocytes was not reversed after transplantation (Whitley et al. 1986). The lack of beneficial effects on skeletal abnormalities was also recorded in other mucopolysaccharidoses, like Hunter-, Morquio-, and Maroteaux-Lamy disease. (Hobbs 1987, 1988, Krivit and Paul 1986).
It is not sure whether an early pre-symptomatic bone marrow transplantation can prevent bone lesions. Six years post-grafting the first transplanted Hurler patient (Hobbs 1981, 1987) started to show some dysostosis of the wrists.
Since storage of glucocerebroside in Gaucher disease occurs predominantly

TABLE 3

Bone marrow transplantations for lysosomal storage diseases

Disease	BMT (n)	alive (n)	follow up	hepato (spleno) megaly	skeletal abn.	CNS functions	Ref.
Hurler	23	13	-6y	i	s/d	s/i	(1,2)
	9	7	6m-5y	i	s/d	i	(3)
Hunter	4	2	?	i	d	d	(1)
	1	1	6m			s	(1)
Gaucher	7	6	-4y	s/i	s/i	?	(2,4)
	1	0	11m	d	d	d	(5)
	3	3	1,3,5y	i	s/d	i/d	(6)
GSDII	3	0	1-6m				(2,7,8)
Morquio	2	2	4y	i	d		(2,9)
MLD	1	1	4y			s/i	(3,10)
	1	1	33m			s	(11)
Krabbe	2	2	1-2y			?	(3)
Mannosidosis	1	0	4m	i		d	(12)
Maroteaux-Lamy	1	1	5y	i	d		(3,13)
Sanfilippo A	1	1	18m			?	(3)
	2	1	3y	i		d	(2,14)
Sanfilippo B	2	2	5y	i		s	(2)
	1	1	1y			s	(3)
Wolman	1	0	10d	d	d	d	(2)
GM1 gangliosidosis	1	0	35d	i		d	(2)
Niemann-Pick A	1	1	3m				(3)
Niemann-Pick B	1	1	3m				(2)

m=months; y=years; s=stabilized; i=improved; d=deteriorated

(1) Hobbs 1987, (2) Hobbs 1988, (3) Krivit et al. 1988, (4) Hobbs et al. 1987, (5) Ginns et al. 1984 (6) Rindgen et al. 1988 (7) Watson et al. 1986, (8) Harris et al. 1986 (9) Desai et al. 1983, (10) Krivit et al. 1987, (11) Bayever et al. 1985, (12) Will et al. 1987, (13) Mc Govern et al. 1986. (14) Hugh-Jones et al. 1984.

in macrophages of liver, spleen and bone marrow, much was expected from bone marrow transplantation as treatment. Ginns et al. (1984) transplanted an 8 year old patient with a far advanced stage of Gaucher disease type III. The patient had experienced multiple bone fractures and severe liver fibrosis had developed. After successful grafting Gaucher cells disappeared gradually from the bone marrow. However, no clinical improvement was observed. Hepatomegaly[1] persisted and the patient died 11 months after transplantation. More positive results were obtained in an 8 year old girl with the Norrbottnian type of Gaucher's disease (Svennerholm et al. 1984, Rindgen et al. 1988). Before transplantation she had massive hepatosplenomegaly, but no evidence of skeletal destruction or mental impairment. After transplantation she showed significant clinical improvement and growth acceleration. Hepatomegaly[1] reversed over a 2 year period. However, 3 years after transplantation her intellectual skills started to deteriorate slightly, and some chest deformation started to develop. Therefore, it is dubious whether bone marrow transplantation is effective in Gaucher disease. A constant finding is the definite but extremely slow disappearance of Gaucher cells from bone marrow and liver (6 months unto one year)(Ginns et al. 1984, Rindgen et al. 1988, Hobbs et al. 1987, 1988).
Bone marrow transplantations applied in cases of infantile glycogenosis type II had no beneficial clinical effects. At least three patients varying from 5-15 months of age have been transplanted. No increase of acid α-glucosidase activity was detectable in liver, heart or skeletal muscle. Glycogen storage was not influenced, and the fatal clinical course of the disease was not altered (Harris et al. 1986, Watson et al. 1986, Hobbs 1988).

B.3 Effects of bone marrow transplantations on the CNS

It is still a subject of controversy as to whether bone marrow transplantation can prevent or correct lesions in the central nervous system. For various Hurler patients it has been reported that mental deterioration was stabilized, prevented or even slightly reversed after transplantation (Hobbs 1987, 1988, Hugh- Jones 1986). The exact cause of this phenomenon is unsure. Only one patient has been reported with biochemical evidence for enzyme uptake in brain. An α-L-iduronidase activity of 4% of normal was recorded in a Hurler patient who died 15 months post-transplant (Hobbs 1987). In non-

[1] splenectomy was performed prior to bone marrow transplantation

transplanted Hurler cases virtually no residual enzyme activity was measured in brain. In addition, decrease of ventricular size on CT scan and improvement of the magnetic resonance imaging pattern of the brain was observed in other Hurler patients (Johnson et al. 1984, Hugh-Jones 1986, Whitley et al. 1986). An explanation can be that the increased intracranial pressure and the concomitant hydrocephalus is relieved or does not occur as a result of degradation of glycosaminoglycans in the meninges (Hugh-Jones et al. 1984). A problem in these trials is, that one can not exactly predict how mental skills would have developed without therapeutical intervention. For example, in Hurler-Scheie syndrome the central nervous system is less progressively involved than in Hurler disease, whereas mental retardation does not occur in Scheie disease. These three phenotypes can not be distinguished by different levels of α-L-iduronidase.
Improvement of the mental condition was not only reported for Hurler disease, but also for metachromatic leukodystrophy and Sanfilippo B syndrome (Krivit et al. 1987, Hobbs 1988). In the latter two cases conclusions were drawn by comparing the course of the disease in a propositus of the same family. While the clinical status of the siblings was similar prior to transplantation, the progression of neurological symptoms was significantly different afterwards.
In α-mannosidosis, Krabbe- and Niemann-Pick type A disease bone marrow transplantation remained without effect on CNS functions (Will et al. 1987, Hobbs 1987, 1988, Krivit et al. 1988).

B.4 Studies in animal models

To elucidate the effect of bone marrow transplantation on development of CNS lesions, studies have been performed in animal models. Transplantation applied in feline Maroteaux-Lamy syndrome and murine Niemann-Pick disease did not result in increase of enzyme activity in brain (Wenger et al. 1986, Sakiyama et al. 1983). Taylor et al. (1986, 1988) reported a marked increase of enzyme activity in brain of fucosidosis dogs after bone marrow transplantation. Delay or absence of CNS symptoms was only observed in dogs transplanted before 6 months of age. Shull et al. (1987, 1988) reported expression of α-L-iduronidase in brains of dogs with mucopolysaccharidosis I, and prevention of symptoms. But the follow-up period is still too short to be conclusive. Hoogerbrugge et al. (1988) demonstrated the presence of donor

derived macrophages and decreased psychosine levels in brain of Twitcher mice, an animal model for Krabbe disease, when transplanted at day 7. However, development of neurological symptoms occurring at 3-4 weeks of age was not prevented. The average life span of transplanted animals was significantly prolonged, from 4-5 weeks to 14-15 weeks (Hoogerbrugge 1988).

B.5 Remarks

Beneficial effects have been obtained with bone marrow transplantation as treatment for lysosomal storage diseases. Reversal of hepatosplenomegaly, improvement of corneal clouding and increased joint mobility were the most consistent findings. For various patients an improved general condition has been reported. Skeletal malformations were not corrected. Also the pathology in other tissues like heart and skeletal muscle did not change after transplantation. Whether symptoms originating from the CNS can definitely be prevented, reversed or stabilized is still unsure. Follow-up studies and results on more patients are required. Facts which render the interpretation of the results difficult are:

- The occurrence of clinical heterogeneity.
- Improvement of the clinical condition and visual acuity can increase intellectual performance.
- Children frequently subjected to IQ-tests may develop some skills in performance.
- Children which have been transplanted are surrounded by much care.
- Patients eligible for bone marrow transplantation are carefully selected.

It is generally assumed that bone marrow transplantation is most efficacious when performed at young age. It has to be realized, however, that bone marrow transplantation are associated with high risks. About 80% of the patients survives the procedure when bone marrow of an HLA identical, MLC-non reactive sib is available (Hobbs 1987). Also in this group of patients graft versus host disease (GHVD) often occurs. Moreover, only 1 in 3 patients has a completely matching sibling. In the other cases, bone marrow of one of the parents or a matching donor is often used, reducing the survival to 50%. Further there is a much higher chance of debilitating complications

like acute and chronic GVHD, interstitial pneumonia, and other infectious diseases. It is generally assumed that the risks of bone marrow transplantation increase significantly with increasing age. Therefore, transplantation is not advised in adult patients with mild variants of a disease.

II.C GENES AND THERAPY

C.1 Somatic gene therapy

In recent years many genes coding for lysosomal enzymes have been cloned. The applicability of gene therapy is currently under investigation for several diseases, and has also been proposed for the lysosomal disorders (Ledley 1987, Hermans and Bootsma 1987, Choudary et al. 1986). Various techniques have been developed to introduce foreign DNA in cultured mammalian cells (transfection). The most simple method is to add DNA to cells as calcium phosphate precipitate or as complex with diethylaminoethyldextran, procedures mostly used for transient expression of DNA. Other methods for transfection are electroporation, microinjection and retroviral mediated gene transfer.
When it comes to in vivo application of somatic gene therapy, only the latter form of transfer seems of significance. For this purpose, the gene of interest is cloned in a retroviral vector and packaged in a helper virus. The newly created retrovirus has the possibility to infect cells once, but can not replicate. The foreign DNA integrates in the host genome.
At present gene therapy is mostly considered in combination with bone marrow transplantation. Stem cells representing 0.1-0.5% of the cell population are the target. Therefore, diseases which can in principle be corrected by bone marrow transplantations are the first choice for application of gene therapy. In 1980, the first gene transfer was conducted for treatment of human thalassemia, after successful expression of rabbit globin genes in mouse bone marrow. The attempt, however, failed and the early application in patients raised heavy criticism (Cline et al. 1980). In recent years, much effort has been devoted towards gene therapy for ADA deficiency in animal models (Anderson et al. 1986, Kohn et al. 1987). Overall, the results on somatic gene therapy are rather disappointing. Expression of transferred genes is extremely low, probably as a result of down regulation by at random integration of the transferred DNA (Stead et al. 1988). In this context, the results obtained with expression of the β-globin gene are promising (Grosveld et al.

1987). Creation of a minilocus, containing the human β-globin gene and the 5' and 3' flanking regions of the β-globin locus, resulted in a position independent high level of expression in transgenic mice.
It has to be awaited whether gene therapy via hemopoietic stem cells will be applicable for lysosomal storage disorders. In this respect, the outcome of the ongoing clinical trials on bone marrow transplantations will be crucial. Since for gene therapy the patient's own stem cells are used, it has a theoretical advantage over bone marrow transplantation by obviating the many immunological limitations of the latter treatment.
Gene therapy via other cell types may be considered, when bone marrow transplantation per se is ineffectual, as seems the case for glycogenosis type II; for instance via fibroblasts (Garver et al. 1987, Selden et al. 1987), hepatocytes or myoblasts. The future will learn whether such developments will offer new opportunities for treatment of lysosomal storage disorders.
Application of gene therapy is certainly not without risks (Ledley 1987, Hermans and Bootsma 1987). For example, retroviral vectors can recombine with proto-oncogenes, and activate their oncogenic potential. Furthermore, the transfected DNA can randomly integrate in the host genome and create new mutations. In addition, recombination of the defective retrovirus with endogenous viruses can result in creation of new "infectious" viruses. Although, important progress has been made, there is still a long way to go before somatic gene therapy can be applied to humans (Weatherall 1988).

C.2 Germ line therapy

The feasibility of germ line therapy has been explored with success in animal models (Palmiter et al. 1983, Wagner et al. 1981). For this purpose, single cell embryos were injected with the gene of interest. An exquisite example is the creation of transgenic mice expressing the human insulin gene (Selden et al. 1987). In these mice insulin was only produced by the pancreas under the control of the normal physiological parameters. Germ line therapy is however not applicable for correction of genetic defects in humans (Anderson 1986, Ledley 1987). Firstly, a diagnosis can not be made without interfering with the integrity of the early embryo. Secondly, microinjection of the gene of interest often results in cell death or remains without effect. Thirdly, random insertion of the gene results often in mutagenesis of other essential genes. Finally, there are many ethical questions with regard to germ line therapy.

C.3 Genetic engineering in vitro.

Another possibility to take advantage of cloned genes is to use them for the production of large amounts of the missing protein (Schoepke 1987). Thus, not the gene is administered to patients, but the deficient product. For instance, insulin and growth hormone are presently produced via biotechnology (Miller 1981). On the basis of the positive results obtained in our model studies on enzyme replacement therapy in glycogenosis type II, biotechnological systems are currently investigated for large scale production of human phosphorylated acid α-glucosidase. Considering the reported effect of α-galactosidase administration in Fabry disease, and adenosine deaminase in severe combined immune deficiency disease, a similar undertaking seems justified for these diseases.

CHAPTER III

GLYCOGENOSIS TYPE II

III.A INTRODUCTION

A.1 Clinical phenotypes

Glycogenosis type II (GSD II) is a lysosomal storage disorder based on the deficiency of acid α-glucosidase. The clinical presentation of the disease varies considerably, and has led to the distinction of three clinical subtypes (Hers and De Barsy 1973, Howell and Williams 1983). The generalized infantile variant was first recognized and is often defined as Pompe disease (Pompe 1932). Milder late onset variants were later discovered and are distinguished into juvenile and adult subtypes (Engel et al. 1973, Hugson et al. 1968, Loonen 1979).

In patients with infantile GSD II symptoms become apparent within the first three months of life. The infants show poor motor development, dyspnea, and failure to thrive. On clinical examination generalized hypotonia, cardiac enlargement, moderate hepatomegaly and a protruding tongue are obvious. Echocardiography reveals extensive hypertrophy of the myocardium. This leads sometimes to stenosis of the aorta or the pulmonary artery due to disproportional thickening of the interventricular septum. In addition, insufficiency of the mitral valve has been described (Roth and Williams 1967, Rees et al. 1976, Maron and Roberts 1981). A concomitant cardiac defect has been encountered in one Dutch patient, but its occurrence seemed not related to Pompe disease (Loonen, personal communication). The electrocardiogram is characterized by marked left axis deviation, a short P-R interval, large QRS complexes, inverted T waves, and ST depressions (Roth and Williams 1967, Hers and De Barsy 1973, Kohlschütter and Hausdorf 1986). The disease is rapidly progressive and is usually fatal within the first year of life by cardiorespiratory failure. On histological examination excessive storage of glycogen is recorded in many tissues.

Patients with the adult form of GSD II may not experience symptoms within the first two decades of life. In this clinical subtype only skeletal muscles are involved with predilection of those of the limb girdle, the trunk, and the diaphragm. Difficulty in climbing stairs is often the initial complaint. The extent of respiratory problems varies considerably. In some patients they dominate the clinical picture, whereas they are not experienced by others until late in life (Trend et al. 1985). In virtually all cases respiratory support is ultimately needed, and respiratory failure is the cause of death. The eldest patient recorded was referred at 53 and survived till 72 years of age (Loonen et al. 1981).

Besides infantile and adult variants an intermediate juvenile subtype is distinguished. Symptoms in these patients usually appear within the first decade of life, and the disease is mostly fatal before the third decade. As in adult GSD II, skeletal muscle weakness is the major problem, cardiac involvement does not occur. In some rare late onset cases, a basilar artery aneurysm has been reported due to glycogen storage in vascular smooth muscle cells (Makos et al. 1987, Matsuoka et al. 1988).

A rather strict correlation has been found between the clinical severity of the disease and the extent of acid α-glucosidase deficiency. Virtually no acid α-glucosidase activity is measured in cells and tissues from infantile patients, while in late onset cases activities up to 25% of normal have been measured. (Mehler and DiMauro 1977, Shanske et al. 1986, Reuser et al. 1978, 1987, Publications I, II, III and V, this thesis). However, three exceptional cases of adult GSD II with unusually low residual acid α-glucosidase activities in fibroblasts have been encountered (Beratis et al. 1983, Reuser et al. 1987).

Some rare cases have been described with lysosomal glycogen storage and normal activity of acid α-glucosidase (Danon et al. 1981, Riggs et al. 1983). This disease seems to occur predominantly in males, and is characterized by muscle weakness, heart arrhythmias (Wolff-Parkinson White pattern), and mental retardation. The etiology is unknown.

In addition to the few attempts at enzyme replacement therapy, clinical management has been focused on supportive and symptomatic treatment. Inspiratory muscle training (Martin et al. 1983) and high protein diets to enhance muscle renewal have variously been reported as beneficial to adult patients with GSD II (Slonim et al. 1983, Isaacs et al. 1986).

A.2 Acid α-glucosidase

Acid α-glucosidase is a lysosomal hydrolase with specificity for the natural substrates glycogen, maltose and isomaltose. The enzyme hydrolyzes both α-1,4 and α-1,6 glycosidic linkages (Jeffrey et al. 1970, Rosenfeld 1975, Koster and Slee 1977). The enzyme is most active at pH 4-5. The acid α-glucosidase gene is localized on chromosome 17 in the region q21-23 (Solomon et al. 1979, D'Ancona et al. 1979, Martiniuk et al. 1985). The gene comprises 20,000-30,000 nucleotides, the RNA messenger is about 3.6 kb long (Martiniuk et al. 1986, Hoefsloot et al. 1988). Acid α-glucosidase is synthesized as a protein of approximately 100kD. Glycosylation in the RER adds about 10 kD to the molecular mass (Van der Horst et al. 1987). Phosphorylation occurs in the Golgi complex, and the enzyme undergoes extensive proteolytic processing. The exact intracellular site of protein modification is uncertain. Pulse-chase experiments, using ^{3}H-Leucine for labeling, indicate that there are early processing intermediates of 105 and 100 kD (Oude Elferink et al. 1985, Reuser et al. 1985) The major 95, 76 and 70 kD molecular species arise after transit through the Golgi complex (Oude Elferink et al. 1985). The 76 and 70 kD forms of acid α-glucosidase have been identified as mature enzyme, and are the most abundant species in tissues and cultured cells of healthy individuals (Hasilik and Neufeld 1980, Reuser et al. 1985, 1987).
Cloning of the full length acid α-glucosidase cDNA and determination of the N-terminal amino acid sequences of the various biosynthetic forms of this enzyme has revealed that both amino-, and carboxy-terminal processing of the enzyme occurs (Hoefsloot et al. 1988). The carboxy terminal processing involves the loss of about 20 kD, which is rather unique compared to processing of other lysosomal enzymes. For β-glucuronidase and cathepsin D, for instance, the reduction is limited to 3 and 1 kD, respectively (Erickson et al. 1984).
The primary structure of acid α-glucosidase shows a high degree of homology with the membrane bound intestinal isomaltase-sucrase complex (Semenza 1986, Hunziker et al. 1986). Both enzymes are probably derived from the same ancestral gene. Based on this homology the putative active site of acid α- glucosidase has been established (Hoefsloot et al. 1988).
Acid α-glucosidase has been purified from different sources. The enzymes isolated from human placenta (95, 76 and 70 kD) (Reuser et al. 1984), human urine (110 kD) (Oude Elferink et al. 1984) and bovine testis (70kD) (Reuser

et al. 1984) have predominantly oligomannosidic type of sugar chains. Acid α-glucosidase from human urine and bovine testis is phosphorylated and taken up with high efficiency by cultured fibroblasts and muscle cells via the mannose 6-phosphate receptor (Reuser et al. 1984, Oude Elferink et al. 1986, Publication III-V, this thesis). The origin of the phophorylated precursor of acid α-glucosidase in human urine is not exactly known, but secretion by cells of the proximal tubule of the kidney has been suggested. Other lysosomal enzymes in human urine containing mannose 6-phosphate are α-L-iduronidase (Sando and Neufeld 1977), arylsulfatase A (Wiesmann and Hershkowitz 1974) and α-N-acetylglucosaminidase (Von Figura and Kresse 1974). β-Glucuronidase was reported to be predominantly present as non-phosphorylated enzyme (Brot et al. 1974).
The phosphorylated precursor of acid α-glucosidase purified from urine has a 2.5 times lower activity for glycogen than the mature enzyme species (Oude Elferink et al. 1984). When the precursor is administered to cultured cells, endocytosis via the mannose 6-phosphate receptor occurs and the enzyme is processed to lower molecular weight species of 80 and 74 kD, each approximately 4 kD larger than comparable molecular forms of acid α-glucosidase purified from human placenta (Oude Elferink et al. 1986). The difference in molecular mass is due to more extensive glycosylation. Processing of precursor to mature enzyme probably occurs in a post-endosomal compartment.

A.3 Acid α-glucosidase deficiencies

Studying the biosynthesis of acid α-glucosidase in fibroblasts from various patients with glycogenosis type II, many different defects in acid α-glucosidase synthesis were observed (Table 4). At least six distinct defects were recognized among infantile cases, all resulting in residual acid α-glucosidase activities of 1% or less. In most instances, synthesis of mature enzyme protein was not detected by immunoblotting or immunoprecipitation. Immuno-electron microscopy showed absence of cross reactive material in lysosomes (Reuser et al. 1987).
In the majority of adult variants reduced synthesis of precursor and mature acid α-glucosidase was observed, while the specific activity and stability of the enzyme were not altered (Reuser and Kroos 1982). The presence of the mutant enzyme was demonstrated in lysosomes by immuno-electronmicroscopy. The residual activity in fibroblasts of these adult variants ranges from 7-29%.

TABLE 4

Protein defects in acid α-glucosidase deficiency

Clinical subtype	Enzyme precursor		P	Mature enzyme	Enzyme activity	Ref.
	Pulse	Blot				
infantile	–	–			1%	(1)
		–	(+)		1%	(2,3)
	–	?			1%	(4,5)
	(+)	(+)	?	(+)	1%	(3)
	R+	R(+)	+	–	1%	(1)
	?	?	?	+	1%	(6,7)
	+	+	–	–	1%	(2)
	+	+	+	–	1%	(unpubl)
juvenile	+	+	+	–	2%	(2,3)
	(+)	(+)	?	+	5-10%	(1,3)
Adult	+	+	–	–	1-2%	(2,7)
	+	+	+	(+)	3-5%	(3,8)
	(+)	(+)	+	(+)	10-29%	(1,2,3)
	(+)	(+)	?	E(+)	25%	(3)
	+	?	?	D(+)	≤15%	(5)

Pulse=pulse labeling; blot=immunoblotting; P=phosphorylation (+=present, –=absent); +=present in normal quantity; –=absent; (+)=reduced amount; R=reduced size; E=enlarged size; D=enhanced degradation. Enzyme activities are given as percentage of the average wild type activity.

(1) Publication II, this thesis (2) Reuser et al. 1985, (3) Reuser et al. 1987 (4) Hasilik and Neufeld 1980 (5) Steckel et al. 1982, (6) Ninomiya et al. 1984, (7) Beratis et al. 1983, (8) Publication III, this thesis.

Three adult patients were identified with very low acid α-glucosidase activities (Reuser et al. 1987, Publication III this thesis) Immuno-electron microscopy on a muscle biopsy of one of these patients revealed the presence of acid α-glucosidase in lysosomal structures. In contrast, the enzyme was virtually absent in lysosomes of cultured fibroblasts of this patient (unpublished results).

A.4 DNA analysis

In the past it was assumed that patients with the same clinical phenotype would have an identical DNA defect. For instance, it was thought that Hurler and Scheie disease were based on two allelic mutations at the α-L-iduronidase locus, while Hurler-Scheie was considered a genetic compound (Neufeld and McKusick 1983). It is likely, however, that in reality a series of allelic mutations exists. This is indicated by the many distinct protein defects underlying acid α-glucosidase deficiency in the clinical phenotypes of glycogenosis type II.

In Gaucher disease type I, once thought to be genetically homogeneous among Ashkenazi Jews, at least 4 distinct mutations have been encountered (Tsuji et al. 1987, 1988). Also for the classical form of Tay Sachs disease various mutations were identified (Myerowitz and Hogikayan 1986, Myerowitz et al. 1988).

The extensive variety of mutants can help in the understanding of molecular requirements for protein synthesis, transport and function. With respect to lysosomal α-glucosidase, for instance, it is of interest, that the enzyme undergoes extensive proteolytic processing at the carboxy- and amino terminus. Furthermore, phosphorylation mutants have been described, which offers in principle the opportunity to identify the phosphotransferase recognition site. Km mutants of acid α-glucosidase are known with a decreased affinity for the natural substrate glycogen and studies on these mutants may indicate the site for glycogen binding (Swallow et al. 1989, Suzuki et al. 1988). Of further interest is the common evolutionary origin of acid α-glucosidase and sucrase-isomaltase, an enzyme complex anchored in the epithelial brush border membrane of the intestine. Detailed study on the specific composition of both these enzymes may elucidate the mechanism of targeting of these proteins to different locations, and the basis of their distinct functions.

The acquired knowledge can aid to the understanding of clinical heterogeneity and to the development of effective methods for treatment of glycogenosis type II.

III.B THE EXPERIMENTAL WORK

B.1 Clinical diversity in glycogenosis type II

Many studies have been devoted to the question "What is the cause of clinical heterogeneity in glycogenosis type II?" Does the level of residual acid α-glucosidase activity play a major role or is the neutral maltase activity important in determining the clinical phenotype? And what is the contribution of the genetic background together with epigenetic factors? In this respect one family has attracted particular attention. In the pedigree of this Dutch family, adult glycogenosis type II occurred in the first generation, whereas three cases of infantile GSD II were diagnosed in the third. In *Publication I* the basis of this clinical diversity was investigated. The acid α-glucosidase alleles of selected family members were segregated in man-mouse somatic cell hybrids, and the function of the separate alleles was analyzed. It was demonstrated that the adult variant is a genetic compound with two functionally distinct alleles. One associated with virtually complete deficiency of acid α-glucosidase activity ("null-allele"), and the other resulting in 30% to 50% residual activity. In the second generation a carrier of the "null-allele" married another carrier of a "null-allele" Among their offspring were three homozygotes for the "null-allele", resulting in severe infantile GSD II. It is concluded that the level of residual activity, dictated by the mutant acid α-glucosidase alleles, is the primary cause of clinical diversity in this family.
Previous studies have demonstrated various molecular defects in infantile and late onset glycogenosis type II variants (Table 4). In *Publication II* the heterogeneity of GSD II among patients from distinct ethnic origins was studied at the protein and DNA level. In 5 South African families, two rare mutations were discovered. In cultured fibroblasts from two unrelated black baby girls we observed a complete absence of acid α-glucosidase protein and mRNA synthesis. Two patients with infantile GSD II from a consanguineous Indian family were found to produce an acid α-glucosidase precursor of reduced size. The size difference between the mutant and wild type precursor was maintained when enzyme synthesis was studied in the presence of tunicamycin, indicating that the defect resides in the protein core. The mRNA appeared of normal length, whereas digestion of genomic DNA with various restriction enzymes and subsequent hybridization with full length acid α-glucosidase

cDNA revealed quite unexpectedly a fragment of increased length. A polymorphism of this nature is not known to occur in the Caucasian population. Both patients were homozygotes for the unusual allele, whereas the parents had both the abnormal and wild type alleles.
The clinical phenotypes of GSD II among the various South African cases studied in *Publication II* appeared to correlate with the level of residual acid α-glucosidase activity in cultured fibroblasts. This correlation, also observed in previous studies, was further investigated in the *Publications III and V* reporting on the extent of lysosomal glycogen storage as a result of acid α-glucosidase deficiency. The most extensive accumulation of glycogen was found in fibroblasts (*Publication III*) and muscle cells (*Publication V*) of patients with infantile GSD II, and less than 2% residual acid α-glucosidase activity. As much as 70-80% of the total cellular glycogen was contained in lysosomes. The mild clinical phenotype of two adult patients with an unusually low acid α-glucosidase activity could not be explained, since lysosomal glycogen accumulation was recorded in cultured fibroblasts. In fibroblasts of patients with adult GSD II and more than 10% residual enzyme activity no glycogen storage occurred. However, moderate storage was observed in muscle cells from some of these variants.
In the course of these studies, we observed that the lysosomal glycogen content of normal human fibroblasts is a constant percentage (10-15%) of the total cellular amount of glycogen. This suggests that the influx of glycogen into lysosomes is determined by the cytoplasmic glycogen concentration. In control muscle cells, 20% of the cellular glycogen was found to be present in lysosomes, suggesting that lysosomes of muscle cells are more involved in the degradation of glycogen than those of fibroblasts. This may play a role in the development of pathological manifestations in muscle.
The clinical course of GSD II seems not to be influenced by differential expression of neutral α-glucosidases, since the activities of the neutral isozymes are similar in all patients tested (*Publication V*).

B.2 Enzyme replacement studies

The studies on enzyme replacement described in this thesis concern the possible use of the mannose 6-phosphate receptor as target for exogenous acid α-glucosidase. The initial studies were performed with cultured fibroblasts, since the function of the receptor was best defined in this cell type. In *Pub-*

lication III it is described that addition of bovine testis acid α-glucosidase to cultured fibroblasts of various patients with glycogenosis type II results in efficient uptake of enzyme in lysosomes, and in disappearance of glycogen storage. In following studies, the feasibility of enzyme replacement was investigated using muscle cells, the major target in glycogenosis type II (*Publication IV and V*). For this purpose, the phosphorylated acid α-glucosidase precursor of human urine was applied to muscle cells in culture. Similar results were obtained as with cultured fibroblasts. The endocytosed acid α-glucosidase was demonstrated to be present in lysosomal compartments, and to be converted to mature enzyme. Evidence for degradation of lysosomal glycogen was obtained.

Since cells in culture are easily accessible to exogenous enzyme, it was further questioned whether there would be transport of acid α-glucosidase across the capillary walls necessary for reaching the main target tissues. In an immunoelectron microscopic study on skeletal muscle biopsies from control subjects (*Publication VI*) it was demonstrated that high molecular weight species of lysosomal enzymes are present in human plasma. In addition, cross-reactive material was found in plasmalemmal vesicles of capillary endothelial cells, and in pericapillary spaces. The enzyme species co-localized with albumin known to be transported across endothelial cells. These findings suggest that transendothelial transport of lysosomal enzymes occurs.

To obtain more information on the uptake of lysosomal enzymes by target tissues in vivo, isolated rat hearts were perfused with bovine testis acid α-glucosidase, using a Langendorff recirculation-perfusion system (*Publication VII*). Uptake of catalytically active bovine testis enzyme was demonstrated by means of specific immunoprecipitation and immunoblotting. In addition, the lysosomal localization of exogenous enzyme was established by immunocytochemical double labeling procedures.

Publication VIII reports on the results obtained after intravenous administration of mannose 6-phosphate containing bovine testis acid α-glucosidase to mice. The animals were sacrificed various days after enzyme administration and the activity of bovine enzyme in mouse tissues was determined via antibody specific immunoprecipitation. Enzyme uptake was demonstrated in liver, spleen, lung, heart and skeletal muscle. Infused enzyme did not cross the placental nor the blood brain barrier. The uptake appeared dose dependent. The half life of the enzyme was estimated to be 2-4 days. Also injection of acid α-glucosidase purified from human placenta leads to uptake in liver,

spleen and lung, but is significantly less in muscle tissue. It is concluded that there is an advantage in using acid α-glucosidase with phosphorylated carbohydrate chains for enzyme replacement therapy.
It is realized that large quantities of highly purified enzyme will be needed for administration to patients. Recent experiments have shown that cloned cDNA of acid α-glucosidase can in principle be used for enzyme production. Enzyme transiently expressed and secreted by monkey kidney cells (COS), transfected with acid α-glucosidase cDNA, is phosphorylated and corrects the enzymatic defect in cultured fibroblasts and muscle cells from patients with infantile GSD II. These findings make it worthwhile to explore the possibility of large scale production of acid α-glucosidase via cloned cDNA.

B.3 Concluding remarks

Studies on the cause of clinical heterogeneity in glycogenosis type II including a large series of patients, show that the clinical severity of the disease is primarily determined by the level of residual acid α-glucosidase activity. Differences in acid α-glucosidase activity are due to allelic variation. This is reflected at the protein level, where a variety of distinct defects in proper synthesis and processing of acid α-glucosidase are found among GSD II patients. A constant observation is, that a high residual acid α-glucosidase activity is associated with formation of mature enzyme.
Intriguing is the role of the lysosomes in the cellular glycogen metabolism. Our studies reveal, that in cultured skeletal muscle cells and fibroblasts of control subjects a defined percentage of the cellular glycogen is contained in lysosomes. This suggests that the lysosomes have a passive role in the regulation of the cellular glycogen content. In infantile GSD II this regulating function is completely abolished, which can explain the reported additional storage of cytoplasmic glycogen. This assumption is an attractive alternative to the hypothesis that there would be a secondary deficiency of neutral maltase in infantile GSD II. Moreover, we have demonstrated that the neutral maltase activities are normal in cultured fibroblasts and muscle cells of all patients. The relatively high residual acid α-glucosidase activity in late onset GSD II apparently delays lysosomal glycogen storage and onset of clinical symptoms. In addition, slow damage of muscle fibres might be counterbalanced by muscle regeneration.
The low acid α-glucosidase activity in some adult variants with GSD II re-

mains difficult to explain. However, the impression exists that the in vitro conditions do not reflect in all cases the in vivo situation. For instance, in a skeletal muscle biopsy of one of these patients, we found a high amount of acid α-glucosidase cross reactive material in lysosomes, whereas this was not the case in the cultured fibroblasts.

The studies on clinical heterogeneity have provided important information for the treatment of glycogenosis type II. GSD II patients with more than 30% residual acid α-glucosidase activity have not been encountered. Thus, partial correction of the acid α-glucosidase deficiency is expected to be sufficient for preventing clinical symptoms in GSD II. In addition, lowering of the cytoplasmic glycogen stores in skeletal muscle is expected to delay progression of the disease.

Our experiments show that acid α-glucosidase containing mannose 6-phosphate species are significantly more effective when used in enzyme replacement therapy than non-phosphorylated enzymes. The fundamental requirements for successful application appear to be fulfilled. Glycogen accumulation is completely reversed by endocytosed acid α-glucosidase. Infused enzyme can cross capillaries, and reaches the target organs.

For administration of purified acid α-glucosidase to humans, large quantities of phosphorylated acid α-glucosidase will be needed. Experiments in mice indicate that, 200-400 mg of purified enzyme has to be administered to adult patients every 6 days. Such vast amounts can only be produced by biotechnological methods. Development of an efficient method for enzyme production by means of cloned cDNA deserves further investigation.

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PUBLICATION I

submitted

ADULT AND INFANTILE GLYCOGENOSIS TYPE II IN ONE FAMILY EXPLAINED BY ALLELIC DIVERSITY.

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SUMMARY

We have investigated the genetic and molecular background of clinical heterogeneity in a family with infantile and late onset variants of glycogenosis type II (GSD II). The (mutant) acid α-glucosidase alleles of crucial family members were segregated in man-mouse somatic cell hybrids to study their individual function.Two kinds of mutant alleles were identified. The first type of allele leads to complete deficiency of acid α-glucosidase activity. Homozygosity of this allele in three family members is associated with infantile GSD II. The second allele is characterized by reduced synthesis of acid α-glucosidase resulting in partial enzyme deficiency. Compound heterozygosity of the two alleles was demonstrated in the adult variant of GSD II. It is concluded that clinical heterogeneity in this family is primarily caused by allelic diversity of the acid α-glucosidase locus.

INTRODUCTION

Glycogenosis type II (GSD II, Pompe's disease) is one of the more than 30 lysosomal storage disorders presently known (1-4). The disease is caused by inherited deficiency of acid α-glucosidase (EC 3.2.1.3), the enzyme responsible for lysosomal degradation of glycogen. Rapidly progressive damage of heart and skeletal muscles is characteristic of the most severe clinical subtype and leads to death in infancy. In other forms of GSD II the first symptoms do not appear before childhood or adulthood, and pathological findings are limited to skeletal muscle (5).

The genetic and molecular background of clinical heterogeneity in GSD II and other lysosomal storage disorders is not fully understood, but allelic diversity could be an important causative factor. Indications for the occurrence of different mutant alleles at the acid α-glucosidase gene locus were obtained by complementation studies, and analysis of acid α-glucosidase in fibroblasts, muscle cells and muscle biopsies of patients (6-11). On the other hand differ-

ences in epigenetic factors and "genetic background" were suggested to play a major role. In this respect, one family has attracted particular attention (12- 16) with three severely affected sibs in the third generation and one patient with a mild form of GSD II in the first. Danon et al. (17) reported on a similar interesting family.
To resolve the important issue concerning the origin of clinical heterogeneity, we analyzed the molecular and functional characteristics of the enzyme products of the different acid α-glucosidase alleles in the family.

MATERIALS AND METHODS

Family history

The pedigree of family S. is illustrated in figure 1. The index patient was diagnosed at the age of 3 months with typical symptoms of infantile GSD II. She died at 16 weeks of age. Lysosomal α-glucosidase activity in skeletal muscle, liver and cultured fibroblasts was less than 1% of normal. Two more cases of severe infantile GSD II occurred in this generation. One child (III.2) was born prematurely after 25 weeks of gestation and lived for half an hour. Virtually no acid α-glucosidase activity was found in fibroblasts cultured from a skin biopsy taken post-mortem. The third incidence was an affected fetus (III.4) diagnosed prenatally with complete deficiency of acid α-glucosidase activity in cultured amniotic fluid cells. The diagnosis was confirmed after termination of pregnancy (14).
The parents are at present 43 (II.1) and 41 (II.2) years old, without clinical signs of GSD II. According to the autosomal recessive mode of inheritance of GSD II both parents must carry at least one mutant acid α-glucosidase allele. The levels of enzyme activity measured in skeletal muscle, lymphocytes, cultured fibroblasts, and urine of both parents were about 50% of normal (14, 18).
The grandfather (I.1) did not experience symptoms of GSD II until the age of 53. At 59 years the diagnosis of adult GSD II was established by the observation of lysosomal glycogen storage in a muscle biopsy, and reduced acid α-glucosidase activity in muscle tissue (16%), lymphocytes (17%), cultured fibroblasts (21%), and urine (13%) (14). He died at the age of 72 of respiratory failure.
On the basis of clinical and biochemical criteria the spouse of I.1 is neither a

patient nor carrier of GSD II (14, 18).

Cell culture procedures and selection of hybrids

Cells were cultured in Dulbecco's modified Eagle's medium (Flow Lab. Inc. McLean, VA) supplemented with 10% fetal calf serum and antibiotics. Human fibroblasts were fused with thymidine kinase (TK) deficient mouse L cells (LTK^-) using β-propiolactone-inactivated Sendai virus as described (19). Human-mouse somatic cell hybrids were selected in HAT-medium (20). After 2-4 weeks cell clones originating from each fusion event were isolated at random and propagated in HAT medium.

Assay of human acid α-glucosidase activity

Exactly the same procedure was used as described before (21). In short, human acid α-glucosidase was precipitated from cell homogenates with antibodies raised in Swiss mice against the enzyme purified from human placenta. The antibodies do not cross-react with mouse acid α-glucosidase. The activity of human acid α-glucosidase was measured in the immunoprecipitate with the artificial substrate 4-methylumbelliferyl-α-D-glucopyranoside.

Immunoblotting

Different molecular species of acid α-glucosidase were separated electrophoretically in 10% polyacrylamide gels containing sodiumdodecyl sulphate (22), and blotted onto nitrocellulose filters (23). The human and mouse enzymes were visualized using rabbit anti-human acid α-glucosidase serum in combination with ^{125}I protein A.

Southern blotting

DNA was isolated from fibroblasts and hybrid cell lines using standard methods (24). Following digestion with the restriction enzyme Hind III, DNA fragments were separated in 0.7% agarose gels. After blotting onto nitrocellulose (24), filters were hybridized with a full length acid α-glucosidase cDNA probe (25) radioactively labeled as described by Feinberg and Vogelstein (26). Filters were washed after 16 hours till a stringency of 0.3 times

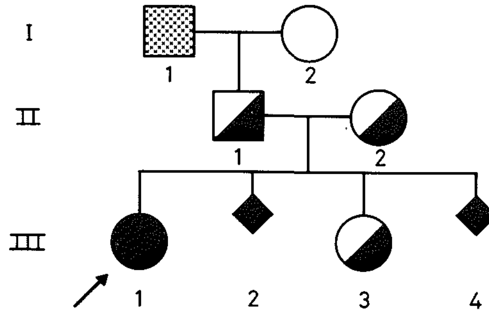

Figure 1: *Pedigree of family S. Dotted gray symbol, adult GSD II; semisolid symbol, GSD II carrier; solid symbol, infantile GSD II. Arrow points to index patient.*

SSC (1 time SSC is 0.15 M sodium chloride, 0.015 M sodium citrate), and exposed to Fuji X-ray film for one to four days.

RESULTS AND DISCUSSION

Segregation of human acid α-glucosidase alleles

Fibroblasts from I.1, II.1, II.2 (Fig. 1), and a healthy control were fused with thymidine kinase (TK) deficient mouse L cells. The resulting man-mouse somatic cell hybrids tend to loose human chromosomes spontaneously, but culturing the cells in HAT-medium provides a condition for selective retention of human chromosome 17 carrying the TK locus (20). Since the gene loci for acid α-glucosidase and TK are closely linked (21, 27-29) this procedure can be used for the selection of somatic cell hybrids containing a human acid α-glucosidase allele.

Analysis of acid α-glucosidase activity in man-mouse somatic cell hybrids

Man-mouse hybrid clones of each individual were isolated at random and the lysosomal acid α- glucosidase activity was measured. Human and mouse activities were distinguished using an antiserum against human acid α-glucosidase which was raised in mice. This antiserum precipitates more than 95% of hu-

man enzyme, whereas less than 1% of mouse acid α-glucosidase is bound (21). The results are illustrated in figure 2. The average background activity of the mouse LTK− cell line is set at 1 (Fig. 2, LTK−). The acid α-glucosidase activity in the man-mouse somatic cell hybrids is expressed as multiples of this value. The thirteen hybrids originating from fusions of normal human

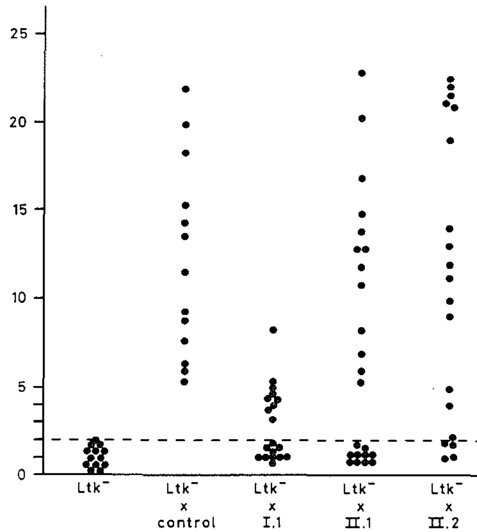

Figure 2: *Acid α-glucosidase activity in LTK^- cells and man- mouse somatic cell hybrids. Each dot represents the acid α-glucosidase activity of a single clone measured at least in duplicate. The average acid α-glucosidase activity in LTK^- is set at 1.*

fibroblasts with LTK− cells had on average an activity of 9.9 ± 5.2. The hybrid cell lines from I.1 formed two groups. Ten clones had similar activities as unfused LTK− cells and thus were completely deficient in human acid α-glucosidase activity. Nine clones, on the other hand, expressed an average enzyme activity of 4.7 ± 1.5. A similar distribution of positive and negative clones was observed with hybrids obtained from II.1 and II.2. Ten (II.1) and 5 (II.2) clones did not express human acid α-glucosidase activity, whereas 13 (II.1) and 14 (II.2) had acid α-glucosidase activities as measured in hybrids obtained after fusion of control fibroblasts with L cells (on average 12.5 ± 5.3 and 14.6± 6.6, respectively).

Immunoblotting was used as a second method to distinguish the products of different human acid α-glucosidase alleles. The molecular species observed in normal human fibroblasts (Fig. 3, control) represent the precursor (110 kD), intermediate (95 kD) and mature (76 kD and 70 kD) forms of acid α-glucosidase (8, 30).

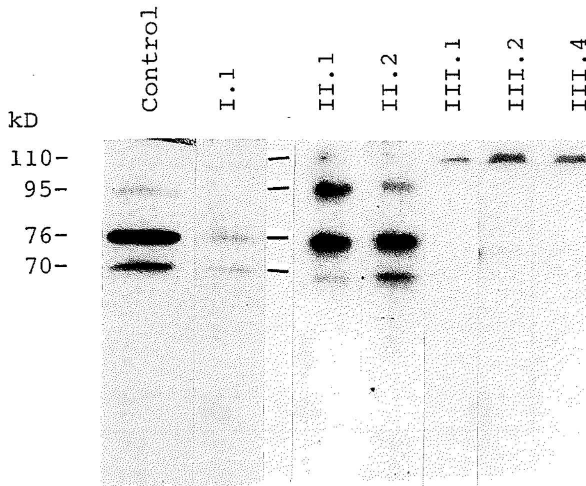

Figure 3: *Molecular forms of acid α-glucosidase in fibroblasts of a control and members of family S. Proteins were separated by PAGE and blotted onto nitrocellulose filters. Acid α-glucosidase was visualized with rabbit polyclonal human acid α-glucosidase antibodies in combination with ^{125}I protein A.*

The other lanes depict acid α-glucosidase of the family members. The immunoblot-pattern of I.1, II.1 and II.2 can not be distinguished from normal. The amount of enzyme, however, is clearly reduced in I.1. A complete lack of mature enzyme is observed in fibroblasts of the three sibs (III.1, III.2 and III.4). Thus, the level of residual acid α-glucosidase activity in fibroblasts of the family members (14) corresponds with the amount of mature enzyme formed. This correlation is preserved in the hybrid cell lines (Fig. 4). Mature human acid α-glucosidase of 76 kD is only detected in hybrid clones with human enzyme activity (+) and not in those without (-).

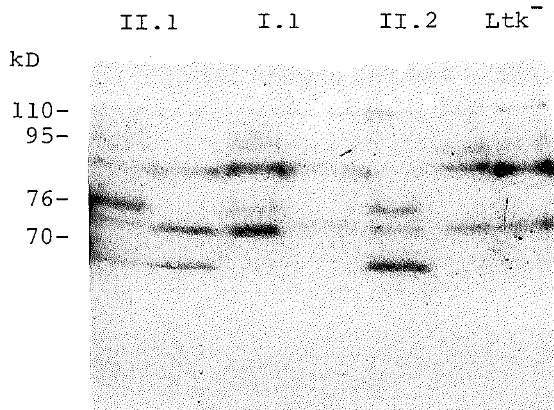

Figure 4: *Immunoblot of acid α-glucosidase species in man-mouse somatic cell hybrids with (+) and without (-) human acid α-glucosidase activity (procedure as in figure 3).*

DNA analysis of man-mouse somatic cell hybrids

The possibility had to be excluded that the absence of human acid α-glucosidase in hybrids is due to loss of linkage of the TK and acid α-glucosidase gene loci. DNA of the different cell lines was digested with HindIII and hybridized with a radioactively labelled full length cDNA clone coding for human acid α-glucosidase (25). Figure 5 shows that human DNA restriction fragments of identical size were detected in all hybrids, establishing the presence of a human acid α-glucosidase gene.

Conclusions

The differential expression of acid α-glucosidase in the hybrids is determined by the nature of the mutant acid α-glucosidase allele that is retained. Patient I.1 with mild clinical symptoms is obviously a genetic compound. One allele does not lead to significant production of acid α-glucosidase (Fig. 4, I.1 (-)) and is associated with complete enzyme deficiency. The other allele is characterized by reduced enzyme synthesis, as judged by the amount of acid α-glucosidase in fibroblasts of I.1 (Fig. 3) and the level of activity in the

positive clones of I.1 (40% compared to control). The reported activity of acid α-glucosidase in fibroblasts and various tissues of the grandfather (I.1) (Materials and Methods) is in accordance with the expected combined effect of the two mutant alleles and can explain the mild clinical phenotype.

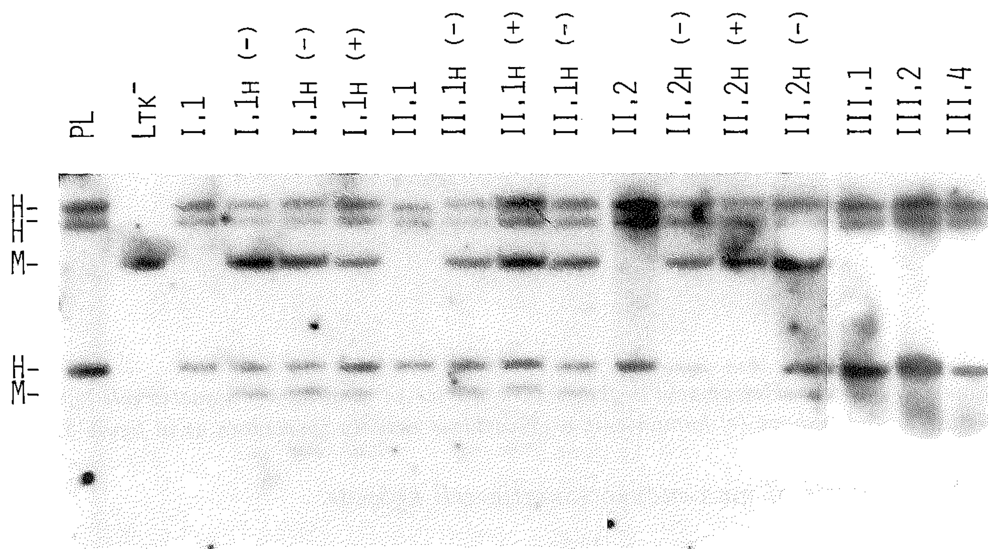

Figure 5: *Hybridization analysis of genomic DNA isolated placenta (PL), mouse LTK⁻ cells, fibroblasts of family members, and man-mouse somatic cell hybrids with (H(+)) and without (H(-)) human acid α-glucosidase activity. DNA was digested with HindIII and hybridized to a human acid α-glucosidase cDNA. Hybridizing genomic DNA fragments from human and mouse origin are indicated with H and M, respectively.*

The allele with the most destructive mutation is transmitted to II.1 as indicated by the complete deficiency of acid α-glucosidase activity and protein in approximately 50% of the somatic cell hybrids derived from II.1. The normal activity in the other hybrid clones confirms that II.1 is a heterozygote. The estimated and actual acid α-glucosidase activity in cells, tissues and urine of II.1 is approximately half normal (14, 18) which is high enough to prevent glycogen accumulation and clinical symptoms. From the pedigree it must be concluded that also II.2 is a carrier of GSD II. Indeed, the average acid α-glucosidase activity in cells and tissues of this family member is in the heterozygous range (14,18). Analysis of somatic cell hybrids of II.2 showed

that only one allele contributes to this activity (Fig. 2). The mutant allele does not lead to enzyme production.
Obviously the three cases of GSD II in the third generation (III.1, III.2, III.4) have inherited the mutant alleles from the parents resulting in an almost complete deficiency of acid α-glucosidase and a severe form of GSD II. Our present analysis does not reveal whether these cases are homo or hetero-allelic. Gross gene abnormalities were not detected (Fig. 5, and unpublished results), but different point mutations can not be excluded. For instance from protein analysis it is known that several allelic mutations can cause infantile GSD II (7, 8, 10). In this respect the situation in GSD II is not different from what is observed in other lysosomal storage diseases. Genetic heterogeneity was even demonstrated in Ashkenazi Jewish Gaucher disease type 1 (31, 32) and in classic Tay-Sachs disease originally thought to be genetically homogeneous (33-35).
Presently, the awareness is growing that various combinations of homo and hetero-allelic mutant genotypes are the basis for clinical heterogeneity in lysosomal storage disorders. However, it is often difficult to resolve the relation between genotype and phenotype, because it requires not only molecular but also functional analysis of the separate alleles. In this rather unique family with GSD II we had the opportunity to perform such ananalysis. Compound heterozygosity was demonstrated and strong support was obtained for the hypothesis that clinical heterogeneity in GSD II is primarily caused by allelic diversity at the acid α-glucosidase gene locus.

Acknowledgments

We thank Prof. Dr. H. Galjaard for helpful advice, P. E. Hupkes for technical assistance, J. G. H. Fengler and M. Kuit for photography, and M. M. C. de Klerck for secretarial assistance. This work was supported in part by the Prinses Beatrix Fonds and the Netherlands Organization for Scientific Research (NWO) under the auspices of the Netherlands Foundation for Medical and Health Research (MEDIGON).

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PUBLICATION II

Am. J. Hum. Genet. (in press)

GLYCOGENOSIS TYPE II: PROTEIN AND DNA ANALYSIS IN 5 SOUTH AFRICAN FAMILIES FROM VARIOUS ETHNIC ORIGINS

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SUMMARY

The molecular nature of lysosomal α-glucosidase deficiency was studied in 5 South African families with glycogenosis type II. Distinct ethnic origins were represented. Two new mutant acid α-glucosidase alleles were discovered. In two infantile patients from a consanguineous Indian family we found for the first time an acid α-glucosidase precursor of reduced size. The mutant precursor appeared normally glycosylated and phosphorylated, but was not processed to mature enzyme. Abnormalities of the mRNA were not obvious, but digestion of genomic DNA with HindIII, BglII and StuI revealed for each enzyme a fragment of increased length. Heterozygosity was demonstrated in the parents.
Complete lack of acid α-glucosidase mRNA, and deficiency of precursor synthesis was observed in two black baby girls from unrelated families. In these cases the length of all restriction enzyme fragments was normal. Reduced enzyme synthesis, but normal processing was registered in juvenile and young adult Cape Coloured patients. The extensive heterogeneity of glycogenosis type II is emphasized in these studies on various ethnic groups. The newly discovered mutants are valuable for the understanding of clinical diversity as a result of allelic variation.

INTRODUCTION

Acid α-glucosidase is a lysosomal hydrolase essential for the degradation of glycogen (Hers 1963). Enzyme deficiency leads to glycogenosis type II, and is inherited as an autosomal recessive trait (Howell and Williams 1983). Three

clinical variants are distinguished (Hers and De Barsy 1973). The infantile subtype has its onset shortly after birth and presents with general hypotonia and muscle wasting. Cardiorespiratory insufficiency leads to death in the first two years of life. The adult variant manifests itself mostly after the second decade of life and is characterized by progressive skeletal muscle weakness. Respiratory distress is ultimately fatal. Often, a juvenile subtype is distinguished as an intermediate variant. A fairly strict correlation exists between clinical severity of the disease, extent of lysosomal glycogen storage, and residual activity of acid α-glucosidase in cultured fibroblasts and muscle cells of patients (Reuser et al. 1978, 1987, Van der Ploeg et al. 1987, 1988). The biosynthesis of lysosomal α-glucosidase is a complex process, whereby a precursor with an apparent molecular mass of 110 kD is gradually converted to mature species of 76 kD and 70 kD (Hasilik and Neufeld 1980, Reuser et al. 1985, Oude Elferink et al. 1985). This posttranslational processing involves modification of oligosaccharide side-chains and proteolytic trimming of the precursor at both the amino- and carboxyterminal end (Van der Horst et al. 1987, Hoefsloot et al. 1988). Among the patients with glycogenosis type II, mutants have been found with reduced synthesis of acid α-glucosidase, or with catalytically inactive enzyme. Furthermore, there were variants with defective phosphorylation of the precursor and/or impaired processing and intracellular transport. The latter studies concerned mainly patients of Dutch ancestry (Reuser et al. 1985, 1987). We presently report on genetic heterogeneity of glycogenosis type II in the South African population. Three families, and two individual cases were studied. Interesting new acid α-glucosidase mutations were discovered.

MATERIALS AND METHODS

Cell culture

Fibroblasts were cultured in Dulbecco's modified Eagle's Medium (DMEM, Flow Laboratories) supplemented with 10% fetal calf serum (Boehringer Mannheim) and antibiotics in an atmosphere of 10% CO2 and 90% air. One week confluent cultures were harvested with trypsin and homogenized by sonication in 250 μl distilled water. Cell debris was removed by centrifugation at 10,000 x g for 15 min.

Biochemical assays

Acid α-glucosidase activity was measured with the artificial substrate 4-methylumbelliferyl-α-D-glucopyranoside (Galjaard 1980) or with glycogen (Koster et al. 1972). Protein concentrations were determined according to Lowry et al. (Lowry et al. 1975).

Immunoblotting

Cell lysates (4.10^6 cells/200 μl) were incubated overnight at 4^oC with 100 μl of a 1:1 suspension of Concanavalin A sepharose 4B beads in sodium phosphate buffer, pH 6.2, with a final concentration of 20 mM. Nonspecifically bound proteins were removed by washing the beads 4 times with 1 ml of the same buffer. Bound glycoproteins were dissolved by heating for 10 min at 90^oC in 75 μl of sample buffer (125 mM Tris-HCl, pH 6.6, 2M glycerol, 4% SDS, 0.6% mercaptoethanol, and 0.05% bromophenol blue). Samples were electrophoresed in a 10% polyacrylamide gel (Laemmli 1970) and subsequently blotted onto nitrocellulose (Towbin et al. 1979). Acid α-glucosidase proteins were visualized with a rabbit polyclonal antibody preparation against human placental acid α-glucosidase (Reuser et al. 1985) in combination with ^{125}I labeled protein A (Towbin et al. 1979).

In vivo labeling

Fibroblasts were cultured in leucine- or phosphate-free medium (DMEM) with addition of L-(4,5-^3H) leucine or carrier-free ^{32}P-phosphate, respectively (Reuser et al. 1985). In some experiments tunicamycin (final concentration 10μg/ml) was added three hours before addition of radioactive precursors to inhibit glycosylation of newly synthesized acid α-glucosidase. Cell homogenates were prepared, and acid α-glucosidase was immunoprecipitated with antibodies. Samples were subsequently analyzed in a 10% polyacrylamide gel as described before (Hasilik and Neufeld 1980, Reuser et al. 1985).

Analysis of RNA and DNA

DNA was extracted from cultured fibroblasts following standard procedures and digested with various restriction enzymes. DNA fragments were sepa-

rated in 0.5-1% agarose gels and transferred to Genescreen Plus (Southern 1975, Rigaud et al. 1987). The lithium chloride technique was used to isolate mRNA (Auffray and Rougeon, 1980). The RNA was electrophoresed in 1% agarose/formaldehyde gels and blotted onto nitrocellulose. Filters were hybridized with radioactively labeled acid α-glucosidase cDNA probes (Maniatis et al. 1982, Feinberg and Vogelstein 1983, Hoefsloot et al. 1988). After 16h the filters were washed till a stringency of 0.3 x SSC and autoradiographed.

CASE REPORTS

Family A

This Cape coloured family (Fig.1A) of Dutch ancestry was referred because of severe skeletal muscle weakness in the first proband of the second marriage of the mother. He was a thin and weak 25 year old man who had not been able to work for the last five years due to muscle wasting. At home he needed assistance to get out of bed, and support in walking. Chest X-ray and ECG were normal. Pompe's disease was suspected and diagnosed on skeletal muscle biopsy and fibroblast α-glucosidase assay. Urine samples of the 8 siblings were tested by thin layer chromatography, and revealed that two younger brothers of 11 and 13 years old were also affected (Sewell 1979, Blom et al. 1983). Till then no gross abnormalities had been noted by the parents. On clinical examination a positive Gower's sign was found in the elder boy. The only possible indication for Pompe's disease in the younger proband was his statement that he could not run as fast as other boys in his class. Skin biopsies were obtained from the two younger boys and the parents for growth of fibroblasts.

Family B

This was a consanguineous Cape coloured family (Fig.2A). The oldest son was admitted at the age of 4 with severe muscle weakness and an abnormal gait. A myopathic EMG and raised CPK were found, whereas echocardiogram and ECG were essentially normal. Glycogen storage was demonstrated in skeletal muscle tissue, and acid α-glucosidase deficiency was measured in cultured fibroblasts. At the time that Pompe's disease was diagnosed the

mother was in her third pregnancy, but too late for prenatal diagnosis. Since the family lives in a remote country area follow-up was not possible.

Family C

The 4 months old female proband of this consanguineous Indian family (Fig. 3A) was referred because of poor feeding and failure to thrive (Bonnici et al. 1980). Motor development was delayed. She lay virtually immobile and was crying incessantly in a weak voice. A severe generalized hypotonia, severe tachypnea and a prominent left hemithorax were noticed. Macroglossia was not present and liver enlargement was moderate. Tendon reflexes were absent. The chest X-ray revealed massive cardiac enlargement. The ECG showed a short P-R interval and gigantic QRS complexes in leads S_1 and S_2 and the left chest leads. The axis was $+45^o$. Pompe's disease was diagnosed on cultured fibroblasts of the patient. The family decided against prenatal diagnosis and a second affected infant was born. A following pregnancy was monitored and an unaffected child was born.

Case D

This was a black Zulu baby girl from the Durban area on the east coast of South Africa. She was referred at the age of 7 months with severe hypotonia, cardiomegaly and liver enlargement. The muscle biopsy showed a morphology characteristic of Pompe's disease. No family history is available.

Case E

This black Ovambo baby girl from South West Africa (Namibia) was at admission moribund with gross respiratory distress syndrome, cardiomegaly and hypotonia. Skeletal muscle biopsy was suggestive for glycogen storage, and acid α-glucosidase deficiency was measured in cultured fibroblasts and EBV transformed lymphoblasts. No family history is available.

RESULTS

Acid α-glucosidase activities were measured in cultured fibroblasts of the various patients and some of the parents under standardized conditions (Table

1). The enzyme deficiency was nearly complete in the severe infantile cases (C.III.3, C.III.4 and Cases D and E), whereas, in the young adult and juvenile cases (A.II.6, A.II.7 and B.III.1) a residual acid α-glucosidase activity of 9-13% of the lowest control value was measured. In fibroblasts derived from the parents enzyme activities were within or slightly below the control range.

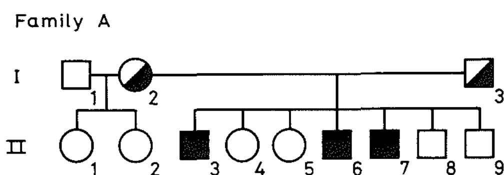
TABLE 1

Acid α-glucosidase activities

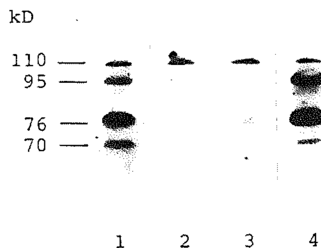
Family		nmol MU/mg protein.h	glucose/MU
Family A	I.2	58.6	6.8
	I.3	95.6	9.2
	II.6	9.1	8.0
	II.7	7.5	7.7
Family B	III.1	6.9	5.0
Family C	I.3	77.9	9.0
	II.1	78.5	8.0
	III.3	0.5	11.0
	III.4	0.65	6.3
Case D		0.67	9.1
Case E		0.51	10.4
Control (n=10)		70-150	

In order to analyse the nature of the defect in the various cases, the biosynthetic forms of lysosomal α-glucosidase were separated by SDS-PAGE and visualized immunologically after transfer to nitrocellulose. Fig.1B shows the pattern obtained for family A. In both parents (lane 1 and 4) and the affected children (lane 2 and 3) acid α-glucosidase is processed normally from a 110 kD precusor via a 95 kD intermediate to mature 76 kD and 70 kD enzyme. However, the amount of protein in both patients appears to be very much reduced. An identical picture is obtained for mutant B.III.1 (Fig.2B).

In family C another defect in acid α-glucosidase synthesis is observed. Instead of a 110 kD precursor, a protein of slightly lower molecular weight (approximately 105 kD) shows on the immunoblot of the probands (Fig.3B;

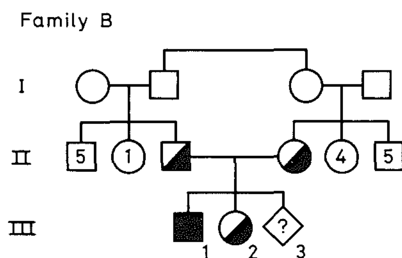


A

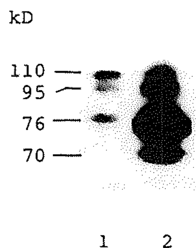


B

Figure 1: *A. Pedigree of family A. Symbols filled: glycogenosis type II patients. Symbols half filled: obligate carriers. Open symbols: healthy individuals. B. Immunoblot of the biosynthetic forms of acid α- glucosidase in fibroblasts from members of family A. Lane 1: A.I.2 (mother), lane 2: A.II.6 (patient), lane 3: A.II.7 (patient), lane 4: A.I.3 (father).*



A



B

Figure 2: *A. Pedigree of family B. Open diamond with question mark: outcome of pregnancy unknown. Figures in symbols indicate number of siblings. B. Immunoblot of the biosynthetic forms of acid α-glucosidase in fibroblasts. Lane 1: patient B.III.1, lane 2: healthy individual.*

lane 4 and 5). This aberrant molecular weight species is also present in cells from the consanguineous parents, but in addition to normally processed enzyme (lane 3 and 6). Pulse labeling of acid α-glucosidase with ^{3}H-leucine for three hours confirms that this 105 kD protein is indeed the first precursor synthesized in cells from the patients. (Fig.3C; lane 4). A subsequent chase period of three hours reveals that the mutant protein is not further processed (lane 8). This is also observed in cells from the parents (lane 2, 3, 6 and 7). The aberrant acid α-glucosidase band remains visible, whereas the normal precursor is processed as in control cells. In the presence of tunicamycin both the normal and the mutant precursor are reduced in size due to inhibition of glycosylation, but the difference in apparent M_r is maintained (not shown). Labeling with carrier-free ^{32}P showed that the aberrant 105 kD acid α-glucosidase precursor is normally phosphorylated (not shown).

Since the abnormal size of the mutant precursor does not seem to reflect a processing defect, it may result from a gene deletion. To test this hypothesis DNA from the patients and their parents was digested with several restriction enzymes and hybridized with full length cDNA of human acid α-glucosidase. Digestion of genomic DNA with the restriction enzyme HindIII reveals in control cells fragments of 12, 10 and 4.6 kb (Fig.3D, lane 1). In the patient cell lines the 12 and 10 kb fragments were normally present, but the 4.6 kb fragment was replaced by a fragment of 5.2 kb (lane 4 and 5). In cells from the parents the 4.6 as well as the 5.2 kb fragments were detected (lane 2 and 3). Larger DNA fragments were also observed when the genomic DNA was digested with the restriction enzymes BglII and StuI, but not with EcoRI (not shown). Size abnormalities of α-glucosidase mRNA were not obvious on Northern blots from patients and parents (not shown). The amount of mRNA appeared also normal.

In neither of the unrelated black baby girls (Cases D and E) was acid α-glucosidase protein detectable on immunoblots. Nor could biosynthetic forms of acid α-glucosidase be visualized by means of radioactive labeling of cells from the two infants (Fig.4, lane 4 and 5). Fig.4 shows for comparison in lane 2 labeling of the 110 kD precursor from the young adult patient A.II.6, and in lane 3 the shorter precursor of infantile patient C.III.3. A Northern blot analysis of RNA extracted from fibroblasts of cases 4 and 5 indicates complete deficiency of α- glucosidase mRNA, while no gross gene deletion is observed on Southern blots.

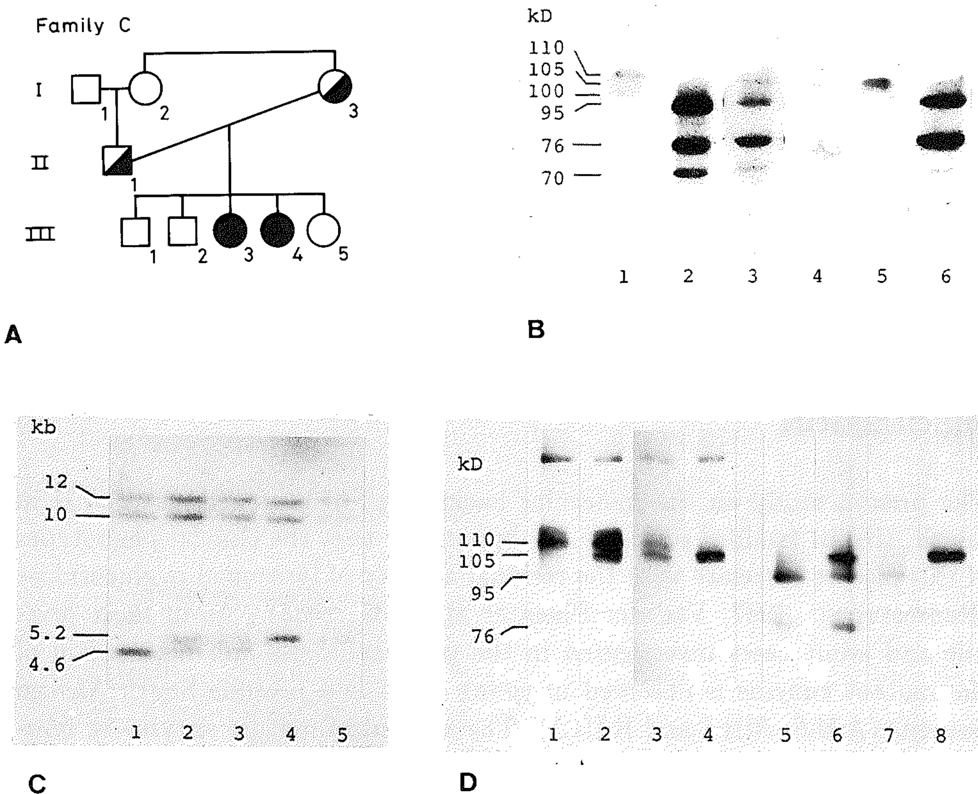


Figure 3: *A. Pedigree of family C. B. Immunoblot of the biosynthetic forms of acid α-glucosidase in fibroblasts. Lane 1: infantile Italian glycogenosis II patient, lane 2: healthy individual, lane 3: C.I.3 (mother), lane 4: C.III.3 (patient 1), lane 5: C.III.4 (patient 2), lane 6: C.II.1 (father) C. Labeling of acid α-glucosidase in fibroblasts of family C with ^{3}H leucine. Cells were labeled for 3h (lane 1-4) and chased for another 3h (lane 5-8). Lanes 1 and 5: control, lanes 2 and 6: mother, lanes 3 and 7: father, lanes 4 and 8: patient 1. D. Southern blot of HindIII digested fibroblast DNA of family C hybridized with radioactively labeled full length cDNA of acid α-glucosidase. Lane 1: control, lane 2: mother, lane 3: father, lane 4: patient 1, lane 5: patient 2.*

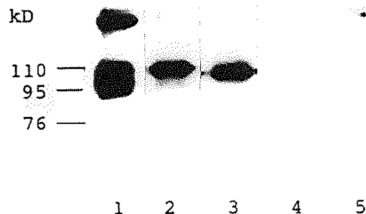


Figure 4: *Pulse labeling of acid α-glucosidase with ^{3}H-leucine for 6h. Lane 1: control, lane 2: patient A.II.6, lane 3: C.III.3, lane 4: case D, lane 5: case E.*

DISCUSSION

The present study on the molecular background of glycogenosis type II in South African families supports our hypothesis that the clinical phenotypes of this disease correlate with the residual activity of lysosomal α-glucosidase (Reuser et al. 1987, Van der Ploeg et al. 1987, 1988). As in most juvenile and adult cases investigated in the past, synthesis and maturation of the mutant enzyme is observed in young adult and juvenile South African patients (A.II.6, A.II.7 and B.III.1). The amount of mature enzyme is, however, clearly reduced which accounts for the decreased acid α-glucosidase activity in the patient's cells. The specific activity of the mutant enzyme is estimated to be close to normal. Also the ratio of acid α-glucosidase activity for glycogen over activity for the artificial substrate 4-methylumbelliferyl-α-D-glucopyranoside is not significantly abnormal (Reuser et al. 1982, 1985, 1987). The fact that patient B.III.1 was born of consanguineous parents makes it likely that he carries two identical mutant alleles. This true homozygosity will facilitate molecular analysis of one of the mutations that predisposes for juvenile glycogenosis type II.
In a second consanguineous marriage of parents from Indian extraction we identified for the first time a mutant α-glucosidase precursor of reduced size. Since the mutant enzyme is also shorter when synthesized in the presence of tunicamycin, it is likely that the abnormality which inhibits further processing resides in the protein core. The type of mutation in this particular family is not easily explained. In seeming contradiction to the nature of the

protein defect a longer DNA fragment of 5.2 kb instead of 4.6 kb is observed when DNA of the patients is digested with HindIII. DNA fragments of abnormal length are also detected with the enzymes BglII and StuI. The single EcoRI fragment seems unaltered, but this may be due to its large size of 20 kb. Among 20 Caucasians we did not encounter a polymorphism of the acid α-glucosidase locus that leads to the unusual fragment size (unpublished results). Therefore, the abnormalities seen at the protein and DNA level may very well be related, but more detailed analysis will be needed before firm conclusions can be drawn.
The second rather unique mutation which was observed in the present study is the complete lack of precursor synthesis in fibroblasts from the two black baby girls. Also the amount of acid α-glucosidase mRNA was below detection. Lack of mRNA has once been described by Martiniuk et al. (1987), but the ethnic origin of this latter patient was not mentioned. At the DNA level no gross deletions were observed using various restriction enzymes. This suggests the occurrence of a point mutation or very small deletion, which blocks transcription or gives rise to an unstable mRNA.
In a random selection of 5 families from South Africa (various ethnic origins) in which glycogenosis type II occurred, we found extensive heterogeneity and interesting mutations. These can provide important information about the molecular prerequisites for synthesis and posttranslational modification of lysosomal acid α-glucosidase. It seems worthwhile to extend these studies to other distinct ethnic groups.

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Breakdown of lysosomal glycogen in cultured fibroblasts from glycogenosis type II patients after uptake of acid α-glucosidase

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SUMMARY

Fibroblast cultures from patients with different clinical subtypes of glycogenosis type II were compared with respect to residual acid α-glucosidase activity and lysosomal glycogen content. Lysosomal glycogen storage was most pronounced in fibroblasts from patients with the rapidly progressive infantile form of the disease, and the most severe enzyme deficiency. In fibroblasts from adult patients with more than 10% of the control activity storage did not occur, and 15% of the total cellular glycogen was found in the lysosomes as in control cells. The strict correlation between residual acid α-glucosidase activity and lysosomal glycogen accumulation was further illustrated in two adult Pompe patients with an unusually low enzyme activity. The mild clinical course is unexplained in these particular cases. The enzyme deficiency in all the different mutant cell lines was corrected by the uptake of bovine testis acid α-glucosidase from the culture medium. As a result of this, the lysosomal glycogen storage disappeared, and the balance between lysosomal and cytoplasmic glycogen was restored to normal. The implications of this study as a model for enzyme replacement therapy are discussed.

Key words: Glycogenosis type II; α-Glucosidase; Endocytosis; Lysosomes; Enzyme therapy

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INTRODUCTION

Pompe's disease or glycogenosis type II is a lysosomal glycogen storage disorder, which is inherited as an autosomal recessive trait (Pompe 1932). The primary defect is a deficiency of acid α-glucosidase (Hers 1963). Extensive heterogeneity in clinical and pathological features is observed. Generally, a subdivision in three clinical forms is made (Howell and Williams 1983).

The infantile variant is characterized by failure to thrive, severe hypotonia, cardiac hypertrophy, moderate hepatomegaly and enlargement of the tongue (Cardiff 1966; Engel et al. 1973). Symptoms are present at birth or shortly after. The patients die within the first or second year of life by cardiorespiratory failure. In this infantile subtype of the disease glycogen storage is most severe and residual activity of acid α-glucosidase is hardly detectable in any tissue (Hers and De Barsy 1973).

The adult form has its onset mostly after the second decade of life (Engel 1970; Trend et al. 1985). In general, only the skeletal muscles are impaired, especially those of the pelvic girdle, proximal part of the legs and thorax. The disease is slowly progressive, and respiratory failure is the major cause of death. Patients over 60 years of age have been described (Loonen et al. 1981). A residual acid α-glucosidase activity between 10 and 20% is usually measured in cultured fibroblasts of adult patients (Reuser et al. 1978).

The juvenile form is often referred to as an intermediate variant with onset of symptoms in early or late childhood (Swaiman et al. 1968; Danon et al. 1986).

In the past, attempts were made to reduce glycogen storage and clinical symptoms, by intravenous or intramuscular injection of α-glucosidase purified from *Aspergillus niger* (Baudhuin 1964; Hug and Schubert 1967) and human placenta (Hers and De Barsy 1973). No beneficial effect was found. In model experiments, however, it was demonstrated that acid α-glucosidase purified from bovine testis was very efficiently taken up by cultured fibroblasts and muscle cells via mannose 6-phosphate receptors, present at the cell surface. Enzyme deficient cells could thus be corrected enzymatically (Reuser et al. 1984). The question remained unanswered as to whether the administered enzyme would reach the secondary lysosomes and degrade the accumulated glycogen.

We have tried to answer this question by studying the effect of endocytosed bovine testis acid α-glucosidase on the cytoplasmic and lysosomal glycogen content of cultured fibroblasts from patients with Pompe's disease. Cell lines from different variants were chosen in order to investigate simultaneously the correlation between residual enzyme activity and glycogen storage. In particular, two cell lines from adult patients were included because of their unusually low acid α-glucosidase activity.

MATERIALS AND METHODS

Cell culture procedures

Fibroblast cell lines originating from skin biopsies of 4 infantile, 2 juvenile and 6 adult glycogenosis type II patients, and 4 control subjects were used (Table I). They

were obtained from the cell repository at the Department of Clinical Genetics, University Hospital Rotterdam (Dr. M.F. Niermeyer), except for cell line GM1935, which was supplied by the Human Mutant Cell Repository (Institute for Medical Research, Camden, NJ). The fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum and antibiotics, in an atmosphere of 10% CO_2 and 90% air.

Cells grown in 24 well plastic culture dishes were used for measurement of acid α-glucosidase activity and their glycogen content. Cell homogenates were prepared by sonication in distilled water.

Fibroblasts cultured in 25-cm^2 flasks were used for isolation of lysosomes.

Isolation of lysosomes

Fibroblasts were homogenized in 800 μl of 250 mM mannitol with 10 mM EDTA and 20 mM imidazol, pH 7.4. A Potter-Elvejhem homogenizer with a 2-ml capacity was used. Seventeen up and down strokes at 1300 rpm were applied. The homogenate (A) was centrifuged at 100 × *g* to remove cell nuclei and intact cells. The supernatant was saved and the pellet washed with 200 μl of buffered mannitol solution. The first and second supernatants were pooled (B) and lysosomes were spun down (1000 × *g*, 15 min, 4 °C). The supernatant (C) was saved and the enriched lysosomal preparation washed once and finally taken up in 400 μl buffered mannitol solution (D). Samples from A, B, C and D were taken for measuring the glycogen content, and acid α-glucosidase and β-hexosaminidase activities. Before assays were done, the samples were frozen and thawed 4 times. The β-hexosaminidase activity was measured in the cytosol and lysosomal pellet to calculate the latency of lysosomal enzymes during the isolation procedure.

Uptake of enzyme in culture

Four weeks after seeding, purified bovine testis acid α-glucosidase (Van Diggelen et al. 1982) with a specific activity of 346 μmol per mg protein per hour was added to one set of cultures in a final concentration of 1 μmol MU/h/500 μl medium. The medium used for uptake was Ham's F10 medium supplemented with 10% fetal calf serum and antibiotics. Piperazine *N,N'*-bis-2-ethanesulfonic acid (Pipes, BDH, Chemicals LHD Poole, Dorset, England) was added in a concentration of 3 mM to maintain the pH between 6.8 and 7.0. A second set of cultures obtained the same medium without acid α-glucosidase.

Enzyme activity

For measuring the acid α-glucosidase and β-hexosaminidase activity, a 10-μl sample was incubated with 20 μl of 4-methylumbelliferyl-α-D-glucopyranoside or 4-methylumbelliferyl-β-D-*N*-acetylglucosaminide substrate for 1 h at 37 °C. The reaction was terminated by adding 500 μl of 0.5 M sodium carbonate buffer, pH 10.7, as described by Galjaard (1980).

Protein assays

The protein concentration of the fibroblast homogenates from the 24-well culture dishes was determined according to Lowry et al. (1951).

In the samples drawn during the isolation of the lysosomes the protein concentrations were measured using fluorescamine according to Udenfriend et al. (1972), because imidazol interferes with the Lowry method.

Glycogen assay

Glycogen levels were determined by a modified procedure of Huijing (1970). Cell homogenates were heated for 1 min at 100 °C. After cooling, a 15-μl aliquot was incubated with a 15-μl mixture of α-amylase (0.2 mg/ml) and α-amyloglucosidase (0.05 mg/ml) in acetate buffer (pH 4.8) for 1 h. The samples were then heated for 1 min at 100 °C and the amount of liberated glucose was determined with the glucose-oxidase method using 2,2′-azino-di(3-ethylbenzthiazolinsulphonic acid-6) ammonium salt (ABTS) as acceptor (Koster et al. 1972).

RESULTS

Fibroblasts from control subjects and patients with various clinical forms of glycogenosis type II were cultured under standardized conditions. Their total glycogen content and acid α-glucosidase activity were compared during 4 subsequent weeks (Fig. 1).

One week after seeding all cultures were confluent and the amount of protein per well remained constant. The acid α-glucosidase activity of control fibroblasts showed a steady increase during 4 weeks (Fig. 1). A similar increase of activity was observed in fibroblasts from most adult patients and from one juvenile variant (77RD84), but the activity did not exceed 20% of the control value. The highest residual activity reached in a second patient with the juvenile phenotype (124LAD) was about 2%, while virtually no activity could be measured in any of the cell lines from the infantile subtypes. In two adult variants, especially selected for their low residual acid α-glucosidase activity (GM1935 and 84RD390) 1 and 3%, respectively, were measured as the highest values.

When the total glycogen content of the different fibroblast lines was compared during the same period, no distinction could be made between patients and control subjects. In one control line, TRI, even pronounced storage of glycogen seemed to occur.

After 4 weeks, bovine testis acid α-glucosidase was added to one set of wells of each cell line and the effect on the glycogen level was measured at day 1, 3, 6 and 9 after addition. Enzyme activities above control level were reached in all cultures (data not shown). The half life of the endocytosed enzyme was about 9 days. A second set of wells without enzyme added served as control.

Addition of enzyme resulted in a significant decrease of the glycogen content of fibroblasts from patients with the infantile subtype of the disease (the average decrease is shown in Fig. 1, dotted line). Degradation of glycogen was also observed in fibroblasts from juvenile patients, but more so in cell line 124LAD than in 77RD84. The amount

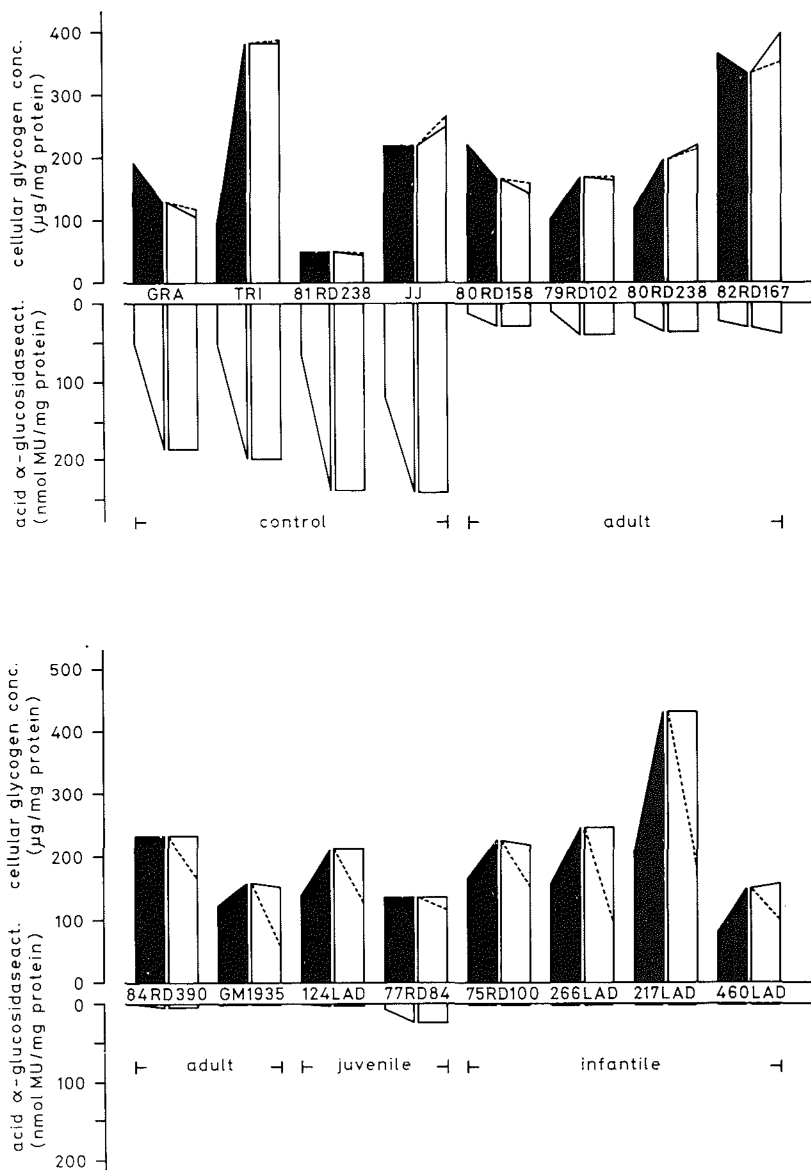

Fig. 1. Changes in glycogen level and activity of acid α-glucosidase over 2 periods: first period (black bar) spans 4 weeks immediately after seeding. Each week 2 wells per cell line were harvested. The second period (open bar) spans 9 days following enzyme addition; cells were harvested at days 1, 3, 6 and 9. Average calculated changes in glycogen levels and acid α-glucosidase activities are indicated by the slope of the line connecting week 1 and week 4 in the first period and day 1 and day 9 in the second period. ——, slope without enzyme addition; ---, slope after enzyme addition.

of glycogen in cells from control subjects and three adult patients (80RD158, 79RD102 and 80RD238) did not decrease. However, in cultures from the two adult patients with low residual enzyme activity (84RD390, GM1935), degradation of glycogen was clearly measured.

To obtain more accurate information on the relationship between residual acid α-glucosidase activity, glycogen storage and clinical heterogeneity, a distinction was made between lysosomal and total cellular glycogen. Lysosomes were isolated during 4 successive weeks after seeding. Fig. 2 shows which part of the glycogen is localized in the lysosomes of some representative cultures. Table 1 gives the lysosomal glycogen content as a percentage of the total for each of the cell lines, and acid α-glucosidase activity, age of the patients and clinical subtype are also summarized.

In all infantile cases of glycogenosis type II an excessive storage of glycogen was observed in the lysosomes (e.g. 217LAD, Fig. 2). An increase from about 38% in week 1 to 80% in week 4 was found (Table 1). The accumulation of glycogen was less in fibroblasts from juvenile patients (Table 1 and Fig. 2). Cell lines from adults with 10–20% residual α-glucosidase activity had an average lysosomal glycogen content of 15%, which is comparable with the amount found in control fibroblasts (14%, Table 1). Interestingly, the two remaining adult fibroblast lines (84RD390 and GM1935) showed an accumulation of glycogen in accordance with their relatively low residual acid α-glucosidase activity. The rather high absolute amount of lysosomal glycogen in control line TRI was also noteworthy. However, in terms of percentage it was the same as in other controls.

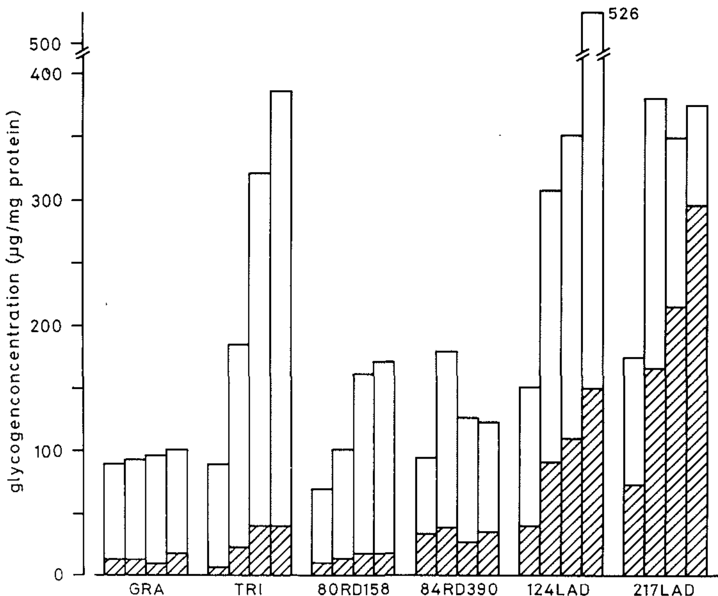

Fig. 2. Absolute amount of lysosomal glycogen of some representative cell lines expressed as part of total cellular content in 4 subsequent weeks after seeding.

TABLE 1

LYSOSOMAL GLYCOGEN CONTENT AS PERCENTAGE OF TOTAL AMOUNT

Asterisk indicates age of death. Percentage of lysosomal glycogen was measured 1, 2, 3, and 4 weeks (wk 1, wk 2, wk 3, wk 4) after seeding.

Clinical subtype	Average α-glucosidase act. (%)	Cell line (code)	Lysosomal glycogen (% of total)				Age
			wk 1	wk 2	wk 3	wk 4	
Control	Normal	GRA	13	14	11	19	27 yrs
	Normal	JJ	11	14	9	10	35 yrs
	Normal	TRI	8	12	12	10	39 yrs
	Normal	81RD238	19	11	21	25	5 yrs
Adult	14	80RD158	14	10	10	13	45 yrs
	15	79RD102	11	18	–	12	53 yrs
	19	80RD238	13	29	16	17	59 yrs
	18	82RD167	13	13	18	–	69 yrs*
	3	84RD390	31	17	23	29	44 yrs
	1	GM1935	26	–	49	66	30 yrs
Juvenile	10	77RD84	16	10	29	39	20 yrs
	2	124LAD	26	33	31	29	18 yrs*
Infantile	<1	75RD100	37	47	61	81	18 mth*
	<1	460LAD	36	44	55	–	8 mth*
	<1	266LAD	37	23	–	75	1 mth*
	<1	217LAD	42	44	61	79	6 mth*

TABLE 2

EFFECT OF ENDOCYTOSED ACID α-GLUCOSIDASE ON THE LYSOSOMAL GLYCOGEN CONTENT

Clinical subtype	Cell line (code)	Enzyme addition	Lysosomal glycogen (% of total)
Control	GRA	–	15
		+	14
Adult	80RD158	–	14
		+	14
Infantile	217LAD	–	56
		+	20
	460LAD	–	33
		+	12

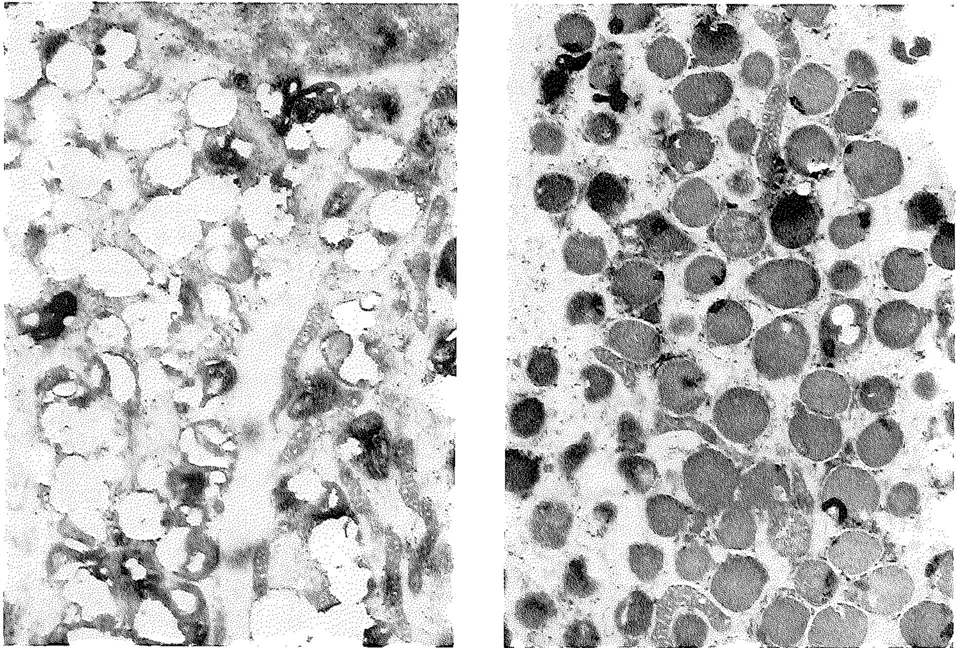

Fig. 3. Transmission electron micrographs of cultured fibroblasts from a patient with the infantile form of glycogenosis type II. The picture at the left was taken 6 days after addition of bovine testis acid α-glucosidase. The picture at the right shows the cells at the same day without addition of enzyme.

As in the previous series of experiments, bovine testis acid α-glucosidase was added to some of the cultures 4 weeks after seeding, to study more specifically the effect on the lysosomal pool of glycogen. The decrease of lysosomal glycogen content appeared most pronounced on day 6 to 9 after enzyme addition. The lysosomal glycogen content expressed as percentage of the total cellular amount, with or without addition of enzyme, is shown in Table 2. As expected, no effect on the lysosomal glycogen content was seen in the control line (GRA) or in the adult culture (80RD158). However, a significant decrease was found in the infantile cases. In 460LAD the lysosomal glycogen content was even reduced to control level.

The clearance of glycogen from the lysosomal compartment is further illustrated in Fig. 3 showing electron micrographs of fibroblasts from a patient with the infantile subtype of glycogenosis type II. Pictures were taken 6 days after addition of enzyme.

DISCUSSION

Cultured fibroblasts from 12 patients with various clinical forms of glycogenosis type II were used as a model system to study the balance between acid α-glucosidase activity and lysosomal glycogen, and furthermore to investigate the potential value of enzyme replacement therapy.

Our data demonstrate that the lysosomal accumulation of glycogen in fibroblasts

is strictly determined by the level of residual activity. The most pronounced storage occurs in fibroblasts from patients with the lowest enzyme activity. These patients all have the rapidly progressive infantile form of the disease. Glycogen storage is less in two juvenile variants with a higher enzyme level and not detectable in fibroblasts from adult patients with more than 10% of the normal activity. Thus, the fibroblast model mimics the in vivo correlation between residual enzyme activity and clinical severity (Mehler and DiMauro 1977). However, it is not representative in all instances. For example, 3% residual activity is measured in fibroblasts from a 44-year-old patient (84RD390) and 1% in another adult case of glycogenosis type II (GM1935). These patients are exceptional and only one other adult case with such a low activity has been described in the literature (Reuser et al. 1987). An explanation for the mild clinical phenotype could not be given. The present data illustrate that, also in fibroblasts from these patients, the low enzyme activity results in accumulation of glycogen. This apparent discrepancy between metabolic defect and clinical phenotype may be due to a different expression of acid α-glucosidase deficiency in muscle cells compared to fibroblasts. Therefore, studies on (cultured) muscle cells are essential in these particular cases.

With respect to glycogen storage there is another, more general, difference between the in vivo situation and the fibroblast model system. In cultured fibroblasts a residual activity of 10–20% appears to be enough to prevent glycogen storage. However, patients with such activity present muscle weakness. This suggests that the lysosomal system is challenged more in muscle cells than in fibroblasts, by the influx of glycogen from the cytoplasm. One can envisage that the delicate balance between residual enzyme activity and glycogen is disturbed with increasing age when subtle metabolic changes occur and the regeneration capacity of muscle decreases. This could be one of the reasons for the late onset of symptoms.

Distribution of glycogen over the cytoplasm and lysosomes is remarkably constant in fibroblasts from healthy individuals and adult patients with a relatively high enzyme activity. Approximately 15% of glycogen is found in the lysosomal fraction. Similar results were reported by Geddes and Stratton for normal rat liver (1977). This percentage of lysosomal glycogen cannot be reduced by adding acid α-glucosidase to the cultures. Also the total glycogen level remains constant. In contrast, enzyme uptake leads to a significant degradation of glycogen in all cell lines with low endogenous enzyme activity. This is most pronounced in the infantile variants. The receptor mediated uptake of acid α-glucosidase in our model system is so efficient that enzyme activities above control level are reached. As a result of correcting the primary defect, the balance between lysosomal and cytoplasmic glycogen is completely restored. We conclude that it is worthwile to further investigate the fundamental aspects of enzyme replacement therapy.

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Receptor-Mediated Uptake of Acid α-Glucosidase Corrects Lysosomal Glycogen Storage in Cultured Skeletal Muscle

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ABSTRACT. Attempts at treatment of glycogenosis type II and other lysosomal storage disorders by enzyme replacement have been reported. Parenteral enzyme administration has been ineffectual. Treatment by bone marrow transplantation is currently under investigation. We have used cultured skeletal muscle cells from a patient with infantile glycogenosis type II to study fundamental aspects of enzyme replacement therapy. Efficient uptake of acid α-glucosidase was achieved by using the mannose-6-phosphate receptor on the cell surface as a target for an enzyme precursor with phosphorylated high-mannose types carbohydrate chains purified from human urine. We found that the enzyme was channeled to the lysosomes and converted to mature acid α-glucosidase. Glycogen storage was reversed. The results are discussed in relation to treatment of glycogenosis type II. (*Pediatr Res* 24: 90–94, 1988)

Abbreviation

IGF, insulin-like growth factor

Pompe's disease or glycogenosis type II is an autosomal recessive disorder (1, 2). Deficiency of acid α-glucosidase is the primary defect, leading to lysosomal accumulation of glycogen (3). Cardiomegaly, hypotonia, and moderate hepatomegaly are characteristic of the rapidly progressive infantile form of the disease. Death occurs in the first or second year of life as a result of cardiorespiratory failure (4). In late onset forms of the disease (juvenile and adult variants) impairment of skeletal muscle function is usually the only symptom (5). Patients more than 60 yr old have been described (6).

In the past, enzyme replacement has been attempted in various lysosomal storage disorders by parenteral administration of purified lysosomal enzymes (7, 8). Infantile Pompe patients received acid α-glucosidase purified from *Aspergillus niger* (9, 10) or human placenta (4). None of these attempts was clinically successful. At present, this form of therapy has been abandoned, and treatment of lysosomal storage disorders by bone marrow transplantation is currently under investigation (11–16). Some beneficial effects have been reported in the mucopolysaccharidoses. However, the few attempts at treatment of glycogenosis type II with bone marrow transplantation have not been successful (15, 16). No increase of acid α-glucosidase activity was found in muscle tissue. Elevated enzyme levels were only measured in blood cells, which in fact reflected the presence of donor cells.

The lack of an efficient treatment for lysosomal storage diseases in general and glycogenosis type II in particular has stimulated us to investigate fundamental aspects of receptor-mediated enzyme replacement therapy.

During the last decade much has been learned about the role of receptors as signal transducers, and as mediators of selective transport of macromolecular compounds. The mannose-6-phosphate receptor was recognized by its function in endocytosis of high-uptake forms of lysosomal enzymes in cultured fibroblasts (17, 18). Later it became evident that the receptor is predominantly localized intracellularly, and is mainly involved in selective transport of lysosomal enzymes from the Golgi complex to lysosomes (19). Various cell types appear to contain the receptor (20), but a systematic study of the expression of the mannose-6-phosphate receptor on the plasma membrane of different cell types has not been performed.

In a previous study, we have shown that mannose-6-phosphate receptors are present at the cell surface of myotubes and mediate efficient uptake of lysosomal enzymes containing carbohydrate chains with mannose-6-phosphate residues (21). However, the most important question remained unanswered: does exogenously supplied enzyme reach the glycogen storage vacuoles of skeletal muscle cells and does it degrade the accumulated glycogen. To answer this question cultured skeletal muscle cells from an infantile glycogenosis type II patient were used as a model system, and supplied with a high-uptake precursor of acid α-glucosidase purified from human urine.

METHODS

Cell culture procedures. Muscle cell cultures were obtained from an infantile glycogenosis type II patient by dissociation of a biopsy from the quadriceps muscle (0.1 g wet weight, Fig. 1) as described by Yasin *et al.* (22). Primary cultures were preplated once to select against fibroblasts (23). Dulbecco's modification of Eagle's medium supplemented with fetal calf serum (20%), chicken embryo extract (2%), and antibiotics were used as growth medium. An atmosphere of 10% CO_2 and 90% air was maintained. Experiments were performed in 24-well plastic tissue culture plates. At a density of 10^4 cells/cm^2 myoblast fusion was stimulated by a change of medium. The fusion medium consisted of Dulbecco's modification of Eagle's medium supplemented with horse serum (2%), in an atmosphere of 5% CO_2 and 95% air. In the next 4 days more than 90% of the mononuclear cells fused to form myotubes.

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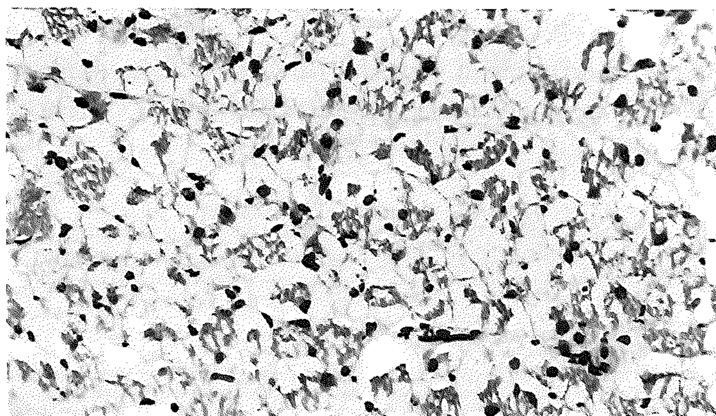

Fig. 1. Light micrograph of muscle tissue derived from the severely damaged quadriceps of the patient at 9 months of age (hematoxylin and eosin, × 630).

A high-uptake 110 kDa precursor of acid α-glucosidase (sp. act. 180.2 μmol 4-methylumbelliferone/mg protein/h) was purified from human urine as described by Oude Elferink *et al.* (24). Myotubes were incubated with enzyme in a final concentration of 1 μmol 4-methylumbelliferone/500 μl/h during 16 h as described previously (21).

Miscellaneous. Acid α-glucosidase activity was measured with the artificial substrate 4-methylumbelliferyl-α-D-glucopyranoside or with glycogen (25, 26). Glycogen content of tissues, cells, and lysosomes was determined as described by Koster *et al.* (27). A crude lysosomal fraction was prepared by homogenizing muscle cells in a Potter-Elvejhem homogenizer at 1300 rpm in 250 mM mannitol with 10 mM EDTA and 20 mM imidazol, pH 7.4. Nuclei and intact cells were removed by centrifugation at 100 × *g*. Lysosomes were subsequently spun down from the supernatant at 1000 × *g* and washed once with homogenization buffer. Protein concentrations were determined according to Lowry *et al.* (28) and creatine kinase levels were measured as described previously (23). All assays were carried out in duplicate.

Immunocytochemistry was performed according to Van Dongen *et al.* (29), using mouse monoclonal antibodies (43G8) (30) raised against acid α-glucosidase and rabbit polyclonal antibodies against β-hexosaminidase (31). Immune complexes were visualized with goat anti-mouse IgG conjugated to a green fluorescent dye and goat anti-rabbit IgG conjugated to a red fluorescent dye, respectively.

Glycogen was demonstrated by transmission electron microscopy following the procedure described by De Bruijn (32).

Table 1. *Acid α-glucosidase activity**

Cells/tissue	Substrate: Glycogen	Substrate: 4-methylumbelliferyl-α-D-glucopyranoside
Leukocytes		
Patient	10	
Control range (n = 10)	70–220	
Muscle		
Patient	1.1	0.54
Control range (n = 10)	70–350	7–40
Cultured fibroblasts		
Patient		1.1
Control range (n = 10)		40–150
Cultured muscle cells		
Patient		0.83
Control range (n = 5)		50–120
Patient after enzyme uptake		150–180

* Activities are expressed as nmol glucose or methylumbelliferone/h/mg protein.

RESULTS

The clinical diagnosis of infantile glycogenosis type II was confirmed by deficiency of acid α-glucosidase activity in leukocytes, cultured skin fibroblasts, and muscle tissue. The same enzyme deficiency was demonstrated in cultured skeletal muscle cells derived from the patient (Table 1). Using immunocytochemistry, enzymatically inactive acid α-glucosidase was shown to be absent (Fig. 2*A*). However, when human urine acid α-glucosidase was applied to the deficient cells, a bright intracellular labeling pattern was observed after 16 h (Fig. 2*B*). The distribution of the fluorescent spots suggested compartmentalization in the lysosomes. Indeed, when a double labeling was performed for acid α-glucosidase (Fig. 2*C*) and β-hexosaminidase (as lysosomal marker) (Fig. 2*D*), exactly the same localization was obtained.

Subsequently, the effect of endocytosed acid α-glucosidase on the cellular glycogen content was determined. The experiments were performed with muscle cell cultures consisting of more than 90% myotubes. The high creatine kinase activity of the cells (1010–1024 mU/mg protein) was indicative of their advanced stage of differentiation. Uptake of enzyme, measured after 16 h, had been very efficient. Intracellular activity above the control range was achieved (Table 1; Fig. 3, day 1). The half-life of endocytosed acid α-glucosidase varied between 6 and 9 days in different experiments. The effect on the glycogen content of the

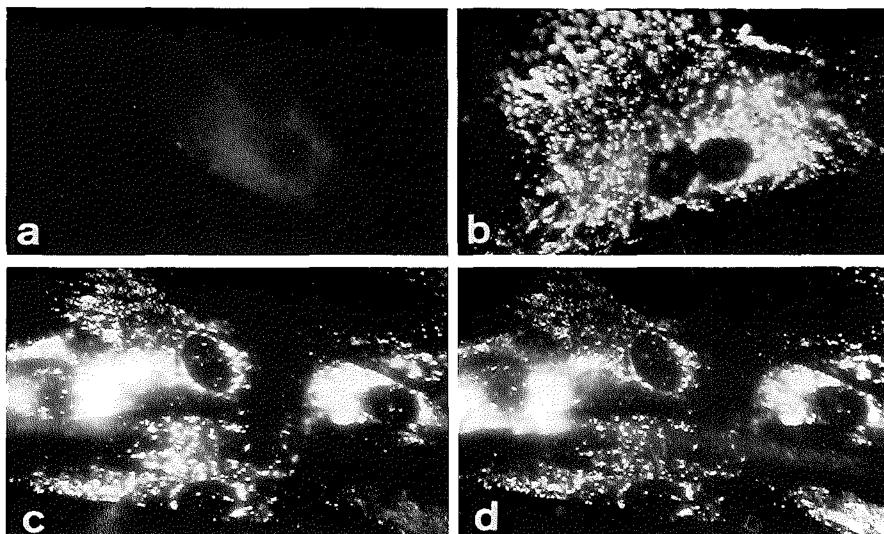

Fig. 2. Immunocytochemical demonstration of acid α-glucosidase of cultured muscle cells from the patient: *A*, multinuclear muscle cell before enzyme addition. *b*, binuclear cell 16 h after enzyme addition. *c*, intracellular localization of acid α-glucosidase after endocytosis. *d*, localization of β-hexosaminidase (as lysosomal marker) in the same cells as in *c*.

cells was most pronounced 6 days after enzyme addition, when the amount of cellular glycogen was approximately 33% reduced (see Fig. 3 for a representative experiment). In four independent experiments the reduction of cellular glycogen varied from 32–35%. In control cells no degradation of glycogen was measured after acid α-glucosidase addition.

Clearance of glycogen was examined by electron microscopy. A striking difference was observed between treated and untreated cultures inspected 6 days after enzyme addition (Fig. 4). Abundant accumulation of glycogen was seen in lysosomes of cells that did not obtain enzyme. In contrast, treated myotubes could not be distinguished from normal. Significant changes in the amount of cytoplasmic glycogen were not observed.

To further substantiate these observations, the distribution of glycogen over cytoplasm and lysosomes was quantitated. In control cells 18–24% of the total cellular glycogen was found in the lysosomal fraction, whereas in cultured muscle cells from the patient the lysosomal glycogen fraction was between 45 and 52% in various assays. Six days after enzyme addition the lysosomal glycogen content of cells from the patient had returned to control values.

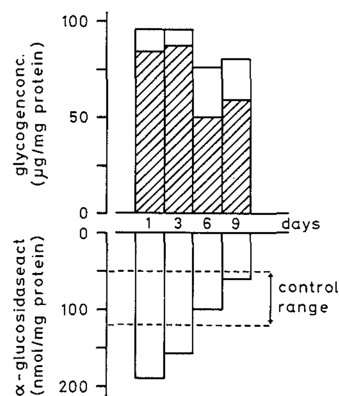

Fig. 3. Acid α-glucosidase activity of cultured myotubes after uptake of human urine enzyme (*open bars downward*). Cellular glycogen content without (*open bars upward*) and after enzyme addition (*hatched bars*). Assays of enzyme activity and glycogen content were carried out in duplicate wells. The average values of the duplicate experiments are indicated. The variation between duplicates was less than 4%.

DISCUSSION

Receptors with different specificities are essential mediators in a variety of cellular and biochemical processes. Two distinct receptors with specificity for the mannose-6-phosphate recognition marker of lysosomal enzymes have been characterized. A 215-kDa cation independent as well as a 46-kDa cation dependent mannose-6-phosphate receptors seem involved in intracellular transport of lysosomal enzymes (33–35). Endocytosis of exogenous lysosomal enzymes seems mainly ascribed to the 215-kDa receptor (36). Recently, it was shown that the latter receptor is identical to the IGF II receptor (37). The exact physiological function of the mannose-6-phosphate receptor as IGF II receptor is not yet understood. IGF II seems to function primarily in fetal and early neonatal development.

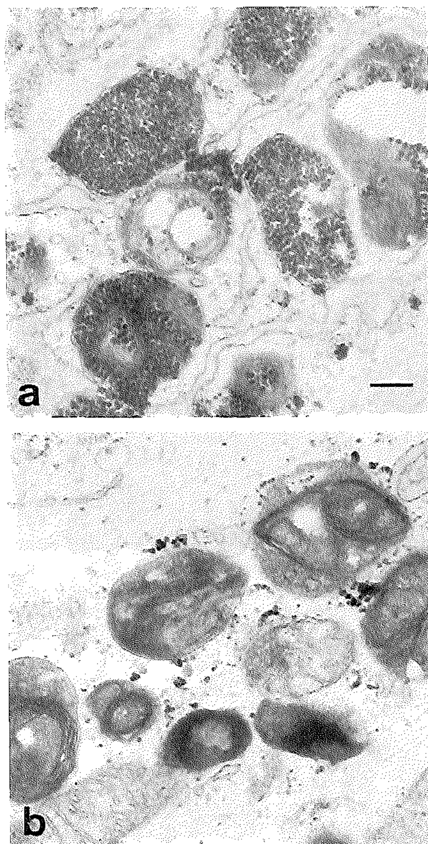

Fig. 4. Transmission electron micrographs: *A*, cultured muscle cells from the patient; *B*, a duplicate culture 6 days after treatment with human urine acid α-glucosidase. *Bar*, 2.5 μm.

Mannose-6-phosphate receptors are transiently present on the plasma membrane of a variety of cell types (17–19, 33, 38). We have previously shown that mannose-6-phosphate receptors on the surface of skeletal muscle cells can be used as targets for acid α-glucosidase species with a high mannose-6-phosphate content (21). The precursor of acid α-glucosidase, which is rather abundant in urine, has this desired characteristic (24, 39), and was used in our study as the enzyme source. After uptake by muscle cells the precursor was converted to fully active mature enzyme. This is concluded from the fact that endocytosed acid α-glucosidase can be visualized with monoclonal antibody 43G8, that only recognizes mature enzyme, and not the administered precursor (30, 40). In addition, the immunocytochemical labeling pattern demonstrates that all lysosomes are reached, and not a selected subset. Uptake of enzyme appeared very efficient. Activities above the control range were reached. Nine days after correction, the acid α-glucosidase activity was still at the lower limit of the control range. Clearance of glycogen from lysosomes, as assessed by transmission electron microscopy and quantitative assays, demonstrates that exogenously supplied acid α-glucosidase can functionally replace deficient endogenous enzyme. Our results suggest that the reported degradation of glycogen in cultured bovine muscle cells after enzyme addition also occurs in lysosomes (41). Thus, receptor-mediated enzyme replacement therapy for glycogenosis type II may be feasible, if high uptake forms of acid α-glucosidase would gain sufficient access to affected muscle tissue.

In the past, results obtained with administration of enzyme to patients with lysosomal storage disorders, and to animals, have been disappointing (7–10). Enzymes administered parenterally appeared to be captured predominantly by the Kupffer cells of the liver (42), and the initial enthusiasm about the applicability of enzyme replacement therapy subsided. However, with the present knowledge about low and high uptake forms of lysosomal enzymes and the occurrence of cell type-specific receptors (8, 17–19, 21, 33–39) it has to be concluded that in these trials the optimal conditions for enzyme replacement therapy were not used. In retrospect, the wrong species of acid α-glucosidase were administered in attempts to correct glycogenosis type II (4, 9, 10). Acid α-glucosidase purified from human placenta does not contain mannose-6-phosphate residues and is very poorly taken up by cultured muscle cells (21, 43). In contrast, mannose-6-phosphate-containing enzyme from urine is rapidly endocytosed by muscle cells. By supplying high uptake forms of acid α-glucosidase muscle cells may compete with macrophages for enzyme capture (38).

At present, the possibilities of bone marrow transplantation and cell and tissue transplantation are being explored (11–16, 44–47). The effectiveness of these procedures depends on transfer of donor enzyme to the affected organs and cell types. Our data also predict that these latter forms of therapy will be more efficient when the transplanted cells supply high-uptake forms of acid α-glucosidase to deficient muscle.

We have shown that cultured skeletal muscle from patients with glycogenosis type II is a suitable model system for fundamental studies on enzyme supplementation. Studies on animal models are in progress to test the *in vivo* feasibility of enzyme replacement therapy by infusion of high uptake forms of acid α-glucosidase. An important question will be whether the enzyme can cross the capillary wall to reach its ultimate goal. Suggestive evidence exists that various macromolecules are actively transported across endothelial cells via plasmalemmal vesicles (48–50).

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PUBLICATION V

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Prospect for enzyme therapy in glycogenosis II variants: a study on cultured muscle cells

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Summary. Impairment of skeletal muscle function is the common feature of distinct clinical forms of glycogenosis type II. In the present study, muscle cultures from different patients were used to investigate the cause of clinical heterogeneity and the feasibility of enzyme replacement therapy. The activity of acid α-glucosidase appears to be the primary factor in determining the extent of lysosomal glycogen storage in muscle, and thereby the clinical severity of the disease. Neutral α-glucosidases do not seem influencial. Correction of the enzymatic defect was achieved in skeletal muscle cultures from patients by administration of a "high-uptake" form of acid α-glucosidase, purified from human urine. The enzyme reaches the lysosomes, including the glycogen storage vacuoles, and the lysosomal glycogen content is reduced to control level. In normal muscle cells 20% of the total cellular glycogen pool is segregated in lysosomal compartments. This percentage is higher than in fibroblasts, which may partly explain why muscles are more prone to store glycogen. The relevance of this study for enzyme therapy is discussed.

Key words: Glycogenosis type II – Lysosomal storage disease – Acid α-glucosidase – Skeletal muscle – Enzyme therapy

Introduction

Replacement of deficient enzymes has been attempted in several lysosomal storage disorders either by direct application of purified enzymes or via transplanted donor cells [6, 10–12, 15, 19]. In particular, bone marrow transplantation is under consideration for future treatment. Encouraging results have been reported in various mucopolysaccharidoses [15, 23]. However, information on the long-term effects and applicability of this therapy in a wide spectrum of lysosomal storage diseases is still required. Target organs vary in the different lysosomal storage diseases and various tissues (bone, muscle, CNS) are not readily accessible to enzymatically competent donor cells [3, 15, 30]. Moreover, bone marrow transplantation is not advised for patients with late-onset forms and relatively mild phenotypes of lysosomal storage disorders, since its risks are high.

A prerequisite for determining therapeutic regimens for distinct clinical variants of a disease is knowledge about the cause of clinical diversity. In this context we have been studying molecular and cellular aspects of acid α-glucosidase (acid maltase) deficiency in glycogenosis type II using cultured fibroblasts from a large series of patients. Distinct molecular defects were discovered interfering with normal biosynthesis of acid α-glucosidase and resulting in a reduced amount of functional enzyme [25, 26]. Accumulation of glycogen in lysosomes appeared to be strictly related to the residual activity of mutant enzyme. Surprisingly, there was no storage of glycogen in fibroblasts from adult patients with more than 10% residual enzyme activity [28], even though these patients had prominent muscle weakness.

It has been demonstrated that cultured skeletal muscle cells can be used to study pathological and therapeutic aspects of glycogenosis type II [2, 7]. In the present study we used muscle cell cultures from different clinical variants to study the role of lysosomes in glycogen degradation and the cause of clinical heterogeneity. Furthermore, these cells were used as a model to investigate the potential value of mannose 6-phosphate receptor-mediated enzyme replacement therapy [13] in distinct clinical variants. To this end, a precursor of acid α-glucosidase with phosphorylated, N-linked, high-mannose carbohydrate chains was purified from human urine [20] and administered to differentiated muscle cells in culture. Delivery to the lysosomal system and degradation of lysosomal glycogen was studied.

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Materials and methods

Cell culture procedures. Skeletal muscle biopsies were obtained from five control subjects and eight patients with glycogenosis type II (Table 1). Part of the biopsy (0.1 g wet weight) was dissociated according to Yasin et al. [32] to initiate myoblast cultures as described before [4]. The cells were grown in Dulbecco's modification of Eagle's medium (DMEM) supplemented with fetal calf serum (20%), chicken embryo extract (2%) and antibiotics, in an atmosphere of 10% CO_2 and 90% air. Preplating of the primary cultures was performed once to select against fibroblasts [31]. After several passages, myoblasts were allowed to reach a density of 10^4 cells/cm^2 and cell fusion was stimulated by changing the medium to DMEM with 2% horse serum. An atmosphere of 5% CO_2 and 95% air was applied. Within 4 days more than 90% of the mononuclear myoblasts had fused to multinuclear myotubes.

Muscle cells were incubated with acid α-glucosidase in an amount equivalent to 1 μmol 4-methylumbelliferone (MU)/h per 500 μl essentially as described elsewhere [24]. The enzyme

Table 1. Glycogenosis type II variants

Clinical subtype	Cell line (code)	Cardio-myopathy	Muscle weakness	Age[a]
Infantile	sp13	+	+++	9 m*
	sp16	+	+++	9 m*
Infantile-juvenile	sp21	?	++	29 m
Juvenile	sp14	–	++	21 y
Adult	sp11	–	+	61 y
	ad1B	–	+	57 y
	ad2B	–	+	62 y
	ad3B	–	+	47 y

[a]In months (m) or years (y); *age at death

added was the 110kDa precursor of acid α-glucosidase purified from human urine according to Oude Elferink et al. [20]. The specific enzyme activity was 180.2 μmol MU per mg protein per h.

Biochemical assays. Acid α-glucosidase and β-hexosaminidase activities were measured with the artificial substrates 4-methylumbelliferyl-α-D-glucopyranoside and 4-methylumbelliferyl-β-D-N-acetylglucosaminide, respectively [8]. Neutral α-glucosidase activities were measured with 4-methylumbelliferyl-α-D-glucopyranoside in sodium phosphate buffer, pH 7.0. Protein concentrations of cell homogenates and lysosomal fractions were determined following the procedure of Lowry et al. [16] and Udenfriend et al. [27], respectively. Glycogen was measured according to Koster et al. [14]. A crude lysosomal fraction was prepared as described elsewhere [28].

Immunocytochemistry and electronmicroscopy. A double labelling method was used to localize acid α-glucosidase after endocytosis [29]. The enzyme was visualized with either mouse monoclonal antibodies (43G8) [21] or rabbit polyclonal antiserum [25] against human placenta acid α-glucosidase, in combination with goat anti- (mouse IgG) or goat anti- (rabbit IgG) conjugated to fluorescein (FITC). For demonstration of β-hexosaminidase as lysosomal marker, rabbit polyclonal antibodies [25] were used in combination with goat anti- (rabbit IgG) conjugated to rhodamine (TRITC). Glycogen was visualized by transmission electron microscopy according to De Bruijn et al. [5] with a Philips 400 electron microscope.

Results

Muscle cells from five control subjects and eight patients with different clinical forms of glycogenosis type II (Table 1) were seeded in 24-well tissue culture plates. Cell fusion and myotube formation were induced 3 days later. At day 8 the acid α-glucosidase activities of the cell lines were measured and followed to day 16 (Fig. 1). A steady increase in enzyme activity was observed in cultures derived from control subjects. To a lesser extent, this increase was also found in muscle cells from adult and juvenile glycogenosis type II variants. The levels of enzyme activity, measured with the artificial 4-methylumbelliferyl substrate as well as with glycogen, appeared to correlate with the clinical severity of the disease. All adult patients had slightly more residual acid α-glucosidase activity than a 20-year-old juvenile variant. Much less activity was measured in cultured muscle cells from a 2 year old patient, while the residual enzyme activity in both infantile cases was hardly above background. The neutral α-glucosidase activities in muscle cells from all the patients were in the control range (41.4–82.4 nmol MU/mg protein per h).

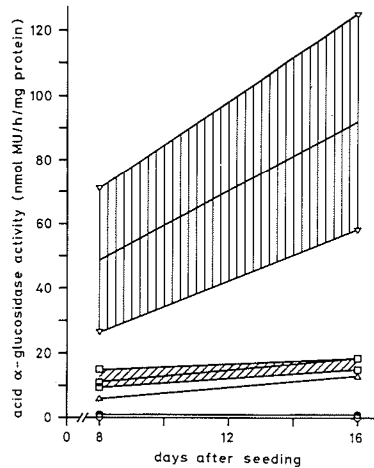


Fig. 1. Acid α-glucosidase activities of muscle cells during culture of cells from controls (▽; $n = 5$; ▥, range) and from subjects with adult (□; $n = 4$; ▨, range) juvenile (△; $n = 1$), infantile-juvenile (●; $n = 1$), and infantile (○; $n = 2$) glycogenosis type II

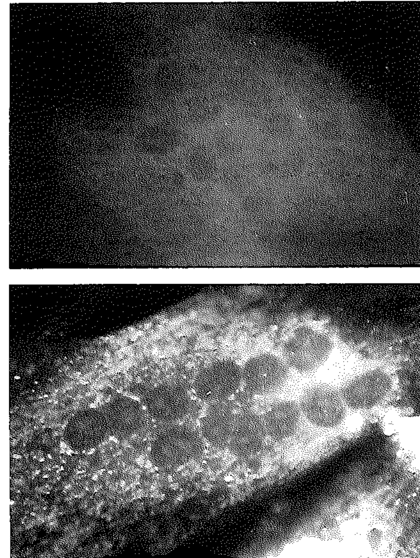
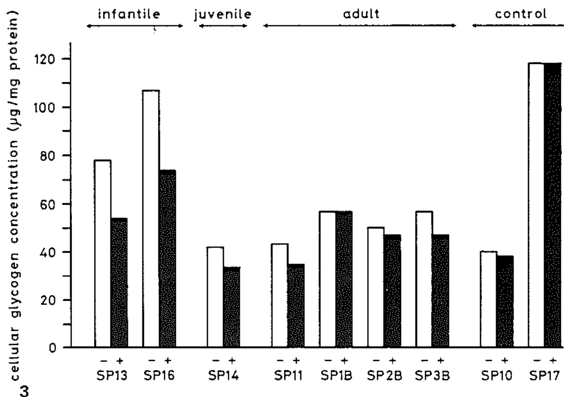


Fig. 2A, B. Uptake of acid α-glucosidase by myotubes from an infantile patient, illustrated by immunocytochemistry. **A** Before and **B** after addition of acid α-glucosidase (magnification 880 ×)



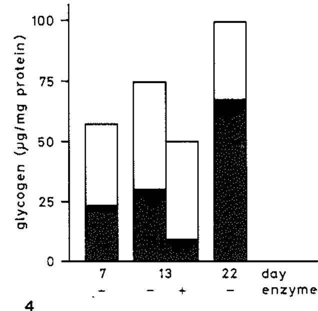


Fig. 3. Effect of human urine acid α-glucosidase 6 days after enzyme addition. The cellular glycogen content without (□) and after (■) enzyme addition is indicated. Measurements were performed in duplicate wells and varied by less than 4%

Fig. 4. Distribution of glycogen over cytosol (□) and lysosomes (■) of myotubes from an infantile glycogenosis type II patient (sp16). Glycogen concentrations were measured on days 7, 13 and 22 in cultures not supplied with enzyme (–), and in cultures 6 days after enzyme addition (day 13, +)

Table 2. Percentage of glycogen present in lysosomes

Clinical subtype	Code	Initial value[a]	Without enzyme[a]	With enzyme[a]
Infantile	sp16	31 (7)	40 (13) 70 (22)	19 (13)
	sp 13	24 (7)	40 (13)	21 (13)
Infantile-juvenile	sp21	29 (15)	52 (21)	23 (21)
Control	sp17	21 (7)	24 (15)	
	sp10	17 (7)	21 (15) 21 (22)	

[a] Day of assay in parentheses

Using immunocytochemistry, it was demonstrated that deficiency of acid α-glucosidase in myotubes from the infantile patients was accompanied by complete absence of immunologically cross-reactive enzyme protein (Fig. 2A). These deficient cells were incubated for 16 h with the 110-kDa precursor of acid α-glucosidase purified from human urine to achieve uptake of enzyme via the mannose 6-phosphate receptor. Two different antibodies were used to localize the enzyme: a rabbit polyclonal antibody that recognizes all molecular forms of acid α-glucosidase (110, 95, 76 and 70 kDa) and a mouse monoclonal antibody (43G8) that recognizes only the intermediate (95 kDa) and the mature (76 and 70 kDa) forms. An identical labelling pattern was obtained with both probes. The result with antibody 43G8 is shown in Fig. 2B. The typical intracellular distribution of label suggests segregation of enzyme in lysosomes. This was confirmed by using an antibody raised against β-hexosaminidase as lysosomal marker (not shown).

To study whether the internalized enzyme would be functional and degrade accumulated glycogen, human urine acid α-glucosidase (1 µmol MU/h) was applied to myotube cultures for 16 h. Enzyme activities rose to 180–400 nmol MU/mg protein per h in muscle cell cultures from patients. The acid α-glucosidase activities in cells from control subjects increased to the same extent. The half-life of the endocytosed enzyme varied from 6 to 9 days. The effect of endocytosed acid α-glucosidase on the cellular glycogen content is illustrated in Fig. 3. After uptake of enzyme a significant degradation of glycogen was observed in myotubes from infantile variants. The effect was most pronounced from day 6 to day 9 after enzyme administration. Less degradation of glycogen was measured in cells from one juvenile and two adult patients. Cell lines from the other two adult variants and control subjects showed no response.

To discriminate between degradation of glycogen in lysosomes or cytosol, a crude lysosomal fraction was prepared and the glycogen concentration was measured in both cellular compartments. For this purpose, muscle cell cultures from the most affected patients were chosen (sp13, sp16: infantile variants; sp21: infantile-juvenile variant) and compared with two control cell lines (sp17, sp10). In control myotubes approximately 20% (17%–24%) of the cellular glycogen appeared to be localized in lysosomes over the experimental period (days 7–22 after seeding; Table 2). In myotubes from the infantile patients a pronounced lysosomal storage of glycogen was observed. On day 13, 40% (sp13, sp16) was found in lysosomes, increasing further to 70% on day 22 (sp16). However, when human urine acid α-glucosidase was administered on day 7, further accumulation was prevented and stored glycogen was degraded. On day 13 a control level was reached (21% and 19%, respectively). Similar results were obtained for cell line sp21. Without enzyme addition, 52% of the cellular glycogen was present in lysosomes as measured on day 21. This figure was reduced to 23% when human urine acid α-glucosidase was added 6 days prior to this date. The absolute amount of glycogen distributed over lysosomes and cytoplasm of sp16 is shown in Fig. 4. It illustrates that the overall decrease of cellular glycogen is caused by degradation of lysosomal glycogen, while the cytoplasmic pool of glycogen is not affected. Clearance of

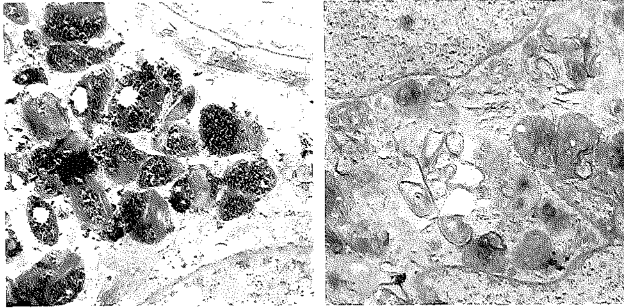


Fig. 5A, B. Electron micrographs of myotubes from an infantile patient (sp16) without enzyme addition (**A**) and 6 days after enzyme addition (**B**) (magnification 13500 ×)

lysosomal glycogen by endocytosed human urine acid α-glucosidase is further illustrated in the electron micrographs shown in Fig. 5. Myotubes derived from an infantile patient (sp16) are shown without (Fig. 5A) and with (Fig. 5B) enzyme addition. In treated cells glycogen storage is completely reversed.

Discussion

Cultured skeletal muscle cells were used to investigate the cause of clinical diversity and the feasibility of enzyme replacement therapy in glycogenosis type II. The results support our view that clinical severity of the disease is primarily determined by the amount of functionally active acid α-glucosidase [18, 26, 28]. The enzyme activity in cultured differentiated muscle cells from adult patients is significantly higher than in those from infantile variants. Muscle cells from juvenile patients show intermediate values. As a consequence, lysosomal accumulation of glycogen is much less in cells from adult than in cells from infantile patients. Data published by Mehler and DiMauro suggest that also in vivo, in skeletal muscle, the same correlation exists between enzyme activity and clinical expression of the disease [17]. However, this view is not generally accepted, and it is contradicted by early observations of Angelini and Engel [1] and more recent findings published by Ninomiya et al. [19]. In both these reports, a difference in the activity of neutral α-glucosidases in skeletal muscle of adult and infantile patients is indicated as the primary cause of clinical diversity. We have no reason to assume that neutral maltases influence the expression of lysosomal α-glucosidase deficiency, since similar activities of the neutral enzyme were measured in all cases. We believe that assays on cultured muscle cells may be more reliable in this respect than assays on biopsy material. The muscle biopsies are mostly taken from severely damaged tissue, whereas all cells in culture are viable.

Correction of the metabolic defect was achieved with the 110-kDa acid α-glucosidase precursor purified from human urine. This enzyme was chosen for two reasons. Firstly, it contains carbohydrate chains with mannose 6-phosphate moieties [20], which are recognized by mannose 6-phosphate receptors at the cell surface of muscle cells [24]. Secondly, this enzyme derived from a human source is not expected to elicit an immune response when administered to humans. Therefore, human urine acid α-glucosidase is more suitable for enzyme replacement therapy in glycogenosis type II than the non-phosphorylated and foreign enzyme species used in former clinical trials [6, 10, 12]. After enzyme administration, cultured muscle cells from patients acquire high intracellular acid α-glucosidase activities, which can be as much as those in cells from healthy individuals, within 1 day.

Importantly, acid α-glucosidase was detected by monoclonal antibody 43G8, which does not recognize the administered 110-kDa precursor, but only mature enzyme [21]. Thus, processing of the human urine precursor occurs after uptake in muscle cells, as in fibroblasts [22]. The enzyme reaches all lysosomes, including the glycogen storage vacuoles. As a result, accumulated glycogen is degraded. This is best illustrated in cells from infantile patients with the most abundant glycogen storage. However, some degradation of glycogen is also observed in cells from two adult variants, suggesting that the amount of glycogen in the lysosomes is above normal. At this point, model studies with fibroblasts gave different results, since glycogen accumulation did not occur in cells from patients with more than 10% residual acid α-glucosidase activity [28]. This indicates that such activity is sufficient to prevent lysosomal glycogen storage in fibroblasts but not in myotubes, nor in muscle. In this context, it may be of significance that in normal fibroblasts on average 14% of the total cellular glycogen is contained in the lysosomes, whereas for muscle cells the corresponding figure is 20%. The lysosomal system of muscle cells seems to be more actively involved in glycogen degradation.

We hypothesize that the late onset and slow progression of the disease in some patients may originate in a subtle imbalance between enzyme activity and lysosomal glycogen supply. Since the glycogen concentration of distinct muscle fibers will be different the absolute amount of glycogen that reaches the lysosomes by autophagy may differ too. As a consequence, one cell may start to accumulate lysosomal glycogen, whereas another is still able to degrade it. One can envisage how one fiber is more affected than the other, as is often observed [2, 9]. Till adult age, degenerated muscle fibers can be replaced by proliferation and differentiation of satellite cells. However, with increasing age of the patient the number of satellite cells and their capacity to proliferate will decrease, which impedes muscle regeneration. We presume that these factors, possibly in combination with metabolic changes in muscle fibers during aging, determine the slowly progressive nature of adult glycogenosis type II.

Our model studies on cultured muscle cells have practical implications. They indicate that enzyme replacement therapy is more promising for adult than for infantile patients with glycogen storage disease (GSD) type II. Since infantile variants have virtually no endogenous acid α-glucosidase activity, they will be totally dependent on uptake of exogenous enzyme. Adult patients, however, have a relatively high residual activity and a small increase by uptake of infused enzyme may be sufficient to prevent pathological symptoms. Therefore, enzyme administration could be a promising treatment for adult patients because the benefits of bone marrow transplantation do not outweigh the risks in adult GSD II. Research on animal models will be required to investigate the ultimate applicability of enzyme replacement therapy.

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PUBLICATION VI

submitted

TRANSENDOTHELIAL TRANSPORT OF LYSOSOMAL ENZYMES AND MANNOSE 6-PHOSPHATE RECEPTOR FRAGMENTS.

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SUMMARY

Immuno-electron microscopy was performed to obtain information on the possible transport of lysosomal enzymes across the endothelial cells of capillaries in human skeletal muscle tissue. To this end, antibodies against the lysosomal enzymes acid α-glucosidase and glucocerebrosidase as well as antibodies against the mannose 6-phosphate/insulin-like growth factor II (M6P/IGFII) receptor were employed. With each of these antibody preparations large quantities of cross reactive material were detected in the capillary spaces. Immunoblot analysis of this material revealed known molecular species as well as high molecular weight degradation products of the proteins under study. In addition, cross reactive material was found in the plasmalemmal vesicles of endothelial cells and in pericapillary spaces. In the plasma, plasmalemmal vesicles and pericapillary spaces the three proteins co-localized with albumin known to be transported across endothelial cells by means of transcytosis. These observations strongly suggest that lysosomal enzymes can pass the endothelial barrier in skeletal muscle tissue.

INTRODUCTION

Over the years much has been learned about the synthesis, intracellular transport and processing of lysosomal enzymes (1,2). In addition, defects in enzyme synthesis and function were identified in various lysosomal storage disorders (see for review Ref.2). Corresponding DNA defects were discovered in some (3-5). Despite this knowledge, the search for an efficacious treatment has been unrewarding. Attempts to correct lysosomal storage disorders have been made by cell and tissue transplantations (6-8), plasma transfusions (6,9), enzyme therapy (6), and bone marrow transplantations (10). The best

results were reported applying bone marrow transplantations as treatment. This therapeutical approach, however, appeared ineffectual for glycogenosis type II (10,11).
For glycogenosis type II, characterized by acid α-glucosidase deficiency (12,13), we have explored the feasibility of enzyme replacement therapy in model systems (14-17), and shown that the mannose 6-phosphate (M6P)/ insulin-like growth factor (IGF) II receptors on cultured skeletal muscle cells can be used as enzyme target (14,16-18). Acid α-glucosidase species containing mannose 6-phosphate are efficiently endocytosed via this receptor and transported to the lysosomes. As a result, the lysosomal glycogen storage in cultured muscle cells from patients with infantile and late onset forms of glycogenosis type II is reversed. It is clear, however, that successful application of enzyme replacement therapy depends on whether lysosomal enzymes can cross the capillary walls and reach muscle fibres, their main target.
In the present study we have investigated the localization of acid α-glucosidase in human skeletal muscle biopsies by means of immuno-electron microscopy in order to find evidence for transendothelial transport. A large amount of enzyme protein was found in capillaries, but protein was also present in plasmalemmal vesicles and pericapillary spaces. Transcytosis of acid α-glucosidase is suggested by its co-localization with albumin, known to be transported from capillaries to pericapillary spaces (19,20). Similar studies were performed for glucocerebrosidase and the M6P/IGFII receptor.

MATERIALS AND METHODS

Antibodies

Rabbit polyclonal antisera against human acid α-glucosidase (21) glucocerebrosidase (22) and albumin (Nordic) were used. Immunoglobulins against the M6P/IGFII receptor were affinity purified from rabbit polyclonal antiserum on a column of protein A sepharose 4B (23).

Immunogold labeling

Skeletal muscle biopsies, taken for diagnostic purpose but found to be normal, were fixed immediately and prepared for ultracryotomy as described before

(24). Ultra-thin frozen sections were made with a LKB NOVA ultratome equipped with the Cryo Nova, at -110°C. Immunolabeling was performed according to Van Dongen et al. (25) Goat anti-(rabbit IgG) antibodies conjugated to 10 nm colloidal gold were used to visualize antigen-antibody complexes in single labeling studies (GAR-10, Janssen-Pharmaceutica). Protein A gold probes of 5 and 10 nm were used to localize two antigens simultaneously (26). Sections were stained with uranylacetate, embedded in 1.5% methyl cellulose (27) and examined with a Philips 400 electron microscope at 80 kV.

Immunoblotting

EDTA-blood (2.5 ml) was centrifuged at 120 g for 10 min, immediately after collection. The supernatant was transferred with a plastic pipet to a siliconized plastic tube. Platelets were spun down at 600 g for 10 min. Plasma was saved. The platelet pellet was washed once with phosphate buffered saline and homogenized by sonication in 1 ml of distilled water. Platelet homogenates and 1 ml aliquots of plasma were incubated, respectively, with 100μl and 50 μl of a 1:1 suspension of concanavalin A sepharose 4B in phosphate buffer, pH 6.2, with a final concentration of 20 mM, overnight at 4°C. The sepharose beads were washed 6 times with phosphate buffer whereafter bound proteins were dissolved by heating for 10 min at 90°C in 75 μl of sample buffer (125 mM Tris-HCL, pH 6.6, 2 M glycerol, 4% SDS, 0.6% mercaptoethanol, and 0.05% bromophenol blue). Glycoproteins were separated in a 10% polyacrylamide gel, and subsequently blotted onto nitrocellulose (28,29). Lysosomal enzymes and the M6P/IGFII receptor were visualized with specific antibodies in combination with ^{125}I labeled protein A (29).

RESULTS

Skeletal muscle biopsies were prepared for ultracryotomy. The structural preservation of microvessels in thin frozen electron microscopic sections is comparable to that in conventional thin epon sections. Especially, the membranes of the endothelial cells are well defined and plasmalemmal vesicles are clearly visible. Fixed material, i.e. blood plasma, can be observed in capillaries.
In these sections we studied the localization of the lysosomal enzymes acid

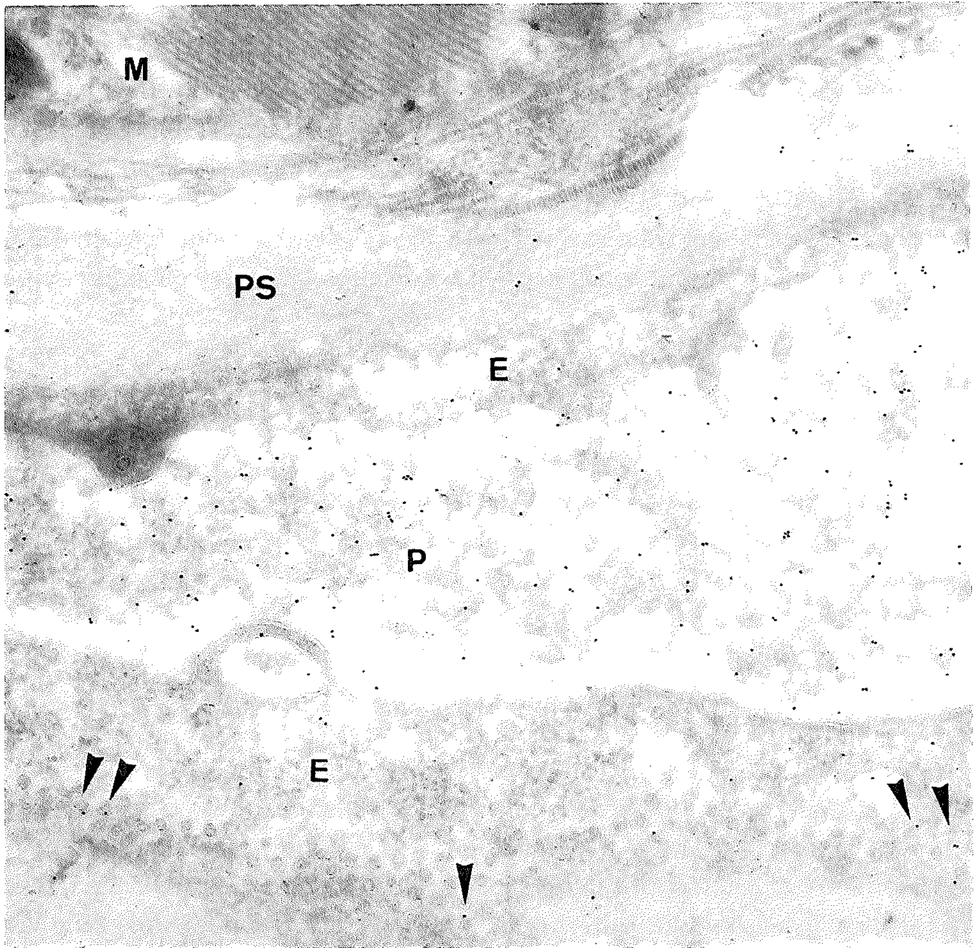

Figure 1: *Ultra-thin cryosection of muscle biopsy incubated with rabbit polyclonal antibodies against acid α-glucosidase followed by goat anti-(rabbit IgG) antibodies conjugated with 10 nm colloidal gold particles. Acid α-glucosidase is observed in bloodplasma (P), in plasmalemmal vesicles (arrow heads) and in peri-capillary spaces (PS). Muscle cell (M); Endothelial cell (E); x 66,500.*

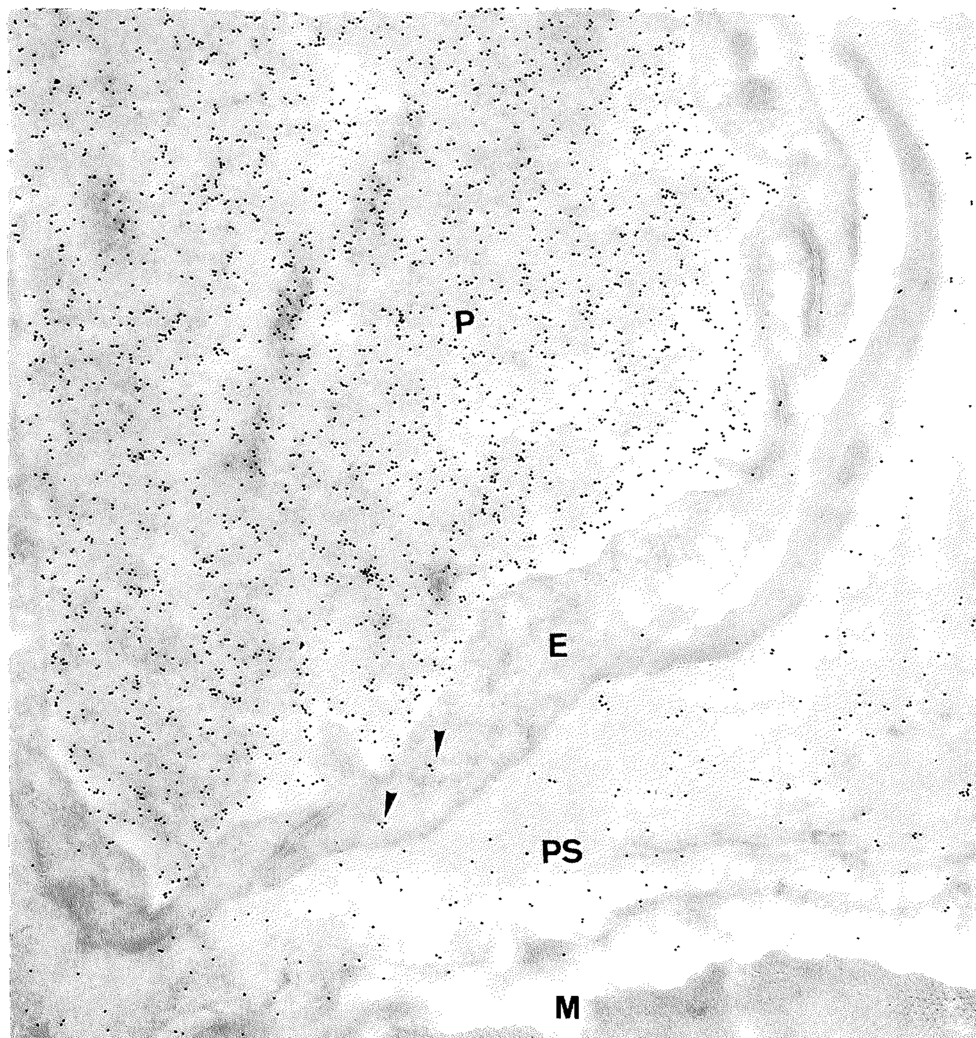

Figure 2: *Immunogold labeling of glucocerebrosidase. The antigen is detected in blood-plasma (P), in plasmalemmal vesicles (arrow heads), and in peri-capillary spaces (PS). Muscle cell (M); Endothelial cell (E); x 66,500.*

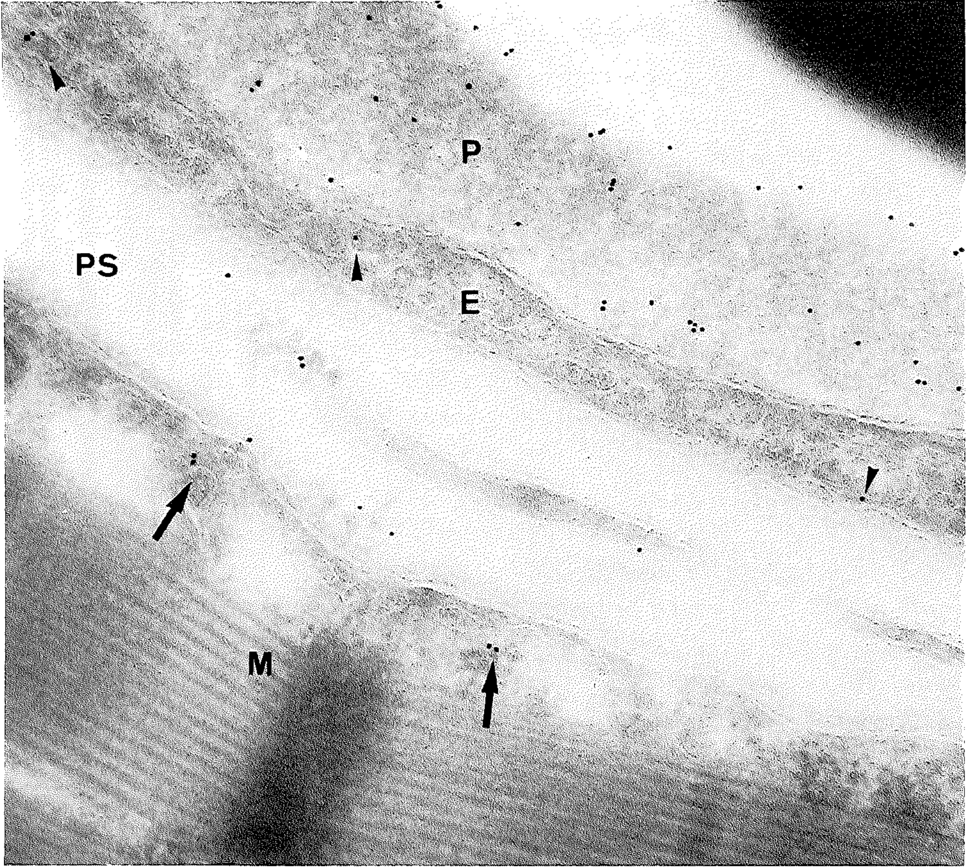

Figure 3: *Immunogold labeling of the M6P/IGFII receptor. Labeling is found in blood-plasma (P), in plasmalemmal vesicles (arrow heads) and in the peri-capillary spaces (PS). In skeletal muscle cells few gold particles are observed at the plasma membrane and in vesicles just below the plasma membrane (arrows). Muscle cell (M); Endothelial cell (E); x 67,500.*

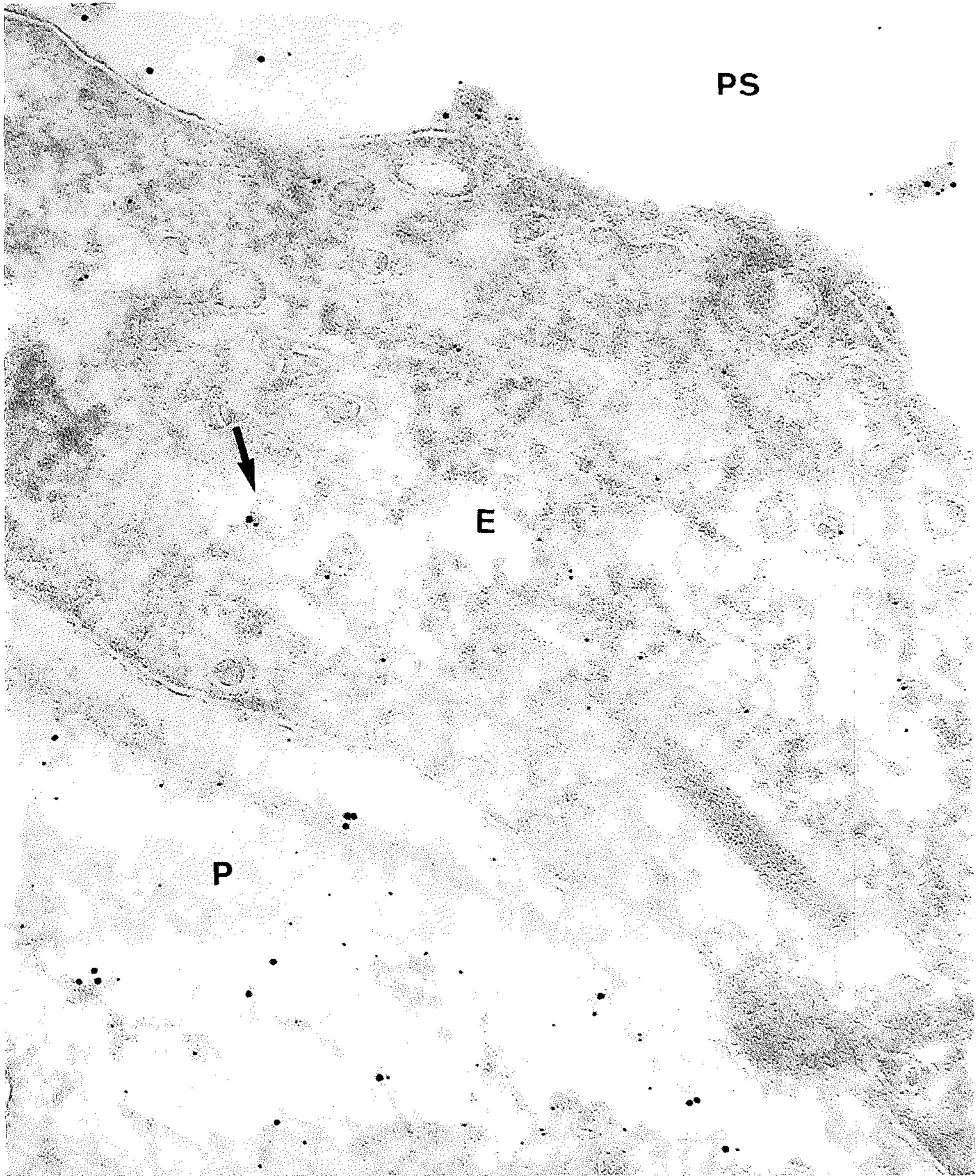

Figure 4: *Simultaneous demonstration of albumin and the lysosomal enzymes acid α-glucosidase and glucocerebrosidase. Albumin (10 nm gold particles) and lysosomal enzymes (both visualized with gold particles of 5 nm) co- localize in bloodplasma (P), in plasmalemmal vesicles (arrow) and in peri-capillary spaces (PS), Endothelial cell (E); x 92,000.*

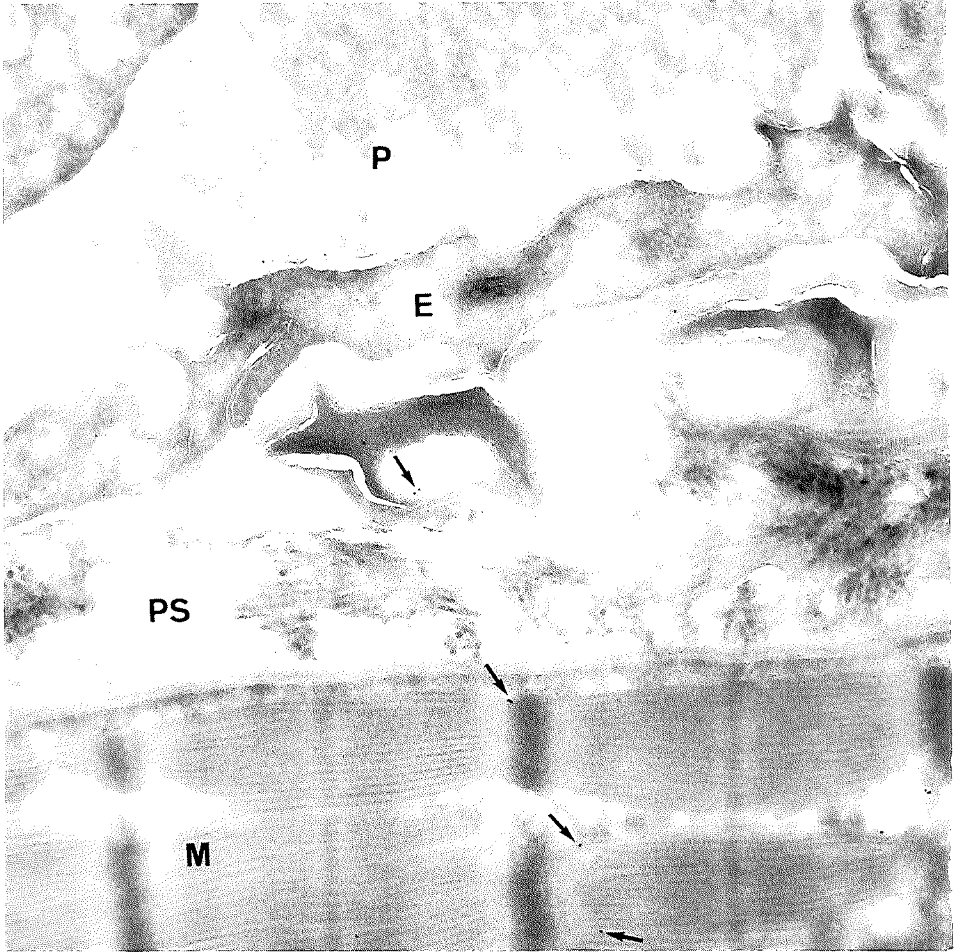

Figure 5: *Control section incubated with normal rabbit serum (instead of antiserum) followed by goat anti-(rabbit Ig G) antibodies conjugated with 10 nm colloidal gold particles as immunomarker. Gold particles (arrows) are detected only occasionally in muscle cells (M) or in peri-capillary spaces (PS). Bloodplasma (P); Endothelial cell (E); x 29,000.*

α-glucosidase and glucocerebrosidase, and the M6P/IGFII receptor by immunogold single labeling. Fig.1 reveals the localization of acid α-glucosidase, while Fig.2 and 3 show similar results for glucocerebrosidase (Fig.2) and the M6P/IGFII receptor (Fig.3). For all 3 antigens the strongest labeling was observed in the capillary lumen, where the gold particles were associated with fixed material. Endothelial cells were labeled at the luminal front, in plasmalemmal vesicles, and at the abluminal front. Furthermore, all three antigens were detected in pericapillary spaces. Skeletal muscle cells were weakly labeled at the plasma membrane and in small vesicles just below. (see Fig.3 for M6P/IGFII receptor).
Since plasmalemmal vesicles are reportedly involved in transport of (exogenous) albumin across endothelial cells, the localization of the three antigens and albumin was compared. For this purpose, a double labeling procedure was employed with 5 and 10 nm protein A gold probes. Albumin , acid α-glucosidase, glucocerebrosidase and the M6P/IGFII receptor were observed together at the same locations in capillaries, in plasmalemmal vesicles of endothelial cells and in pericapillary spaces. Co-localization of albumin with both lysosomal enzymes is shown in Fig.4 .
The specificity of the labeling was tested by incubating sections of muscle tissue with pre-immune serum instead of antiserum. As in the single labeling experiments GAR-10 was used as marker. Fig.5 shows the level of background labeling. Gold particles were occasionally observed on muscle fibres and in pericapillary spaces. As a second test, sections were incubated with antiserum in the presence of excess antigen. The specificity of the labeling for acid α-glucosidase and the M6P/IGFII receptor were analyzed in this way. Also under this condition gold particles were rarely detected (not shown).
In order to obtain more detailed information on the molecular nature of the various antigens tested, human bloodplasma was taken and analyzed by immunoblotting. Platelets were carefully removed from the plasma to prevent release of their enzymes. Glycoproteins were extracted from platelet homogenates and plasma using concanavalin A and separated by SDS-PAGE. Fig.6A shows the immunoblot analysis for acid α-glucosidase. Several molecular forms of the enzyme are present in plasma (lane 2) and platelets (lane 3). A 95 kD form matches in relative molecular mass with an acid α-glucosidase enzyme component purified from human placenta (lane 1). Glucocerebrosidase is predominantly present in molecular forms ranging from 50 kD to 66 kD in plasma and platelets (Fig.6B, lanes 1 and 2, respectively).

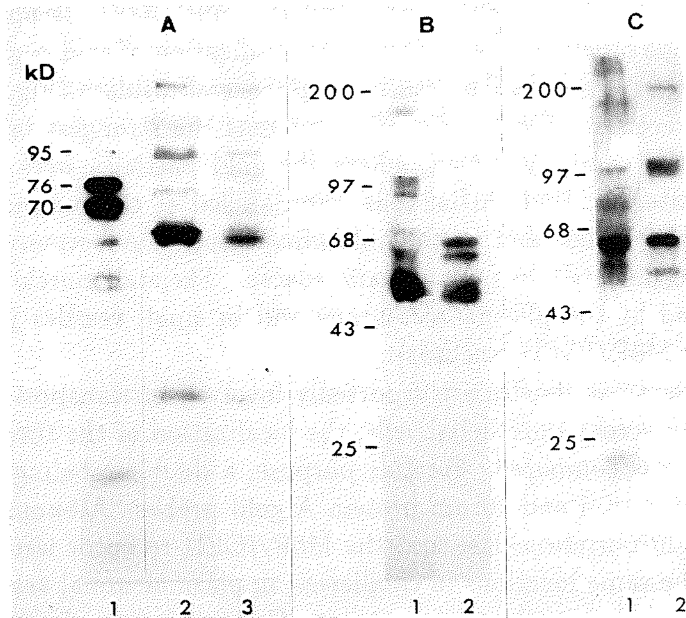

Figure 6: *Immunoblot analysis of molecular weight species of acid α-glucosidase (A), glucocerebrosidase (B) and the M6P/IGFII receptor (C) in plasma (A, lane 2; B, lane 1; C, lane 1) and platelets (A, lane 3; B, lane 2; C, lane 2) and human placenta (A, lane 1).*

The immunoblot obtained with antibodies against the M6P/IGFII receptor reveals various cross-reacting polypeptides in plasma as well as in platelets (Fig.6C, lanes 1 and 2, respectively). These range in size from approximately 250 kD (plasma) to about 55 kD (plasma and platelets).

DISCUSSION

This study conducted on skeletal muscle shows that large amounts of acid α-glucosidase, glucocerebrosidase and M6P/IGFII receptor antigens are present in capillaries. In addition, the antigens are found in plasmalemmal vesicles and pericapillary spaces. At these locations they co-localize with albumin. Since it has been shown that albumin is transported by means of plasmalemmal vesicles from capillaries to the pericapillary spaces (19,20), our results

suggest that an identical transport mechanism exists for antigenic components of the lysosomal enzymes and the M6P/IGFII receptor.

The molecular components of acid α-glucosidase and glucocerebrosidase present in plasma are similar to those in platelets. Some of the acid α-glucosidase species are known to occur also intracellularly as for instance a 110 kD precursor and a 95 kD intermediate (21,30). The 76 and 70 kD species most abundant in placenta and fibroblasts are notably absent in plasma and platelets. Instead a major polypeptide of 65 kD is found. Glucocerebrosidase was analyzed before in platelets, and essentially the same molecular species were observed as in our study (31). The strong labeling of the blood plasma with antibodies against the M6P/IGF II receptor was most surprising.

While our study was in progress Kiess et al. (32) and Causin et al. (33) reported similar observations. In the latter study in which phosphomannan binding proteins were affinity purified from human serum, a major polypeptide of 205 kD was detected. In addition, molecular weight species ranging from 104 to 180 kD were identified. From our experiments in which platelets and plasma were separated we conclude that some of the polypeptides in the serum are probably derived from platelets. The molecular weight products smaller than 104 kD have probably lost their binding capacity for mannose 6-phosphate (33), and IGF II (32) since they were not identified by Causin et al. and Kiess et al. The presence of receptor fragments is probably due to shedding of the receptor from the cell surface (33,34), a putative way of turnover of the receptor. The occurrence of various M6P/IGFII fragments in platelets as observed in our study is not completely understood, but it is conceivable that these fragments are generated in aging platelets after arrest of protein synthesis.

Acid α-glucosidase, glucocerebrosidase and M6P/IGFII receptor fragments with a molecular mass lower than 50,000 were hardly present in human plasma. This suggests that relatively high molecular weight species are transported across endothelium, but the exact mechanism of transport remains at present unclear. For albumin with a molecular mass of 67,000 it has been demonstrated that a low molecular weight binding protein is involved in trans- cytosis (35,36).

The present observations are of particular interest with respect to the possible applicability of enzyme replacement therapy in glycogenosis type II. They suggest that there is a way of transport for lysosomal enzymes across capillary walls by which infused enzymes might reach affected muscle fibres.

Acknowledgments

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PUBLICATION VII

submitted

ACID α-GLUCOSIDASE PERFUSION IN RAT HEART: A MODEL SYSTEM FOR ENZYME REPLACEMENT THERAPY IN GLYCOGENOSIS TYPE II.

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SUMMARY

Cardiac failure and skeletal muscle weakness are the main clinical features of Glycogenosis type II, a lysosomal disease caused by acid α-glucosidase deficiency. Attempts at enzyme replacement therapy with purified acid α-glucosidase species have not resulted in uptake of enzyme by the target organs.

In previous studies we have demonstrated that the mannose 6-phosphate receptor can be used for efficient correction of enzyme deficiency in cultured skeletal muscle cells. In the present study we used a rat heart perfusion recirculation system to investigate whether mannose 6-phosphate containing acid α-glucosidase is taken up from the vascular space by the cardiomyocytes. For this purpose, rat hearts were perfused with acid α-glucosidase purified from bovine testis. A 3-4 fold increase of acid α-glucosidase activity in the rat heart was observed. The increase is due to uptake of bovine testis enzyme as is demonstrated by means of specific immunoprecipitation and immunoblotting. Immunocytochemical evidence is presented that the administered enzyme reaches the lysosomal compartments of cardiomyocytes. The relevance of these findings for enzyme therapy in glycogenosis type II is discussed.

INTRODUCTION

Glycogenosis type II (GSD II) is a lysosomal storage disorder characterized by deficiency of acid α-glucosidase (1,2). The disease is inherited in an autosomal recessive mode, and is clinically very heterogeneous (3,4). The infantile subtype presents with severe hypotonia, cardiomegaly, and moderate hepatomegaly, and is mostly fatal within the first year of life. In the late onset,

juvenile and adult, variants skeletal muscle weakness is the main symptom, and respiratory failure is the major cause of death. It was demonstrated that the severity of symptoms in the various phenotypes correlates fairly strictly with the extent of lysosomal glycogen storage and residual activity of acid α-glucosidase (5-7).

An efficacious treatment for lysosomal storage diseases is not available. In recent years encouraging results have been reported, using bone marrow transplantation as therapy (8). This treatment, however, appeared ineffectual for glycogenosis type II (8-10). Also attempts at enzyme therapy, using acid α-glucosidase preparations from human placenta and Aspergillus Niger were unsuccessful (11-13).

It has been suggested that the effect of enzyme therapy could be improved by using cell surface receptors for efficient delivery of enzyme to the affected tissues (14). One such receptor, recognized for its function in uptake of lysosomal enzymes with phosphorylated carbohydrate moieties, is the 215 kD mannose 6-phosphate receptor (15). The receptor is presently known to be identical to the insulin-like growth factor (IGF) II receptor. (16), and has been demonstrated in a variety of cell types and tissues, including heart and skeletal muscle (17-19). Despite its apparent function in endocytosis of lysosomal enzymes containing mannose 6-phosphate, the receptor has never been used as target in enzyme therapy (20,21).

In previous studies we have investigated the fundamental requirements for treatment of glycogenosis type II by receptor mediated enzyme targeting (6,7,22,23). It was demonstrated that mannose 6-phosphate containing acid α-glucosidases purified from bovine testis, and human urine are taken up by cultured fibroblasts, and skeletal muscle cells with a 100 fold higher efficiency than the enzyme species lacking this recognition marker, and used in the few attempts at enzyme replacement therapy in GSD II. The phosphorylated enzymes are transported to the lysosomes and are able to reverse abundant glycogen storage in cells obtained from the most severely affected infantile patients.

In the present study we have used a rat heart perfusion system according to Langendorff to investigate whether infused bovine testis acid α-glucosidase is transported from the vascular space across the endothelial barrier to the cardiomyocytes.

MATERIALS AND METHODS

Enzyme perfusion

Hearts were obtained from 20 week old male Wistar rats, under anaesthesia with di-ethylether. They were mounted in a perfusion system according to Langendorff, and cannulated in the aorta (24,25). Thus, the coronary vascular system was perfused selectively. Experiments were performed at 37°C, pH 7.4, using a modified Tyrode's buffer (128 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 20 mM $NaHCO_3$, 0.4 mM NaH_2PO_4 and 11 mM glucose, saturated with 95% O_2-5%CO_2). After an equilibration period of 10 min, in which heart contractility and flow stabilized, a recirculation perfusion system was connected, containing 35 ml of Tyrode's buffer to which acid α-glucosidase had been added in an amount equivalent to 40 μmol methylumbelliferone (MU) per h. The enzyme (specific activity 338 μmol MU/mg protein.h.) was purified from bovine testis according to Van Diggelen et al. (26) and is rich in mannose 6-phosphate residues. After 72 min, the recirculation system was disconnected, and the heart was perfused with the initial equilibration buffer for another 25 min. During perfusion samples were taken for measuring acid α-glucosidase activities. At the end of the experiment, the heart was removed from the perfusion system, and
quickly divided in two halves. One part was embedded in tissue-tek (Miles) for immunocytochemical purposes. The other part was frozen in liquid nitrogen for biochemical assays.

Miscellaneous

Rat hearts were homogenized in 400 μl of distilled water with a Potter-Elvejhem homogenizer at 1300 rpm and subsequent sonication. Cell debris was removed by centrifugation at 15,000 x g. Acid α-glucosidase activities were measured with the artificial substrate 4 methylumbelliferyl-α-D-glucopyranoside as described previously (27). Protein concentrations were determined following the procedure of Lowry et al. (28).

Immunoassays

Antiserum against human placenta acid α-glucosidase was raised in Wistar

rats, and antiserum against bovine testis acid α-glucosidase in Swiss mice, essentially as described before (29). Rat heart homogenate, or purified bovine testis acid α-glucosidase was diluted to an acid α-glucosidase activity of 1 μmol MU per h per 20 μl. Samples of this volume were incubated with 10 μl of serial dilutions of rat antiserum in phosphate buffered saline containing 1 mg/ml BSA (PBS/BSA) or with PBS/BSA alone (as infinite antiserum dilution). Staphylococcus A membranes (10 μl of a 1:1 dilution in PBS/BSA) were added to precipitate immunecomplexes. Incubations were performed overnight at 4°C. The membranes were spun down at 15,000 x g, and acid α-glucosidase activities were determined in the supernatant and on the membranes. An immunocytochemical labeling method was used to co-localize bovine testis acid α-glucosidase and endogenous β-hexosaminidase (30). For this purpose, frozen tissue sections (7 μm) were fixed in formaldehyde vapour, and post-fixed in 100% methanol. They were incubated with mouse anti- (bovine testis acid α-glucosidase) serum in combination with goat anti- (mouse IgG) conjugated to fluorescein (FITC, Nordic), and with rabbit polyclonal antiserum against human β-hexosaminidase (29) in combination with goat anti- (rabbit IgG) conjugated to rhodamin (TRITC, Nordic).

Immunoblotting

Rat antiserum against bovine testis acid α-glucosidase was added to 400 μl aliquots of rat heart homogenate (0.2 g wet weight/400 μl), together with 50 μl of protein A sepharose beads (1:1 diluted in PBS) to precipitate immunecomplexes. Incubations were carried out overnight at 4°C. Nonspecifically bound proteins were removed by washing the beads 4 times with 1 ml of PBS. Bound acid α-glucosidase was dissolved by heating for 10 min at 90°C in sample buffer (125 mM Tris-HCl, pH 6.6, 2 M glycerol, 4% SDS, 0.6% mercaptoethanol, and 0.05% bromophenol blue). Proteins were separated by size in a 10% polyacrylamide gel according to Laemmli (31) and blotted onto nitrocellulose filters as described by Towbin et al. (32). Bovine testis acid α-glucosidase was visualized with polyclonal mouse antiserum against bovine testis enzyme in combination with ^{125}I labeled protein A (32).

RESULTS

After an initial equilibration period of 10 min, the heart was perfused with 40

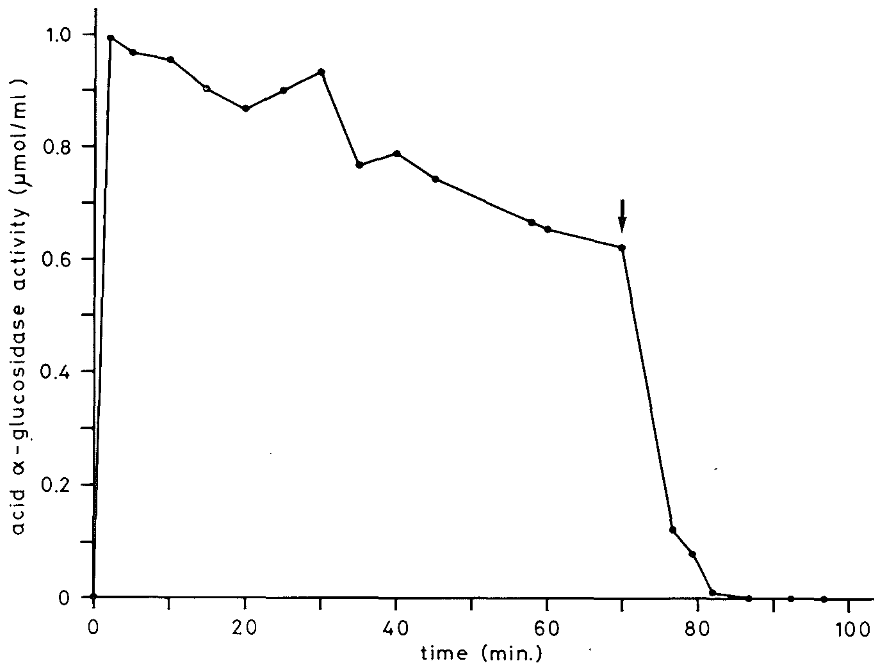

Figure 1: *Acid α-glucosidase activities in the perfusate. Enzyme perfusion was performed with purified bovine testis acid α-glucosidase (40 μmol MU/h in 35 ml Tyrode's buffer). After enzyme perfusion (arrow) the heart was rinsed with Tyrode's buffer for an additional 25 min.*

μmol (MU/h) of purified bovine testis acid α-glucosidase in a total volume of 35 ml Tyrode's buffer, using a perfusion-recirculation system. During the experiment, samples were taken from the perfusate to follow the acid α-glucosidase activities in the circulating fluid (Fig.1). A gradual decline of enzyme activity was observed over the experimental period. After 72 min of enzyme perfusion the heart was rinsed for 25 min with the original equilibration buffer to clear the capillary bed. This resulted in a fast decline of enzyme activity. After 15 min, when the perfusate was completely devoid of acid α-glucosidase, the heart was removed from the perfusion apparatus and quickly frozen.
The acid α-glucosidase activities measured in the perfused hearts are given in Table 1. The experimental conditions were similar for enzyme perfused

and control hearts, except that the latter ones did not receive purified bovine testis acid α-glucosidase. A significant increase in acid α-glucosidase activity was found in the enzyme perfused hearts. An immunological test was carried out to prove that the increment in enzyme activity was due to uptake of bovine testis acid α-glucosidase. For this purpose antiserum raised in Wistar rats against human placental acid α-glucosidase was used. This antiserum binds 90% of bovine testis acid α-glucosidase, but does not cross-react with the rat enzyme (Fig.2). It is shown that approximately 45% of the total acid α-glucosidase activity is precipitable from homogenates of enzyme perfused rat hearts.
Subsequent immunoblotting was performed to check whether the precipitated enzyme had the characteristic molecular mass of the administered 70 kD bovine testis acid α-glucosidase. For this purpose a mouse anti- (bovine testis acid α-glucosidase) serum was used, since the rat antiserum employed for immunoprecipitation did not react with the bovine testis enzyme on an immunoblot. A protein with a molecular mass identical to pure bovine testis enzyme (Fig.3, lane 1) was visualized in enzyme perfused rat hearts (lane 3 and 4), but not in control hearts (lane 2).

Immunocytochemistry was employed to localize the bovine testis enzyme in thick frozen sections of rat heart. Mouse polyclonal antiserum against bovine testis acid α-glucosidase was used for detection. Brightly fluorescent spots were localized intracellularly in sections from enzyme perfused hearts (Fig.4B), whereas control heart sections remained devoid of label. Double labeling for endogenous β-hexosaminidase (Fig.4C), and bovine testis acid α-glucosidase (Fig.4B) showed complete overlap of labeling patterns. No acid α-glucosidase was detectable in capillaries. Fig.4A shows for comparison a similar rat heart section stained with hematoxilin and eosin (HE).

DISCUSSION

Uptake of acid α-glucosidase purified from bovine testis was studied in a rat heart perfusion-recirculation system. We could demonstrate immunocytochemically that acid α-glucosidase is transported from the cardiovascular space to cardiomyocytes. The enzyme reaches the lysosomes, which is concluded from the fact that the endocytosed enzyme is detected at the same intracellular location as endogenous β-hexosaminidase. Apparently, transport across the capillary wall occurs. It is unlikely that this transendothelial

TABLE 1

Acid α-glucosidase activities	nmol MU/mg protein.h
Enzyme perfused hearts (n=5)	37.5 (±7*)
Control (n=3)	10.7 (± 0.9)

* standard deviation

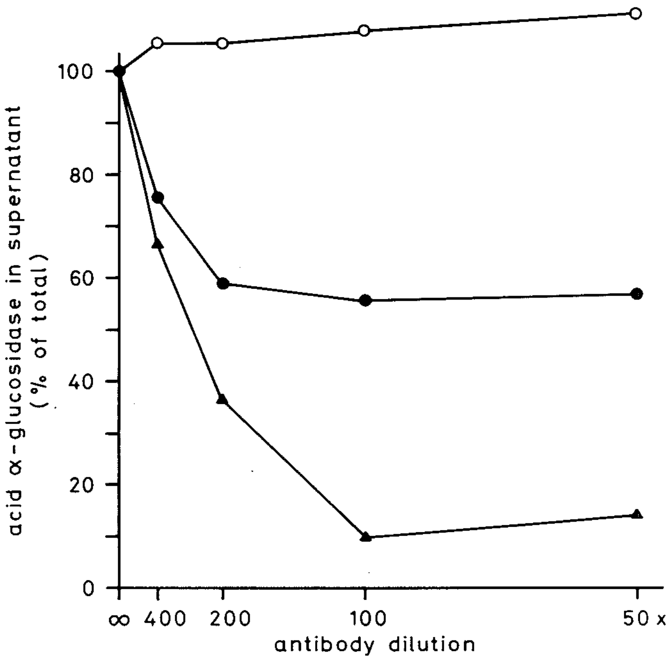

Figure 2: *Immunoprecipitation of bovine testis acid α-glucosidase. Rat heart homogenates and purified bovine testis enzyme were incubated with serial dilutions of rat anti- (human placental acid α-glucosidase) serum in combination with Staphylococcus A membranes. After overnight incubation at 4° C, membranes were spun down and acid α-glucosidase activities were measured in the supernatant.* ○ *Control rat heart,* ▲ *Purified bovine testis acid α-glucosidase,* ● *enzyme perfused rat heart.*

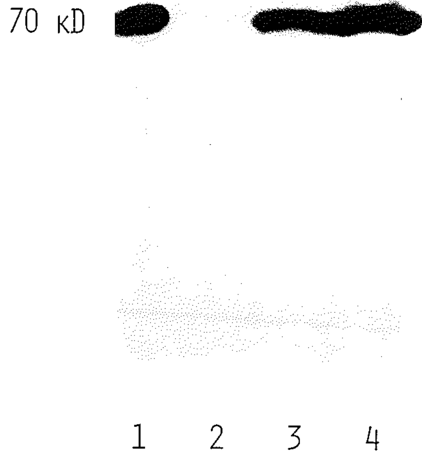

Figure 3: *Immunoblot analysis of bovine testis acid α- glucosidase in perfused rat heart. Acid α-glucosidase was immunoprecipitated with rat anti- (human placental acid α-glucosidase) serum, subjected to polyacrylamide gel, electrophoresed, and immunoblotted. Enzyme protein was visualized with mouse antiserum against bovine testis acid α-glucosidase in combination with ^{125}I protein A. Lane 1: purified bovine testis acid α-glucosidase, lane 2: control heart, lane 3 and 4: enzyme perfused rat hearts.*

transport is mediated by the 215 kD mannose 6-phosphate receptor, since Hasilik et al. demonstrated that endothelial cells do not expose this receptor at the cell surface (33). Suggestive evidence has been presented that various macromolecules, like albumin and transferrin, are transported across endothelial cells via a plasmalemmal vesicular system (34,35). Possibly, this mechanism is also used for transport of infused lysosomal enzymes, but further research is needed before firm conclusions can be drawn.
The enzyme that reaches the lysosomes of cardiomyocytes is not proteolytically degraded during transport, since the molecular mass of 70 kD is not changed after uptake. Moreover, the enzyme remains active after endocytosis. The acid α-glucosidase activity in the perfused hearts increased to 3-4 times the normal activity. On average 1-2% of infused enzyme was recovered

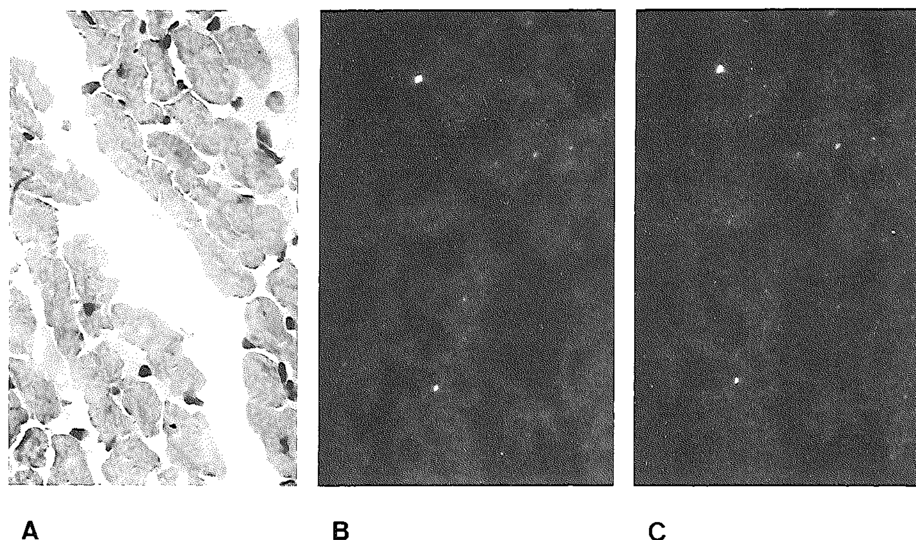

Figure 4: *Immunocytochemical localization of bovine testis acid α-glucosidase. A. Rat heart section, HE 650x, B. Enzyme perfused rat heart section incubated with mouse anti- (bovine testis acid α-glucosidase) serum in combination with goat anti- (mouse IgG) conjugated to fluorescein. C. The same section incubated with rabbit anti- (human placental β-hexosaminidase) serum in combination with goat anti- (rabbit IgG) conjugated to rhodamin to establish the location of lysosomes.*

in heart tissue. In cultured fibroblasts and muscle cells the recovery was 4% for mannose 6-phosphate receptor mediated uptake of acid α-glucosidase purified from bovine testis, and human urine, but only 0.04% when the non-phosphorylated placental acid α-glucosidase was administered (22). Therefore, the uptake of bovine testis acid α-glucosidase by heart tissue is relatively efficient.

Based on these findings one might speculate that mannose 6-phosphate containing acid α-glucosidase species are more suitable for enzyme replacement therapy in glycogenosis type II than the enzymes previously used in clinical trials. The results of our study encourage further investigations on the fate of phosphorylated enzymes in vivo. The efficiency of enzyme uptake in vivo by target organs is not expected to be as high as in the presented rat heart model. The liver will clear part of the infused enzyme from the circulation (13). On the other hand, complete correction of enzyme deficiency in affected tissues will not be needed. Cardiac involvement, for instance, is only

observed in patients with infantile glycogenosis type II, who have less than 2% residual acid α-glucosidase activity, but not in late onset variants with 5-20% of the normal amount of enzyme. Heterozygotes with 50% activity are completely free of symptoms. The outcome of the present study makes it worthwhile to test the rationale for enzyme replacement therapy in animal models.

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PUBLICATION VIII

submitted

INTRAVENOUS ADMINISTRATION OF PHOSPHORYLATED ACID α-GLUCOSIDASE LEADS TO UPTAKE OF ENZYME IN HEART AND SKELETAL MUSCLE OF MICE.

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SUMMARY

The lysosomal storage disorder glycogenosis type II is caused by acid α-glucosidase deficiency. The major clinical features of this disease are skeletal muscle weakness and heart failure. In the present study we have investigated the applicability of mannose 6-phosphate receptor mediated enzyme replacement therapy. It is shown that intravenous administration of phosphorylated bovine testis and non-phosphorylated human placental acid α-glucosidase to mice results in equal uptake of both enzyme species by liver, spleen and lung. However, the uptake of enzyme by heart (70% increase of activity) and skeletal muscle (43% increase) was significantly higher for the mannose 6-phosphate containing bovine testis acid α-glucosidase. Enzyme uptake appeared to be dose-dependent. The bovine enzyme remained detectable in mouse skeletal muscle up to 9-15 days after administration, with a half-life of 2-4 days. Application of mannose 6-phosphate receptor mediated enzyme replacement therapy seems to offer new opportunities for treatment of glycogenosis type II.

INTRODUCTION

Glycogenosis type II (GSD II) is one of the lysosomal storage disorders, and is characterized by deficiency of acid α-glucosidase (1). The clinical presentation of the disease is diverse (2). The infantile variant has its onset immediately after birth, with severe hypotonia and cardiorespiratory failure as major clinical features. Acid α-glucosidase activity is fully deficient in all tissues and body fluids. The late onset juvenile and adult variants present with muscle weakness and respiratory problems occur. In these clinical phenotypes enzyme activities up to 25 % of normal have been reported (3-8).

All clinical forms of GSD II are ultimately fatal by lack of an effective mode of treatment.
For lysosomal storage disorders many attempts at enzyme replacement therapy have been made, once it became evident that lysosomes are accessible to exogenous enzymes (see for review 9,10). Lysosomal enzymes, purified from various sources, were administered to patients. However, infused enzymes appeared to be captured predominantly by liver and spleen (11-13).
We are currently investigating the possible use of the cation independent mannose 6-phosphate receptor as target in enzyme therapy. This receptor was first recognized for its function in endocytosis of mannose 6-phosphate containing lysosomal enzymes (14,15). At present it is known that the receptor is involved primarily in intracellular transport of newly synthesized enzymes to lysosomes (see for review 16). Another quite different function of this mannose 6-phosphate receptor is binding of insulin-like growth factor II (17-19).
In previous studies we have shown that mannose 6-phosphate containing acid α-glucosidases purified from human urine or bovine testis are taken up efficiently by cultured muscle cells and fibroblasts of GSD II patients (7,8,20,21). The enzymes reach the lysosomes and glycogen storage is reversed. Much less efficient is the uptake of human placental acid α-glucosidase, used in former clinical trials, but lacking the mannose 6-phosphate recognition marker.
The results of in vitro experiments need to be interpreted with caution, when it concerns the ultimate applicability of enzyme replacement therapy. Exogenous enzymes have easy access to the target cells in culture, but it remains uncertain as to whether target tissues become sufficiently exposed to administered enzyme in vivo.
In the present study Swiss mice were injected intravenously with acid α-glucosidase purified from bovine testis and human placenta. Evidence for enzyme uptake in skeletal muscle and heart, the major target organs in GSD II, was obtained.

MATERIALS AND METHODS

Enzyme purification and perfusion protocol

Acid α-glucosidase containing mannose 6-phosphate was purified from bovine testis (spec. act. 364 μmol methylumbelliferone (MU)/mg.h), and a non-

phosphorylated form from human placenta (spec. act. 392 μmol MU/mg.h) (20,22). Purified acid α-glucosidase (90-180 μg) was intravenously administered to six week old Swiss mice (30 g) via the tail veins. After 24 hours (or longer periods) the animals were anaesthetized, and thoracotomy was performed (23). A heparinized canula connected to a perfusion apparatus was inserted in the left ventricle, and the right atrium was disrupted. The animals were perfused with 10 ml of a 0.1% procain-HCl solution in phosphate buffered saline (pH 7.2, 37°C). Brain, liver, spleen, kidneys, heart, and muscles (soleus, quadriceps and pectoral muscles) were quickly removed, frozen in liquid nitrogen, and stored at -70°C.

Miscellaneous

Tissue specimens (0.1-0.2 g wet weight) were homogenized in 400 μl of distilled water, using a Potter Elvejem homogenizer at 1300 rpm and a sonicator. Cell debris was removed by centrifugation at 15,000 x g. Protein concentrations were measured according to Lowry et al. (24), and acid α-glucosidase activities were determined as previously described, using 4-methylumbelliferyl-α-D-glucopyranoside as substrate (25). Enzyme activities are expressed in nmol MU per h.

Immunoprecipitation

Antisera against bovine testis and human placental acid α-glucosidase were raised in Swiss mice to discriminate between infused exogenous enzymes and mouse endogenous acid α-glucosidase (26). Tissue homogenates from control and enzyme injected mice were diluted in phosphate buffered saline (10mM sodium phosphate in 0.9% NaCl), pH 6.8, containing 1mg/ml bovine serum albumin (PBS/BSA) to an acid α-glucosidase activity of 1 μmol/h per 20 μl. Purified bovine testis and human placental acid α-glucosidase were diluted in the same way. Samples of 20 μl were incubated overnight at 4°C with 10 μl of a 400 fold antiserum dilution in PBS/BSA or with PBS/BSA only. Staphylococcus A membranes (10 μl, Bethesda Research Laboratories) were added to precipitate immunecomplexes. The membranes were spun down at 15,000 x g, and the acid α-glucosidase activity was measured in the supernatant, and on the membranes after prior washing for 6 times with PBS/BSA.

Immunoblotting

Tissue homogenates from control mice, and mice injected with bovine testis acid α-glucosidase were incubated with mouse antiserum raised against this enzyme. Immunecomplexes were precipitated with protein A sepharose beads (50 μl of a 1:1 suspension in PBS) at 4°C. The beads were washed 4 times with PBS, and bound complexes were dissolved by heating for 10 min at 90 °C in sample buffer (125 mM Tris-HCl, pH 6.6, 2M glycerol, 4% SDS, 0.6% mercaptoethanol, and 0.05% bromophenol blue). Samples were subsequently subjected to electrophoresis in 10 % polyacrylamide gels and blotted onto nitrocellulose (27,28). Bovine testis acid α-glucosidase was visualized with mouse antiserum specifically raised against this enzyme with ^{125} labeled protein A as radioactive probe (28).

TABLE 1.

Dose dependent uptake of bovine testis acid α-glucosidase in mouse organs

	precipitable enzyme act. (%)*		increase in enzyme act. (%)**	
injected dose	90 μg	180 μg	90 μg	180 μg
brain	0	0	0	0
liver	50	70	100	233
spleen	64	75	177	300
heart	17	36	20	56
muscle	14	32	16	44
lung	8	35	9	54
kidney	7	11	8	12

* The activity precipitable with mouse antiserum against bovine testis acid α-glucosidase is expressed as percentage of the total acid α-glucosidase activities in the tissue homogenates.

** The increase of acid α-glucosidase activity in mouse tissues is expressed as percentage of the tissue specific endogenous enzyme activity.

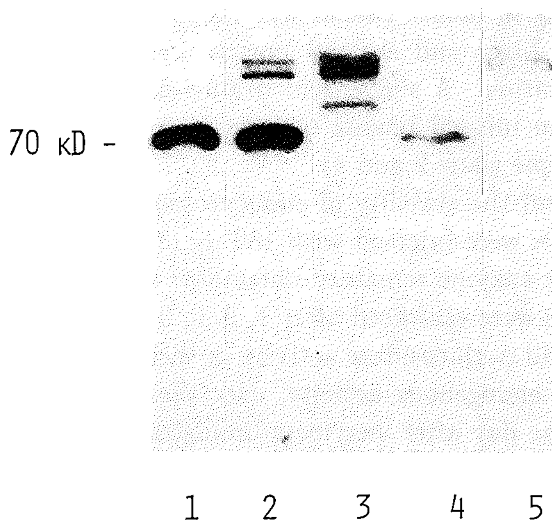

Figure 1: *Immunoblot analysis of bovine testis acid α-glucosidase in mouse liver and skeletal muscle. Tissue homogenates were prepared one day after administration of 180 μg of enzyme. Bovine testis acid α-glucosidase was specifically immunoprecipitated and analyzed by immunoblotting using mouse antiserum raised against the bovine enzyme. ^{125}I protein A was used as radioactive probe. Lane 1, purified bovine testis acid α-glucosidase; lane 2, mouse liver after enzyme administration; lane 3, control mouse liver; lane 4, mouse skeletal muscle after enzyme administration; lane 5, control mouse skeletal muscle.*

RESULTS

In a pilot experiment, two 6 week old mice (30 g) were injected with 90 and 180 μg (equivalent to 32 and 64 μmol MU/h) of bovine testis acid α-glucosidase. After 24 hours the mice were sacrificed and the activity of bovine testis acid α-glucosidase in the mouse organs was determined by means of species specific immunoprecipitation. The antiserum used precipitates 90-95% of acid α-glucosidase purified from bovine testis, but less than 2% of endogenous mouse enzyme. Table 1 shows the percentage of precipitable acid α-glucosidase activity in mouse tissues after enzyme administration. Brain does not contain a significant amount of exogenous enzyme. In all other tissues a dose dependent uptake of bovine acid α-glucosidase is observed. The

effect is most pronounced in liver and spleen, but also noted in muscle and heart. Table 1 shows the increase of acid α- glucosidase activity as a percentage of the endogenous enzyme activity of each tissue.
The precipitable enzyme in mouse tissues was further characterized by immunoblotting (Fig.1). Liver and skeletal muscle were prepared 24 hours after enzyme administration. A single acid α-glucosidase species of 70kD, not distinguishable from infused bovine testis enzyme, is only observed in mice injected with enzyme (lane 2 and 4).
In a following experiment the stability of endocytosed enzyme was studied. For this purpose 13 mice were injected with 180 μg of purified bovine testis acid α-glucosidase. The enzyme remained detectable in serum till one hour after injection. Animals were sacrificed after 1, 3, 6, 9 and 15 days. Table 2 shows the increase of acid α-glucosidase activity in various tissues, expressed as a percentage of the endogenous activity, over the experimental period. The results obtained one day after enzyme administration correspond with those obtained in the pilot experiment. No enzyme uptake was recorded in brain, while acid α-glucosidase activities in liver and spleen increased to 3-4 times the endogenous activity. Enzyme activities in skeletal muscle, heart and lung showed an increment of 43.4, 70.6 and 64.5%, respectively. Uptake of enzyme in kidney was hardly observed. The calculated half-life of bovine enzyme in the different tissues varied from 2-4 days. Even at day 15 some activity of bovine testis acid α-glucosidase remained detectable in liver and muscle (Table 2), while relatively high exogenous enzyme activities were still present in spleen.
A second series of 5 animals were injected with acid α-glucosidase purified from human placenta, which lacks the mannose 6-phosphate recognition marker. The mice were sacrificed after one day, and human acid α-glucosidase was precipitated. The results are included in Table 2 (day 1). It appears that human placental and bovine testis enzymes are taken up with the same efficiency by liver, spleen and lungs. However, in heart and skeletal muscle uptake of bovine acid α-glucosidase is respectively 2 and 3-4 times higher than uptake of placental enzyme. The activities obtained per wet weight are given in Table 3. Targeting of enzyme to liver and spleen is evident also without specific enzyme precipitation.

Finally, it was investigated whether infused acid α-glucosidase could cross the placental barrier. To this end, pregnant mice were injected with the bovine testis enzyme (180 μg), and brain, liver, spleen, heart, skeletal muscle

TABLE 2.

Increase of acid α-glucosidase activity in mouse organs after uptake of exogenous enzyme*

tissue	enzyme source	day 1	3	6	9	15
brain	b.t	0 (n=3)	0	0	0	0
	h.pl	0 (n=2)				
liver	b.t.	282±70 (n=6)	269±47	79±10	100	6.5±1.5
	h.pl.	215±70 (n=5)				
spleen	b.t.	341±74 (n=5)	274±144	118.5±14.5	72	265±0.5
	h.pl.	274±95 (n=5)				
heart	b.t.	70.6±8 (n=5)	39±7	14±9	0	0
	h.pl.	34.6±6.5 (n=5)				
muscle	b.t.	43.4±12.1 (n=6;x=16)	23.6±8.4 (x=10)	16±6.7 (x=6)	2.6±1.4 (x=5)	1.1±0.8 (x=4)
	h.pl.	13.2±5.4 (n=5;x=11)				
lung	b.t.	64.5±24.5 (n=2)	28.5±10.5	11±7	0	0
	h.pl.	49±8 (n=2)				
kidney	b.t.	8±1 (n=2)	6±5	0	0	0
	h.pl.	8±3.6 (n=2)				

* The increase is expressed as percentage of the tissue specific endogenous activity. 180 μg of acid α-glucosidase from bovine testis (b.t) or human placental (h. pl.) was injected. n=number of mice (Two animals were tested on day 3, 6 and 15, and one on day 9); x=number of muscles analyzed including quadriceps, soleus and pectoral muscles.

TABLE 3

Acid α-glucosidase activities expressed per wet weight

	Control	bovine testis	human placenta
Brain	1.68±0.15*	1.92±0.36	2.1 ±0.3
liver	3.83±0.3	13.5±2.8	9.5 ±1.2
spleen	1.38±0.08	7.08±0.87	7.2±0.3
muscle	0.44±0.08	0.68±0.16	0.46±0.06
heart	0.71±0.15	1.23±0.17	0.99±0.18
lung	0.5±0.12	0.65±0.06	0.51±0.08
kidney	3.3±0.09	3.4±0.32	3.0 ±0.1

* Enzyme activities are expressed as nmol MU/h.mg wet weight. Each value represents the average activity measured in 3 mice one day after enzyme administration.

and kidney were obtained 24 hours later from the 17 day old fetuses. Exogenous enzyme could not be demonstrated in any of the fetal tissues.

DISCUSSION

The present study was conducted to investigate the potential applicability of mannose 6-phosphate receptor mediated enzyme replacement therapy, using phosphorylated forms of acid α-glucosidase. It is shown that the appropriate enzyme species isolated from bovine testis is taken up by mouse tissues after intravenous injection. This results in 43% increase of acid α-glucosidase activity in skeletal muscle and 70 % in heart, notably higher than obtained with a similar dose (180 μg) of non-phosphorylated acid α-glucosidase purified from human placenta. The exogenous enzyme remains detectable in heart until 6 days after administration and in skeletal muscle until 9-15 days with a half life of 2 to 4 days. In contrast, the enzyme disappears from the circulation within one hour.
The major fraction of infused enzyme is captured by the liver and spleen, with little distinction between phosphorylated and non-phosphorylated species. The macrophages in these tissues, known to express both mannose and mannose 6-phosphate receptors might be responsible for enzyme uptake (29).

Brain remains completely devoid of enzyme due to protection by the blood brain barrier (30,31). Also the placental barrier separating the maternal and fetal circulation can not be passed.
To evaluate the practical significance of our study for enzyme replacement therapy in GSD II, the following considerations are important. Model experiments with cultured muscle cells of GSD II variants have indicated that lysosomal glycogen storage does not occur when the residual acid α-glucosidase activity is more than 20% (7,8). Most patients with late onset GSD II have enzyme activities of approximately 10% to 20%. Therefore, a slight increase of acid α-glucosidase activity might be sufficient to prevent or reverse lysosomal glycogen storage, and clinical symptoms. In our present study a 43% increase of activity is measured in mouse skeletal muscle one day after enzyme administration. Six days later the level of activity is still 10-20% above normal, which would in principle be sufficient to compensate the enzyme deficiency in adult GSD II. Thus, the results are promising, but a large supply of human acid α-glucosidase containing mannose 6-phosphate will be needed to study the long term effects of enzyme supplementation in clinical trials. The mice used in our studies received 90-180 μg of purified enzyme. Calculated by body weight, 200-400 mg would be the equivalent amount of enzyme to be administered to humans. Based on the estimated half life of exogenous enzyme in mouse skeletal muscle, the required frequency of enzyme administration will be approximately once a week. Isolation of this amount of enzyme from human tissues is not within technical reach, but it may become possible to produce sufficient amounts of enzyme biotechnologically by using human acid α-glucosidase cDNA (32). We are presently exploring the feasibility of this approach.

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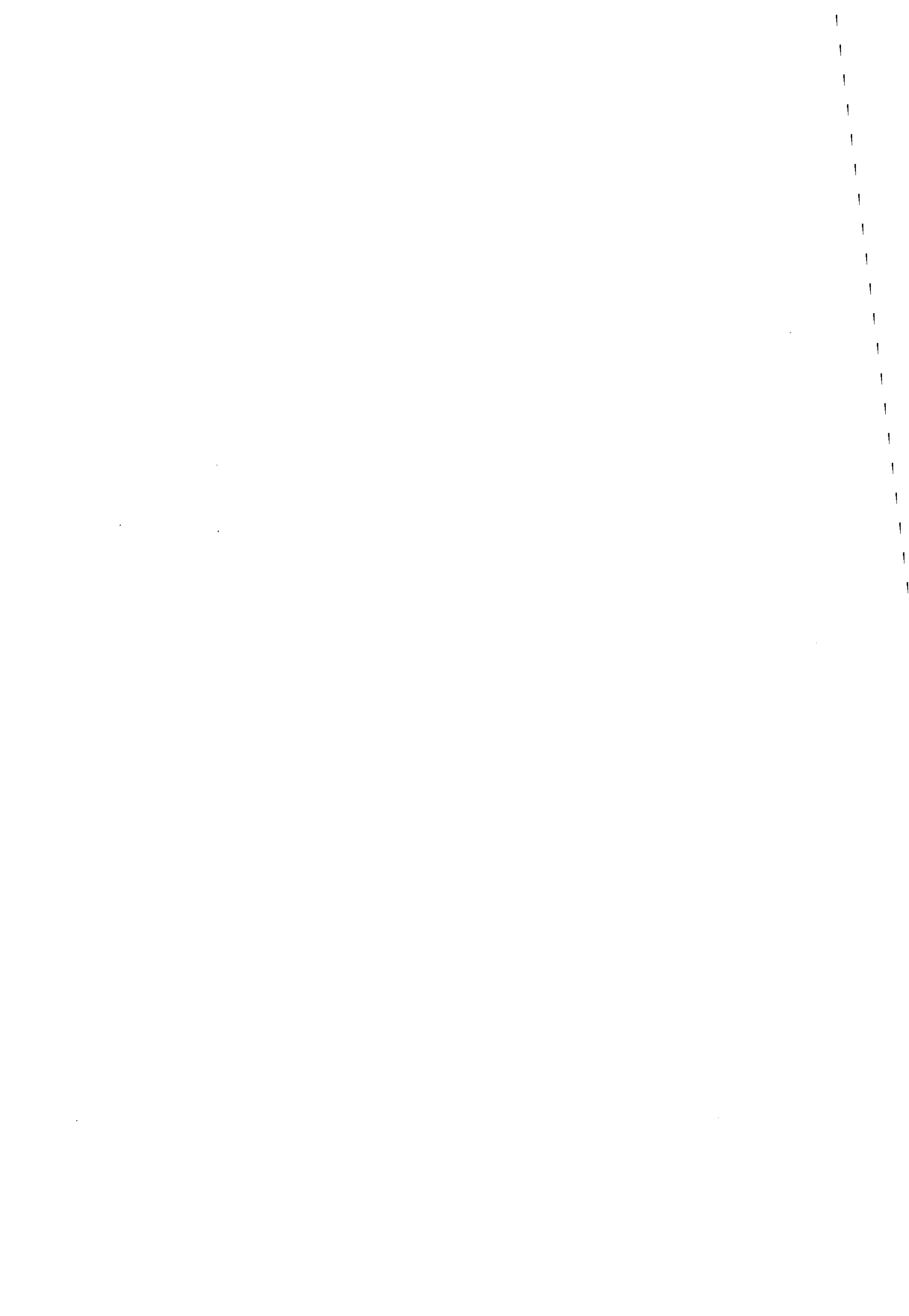

SUMMARY

At present more than 30 lysosomal storage disorders have been identified. Most diseases have a diverse clinical presentation (*Chapter I*), and for none of the diseases does an efficacious treatment exist (*Chapter II*). The experimental work presented in this thesis focuses on two strongly related topics **I.** the cause of clinical heterogeneity and **II.** the applicability of enzyme therapy for glycogenosis type II.
The primary defect in GSD II is acid α-glucosidase deficiency. Three clinical phenotypes are distinguished. The infantile subtype presents with severe hypotonia, cardiomegaly and moderate hepatomegaly, and is fatal within the first two years of life. In late onset GSD II, juvenile and adult variants, skeletal muscle weakness is the main symptom.
Studies on the cause of clinical heterogeneity in a large series of patients reveal a strong correlation between the clinical severity of GSD II and the level of residual acid α-glucosidase activity in cultured fibroblasts and skeletal muscle cells (*Publications I, II, III and V*). In *Publication I* it is demonstrated that the occurrence of two distinct clinical phenotypes of GSD II in one family, (adult GSD II in the first generation and three infantile cases in the third) is based on allelic diversity. Functional analysis of acid α-glucosidase encoded by the separate alleles revealed that the adult patient is a genetic compound. One mutant allele produces 30-50% residual acid α-glucosidase activity, the other does not direct formation of active enzyme. Transmission of the latter allele in the family pedigree is demonstrated. The infantile variants are homozygous for this "null-allele". These findings confirm the presumed correlation between acid α-glucosidase genotype and the GSD II phenotype.
Sofar, three adult patients have been identified with an exceptionally low residual acid α-glucosidase activity. Two of these cases were included in the study presented in *Publication III.* Also during prolonged culture periods the enzyme activities in fibroblasts remained low, and glycogen storage did occur. The basis for the relatively mild clinical course of GSD II in these patients could not be elucidated.
The extent of lysosomal glycogen storage in cultured fibroblasts (*Publication III*) and muscle cells (*Publication V*) of all clinical variants with GSD II was found to be strictly dependent on the level of residual acid α-glucosidase activity.

The extensive molecular and genetic heterogeneity among clinical phenotypes of GSD II is emphasized by studies described in *Publication II.* Among 5 South African families of distinct ethnic origin two new mutations leading to infantile GSD II were discovered.

With respect to enzyme replacement therapy it is investigated (*Publication III-VIII*) whether the mannose 6-phosphate/insulin-like growth factor II receptor can be used as a target for exogenous acid α-glucosidase. For this purpose, phosphorylated acid α-glucosidase species, purified from bovine testis and human urine, were applied to cells in culture. The enzymes are taken up equally efficiently by cultured fibroblasts and skeletal muscle cells (*Publication III-V*), and are transported to the lysosomes. Accumulated glycogen is degraded in cells from all glycogenosis type II variants.
A prerequisite for effective enzyme therapy is that infused acid α-glucosidase is transported across endothelial cells to the target tissues. In an electron microscopic study conducted on skeletal muscle biopsies of control subjects (*Publication VI*), suggestive evidence is presented that lysosomal enzymes can be transported via plasmalemmal vesicles from capillaries to pericapillary spaces. The occurrence of transendothelial transport is sustained by the results described in *Publication VII.* Perfusion of isolated rat hearts with purified bovine testis acid α-glucosidase via the coronary vessels results in uptake of catalytically active enzyme in heart tissue. A lysosomal localization of the bovine enzyme in cardiomyocytes is demonstrated.
To further investigate the importance of mannose 6-phosphate containing enzymes for treatment of glycogenosis type II, phosphorylated bovine testis or non-phosphorylated human placental acid α-glucosidase were administered intravenously to mice. Both enzyme species were taken up with equal efficiency by liver, spleen and lung (*Publication VIII*). However, the uptake in heart and skeletal muscle, the major target tissues in glycogenosis type II, was significantly higher for the phosphorylated enzyme. Uptake appeared dose dependent. The half-life of the bovine enzyme in mouse tissues varied from 2 to 4 days.
For administration of phosphorylated acid α-glucosidase to patients with GSD II, large quantities of highly purified enzyme will be needed. We have recently shown that human acid α-glucosidase with the proper characteristics can be synthesized using cloned acid α-glucosidase cDNA.
The main conclusion drawn from the work presented in this thesis is, that

enzyme replacement therapy for glycogenosis type II is in principle feasible. This type of therapy is expected to be most successful in patients with adult GSD II, since the enzyme deficiency is least pronounced in these cases. Methods for large scale production of enzyme are currently under investigation.

SAMENVATTING

Momenteel zijn meer dan 30 lysosomale stapelingsziekten bekend. Over het algemeen hebben de ziekten een diverse klinische presentatie (*Hoofdstuk I*). Voor geen van de ziekten bestaat een afdoende therapie (*Hoofdstuk II*). Het experimentele werk beschreven in dit proefschrift omvat twee nauw verwante onderwerpen **I.** de oorzaak van klinische heterogeniteit en **II.** de toepasbaarheid van enzymtherapy voor glycogenose type II.
Het primaire defect in glycogenose type II is zure α-glucosidase deficientie. Drie verschillende fenotypen worden onderscheiden. Het infantiele subtype presenteert zich met ernstige spierzwakte, hartvergroting en matige leververgroting, en is fataal binnen de eerste twee levensjaren. In de later optredende vormen van glycogenose type II, de juveniele en adulte varianten, is skeletspierzwakte het belangrijkste symptoom.
Studies met betrekking tot de klinische heterogeniteit bij een groot aantal patienten toonden een sterke relatie aan tussen de klinische ernst van glycogenose type II en het restniveau van zure α-glucosidase in gekweekte fibroblasten en spiercellen (*Publikaties I, II, III, en V*). In *Publikatie I* wordt aangetoond dat het voorkomen van twee verschillende klinische fenotypen van glycogenose type II in één familie (de adulte vorm van glycogenose type II in de eerste generatie en de infantiele vorm van de ziekte in de tweede) is gebasserd op allele diversiteit. Functionele analyse van zure α-glucosidase gecodeerd door de verschillende allelen laat zien dat de adulte patient een "genetic compound" is. Een mutant allel produceert 30-50% residuele zure α-glucosidase activiteit, terwijl het andere allel geen actief enzym maakt. Overdracht van het laatste allel (nul-allel) in deze familie kon worden aangetoond. De infantiele varianten zijn homozygoot voor dit "nul-allel". Deze bevindingen bevestigen de veronderstelde correlatie tussen zure α-glucosidase genotype en glycogenosis type II fenotype.
Tot dusverre werden drie adulte patienten onderscheiden met een exceptioneel lage restactiviteit voor zure α-glucosidase. Twee van deze patienten werden opgenomen in de studie gepresenteerd in *Publikatie III.* Ook als de cellen lang in kweek gehouden werden, bleef de activiteit in gekweekte fibroblasten laag, en trad glycogeen stapeling op. Dientengevolge kon bij deze patienten het relatief milde ziektebeloop niet worden verklaard.
Voor alle klinische varianten van glycogenose type II werd een strikte relatie gevonden tussen de mate van lysosomale glycogeen stapeling en het rest-

niveau van zure α-glucosidase.
De excessieve genetische heterogeniteit onder glycogenose type II patienten werd benadrukt door de studies beschreven in *Publikatie II.* Onder 5 Zuid-Afrikaanse families van verschillende ethnische origine werden twee niewe mutaties gevonden, die verantwoordelijk zijn voor het optreden van de infantiele vorm van glycogenose type II.

In de studies met betrekking tot enzymvervangingstherapie werd onderzocht of via de mannose 6-fosfaat/IGF II receptor efficiente opname van gezuiverd zure α-glucosidase kan worden bewerkstelligd. Voor dit doel werden gefosforyleerde zure α-glucosidase vormen gezuiverd uit rundertestis en menselijke urine en toegediend aan cellen in kweek. Beide enzymsoorten worden met identieke efficientie opgenomen door gekweekte huidcellen en spiercellen van patienten (*Publikatie III-V*), en bereiken hun eindbestemming: het lysosoom. Dientengevolge wordt gestapeld glycogeen afgebroken in cellen van glycogenose type II patienten.
Enzymtherapie zal alleen effect sorteren als intraveneus toegediend enzym de aangedane weefsels bereikt. Daartoe is transport over de vaatwand noodzakelijk. In een electronenmicroscopische studie, uitgevoerd op spierbiopten van gezonde personen, (*Publikatie VI*) werden aanwijzingen verkregen dat lysosomale enzymen inderdaad van de capillairen naar de pericapillaire ruimten kunnen worden getransporteerd. Dit transport vindt plaats via plasmalemmale blaasjes. Het bestaan van een dergelijk transport wordt verder ondersteund door de resultaten beschreven in *Publikatie VII.* Doorstroming van de kransslagaders van geisoleerde ratteharten met gezuiverd rundertestis zure α-glucosidase resulteerde in opname van actief enzym in hartspiercellen. Het runderenzyme kon worden aangetoond in lysosomen van deze cellen.
Om het therapeutisch belang van mannose 6-fosfaat bevattende enzymen verder uit te testen werd zowel gefosforyleerd rundertestis als niet-gefosforyleerd menselijk placenta enzym aan muizen toegediend. Beide enzymsoorten bleken met identieke efficientie te worden opgenomen door lever, milt, en long (*Publikatie VIII*). Echter in hart en skeletspier, de weefsels die het meest aangedaan zijn bij glycogenose type II, was de opname van gefosforyleerd enzym significant hoger. De opname bleek dosis afhankelijk. De halfwaardetijd van het runderenzym in muizeorganen varieerde van 2 tot 4 dagen.
Voor toepassing van enzymtherapy met gefosforyleerd zure α-glucosidase bij patienten met glycogenose type II zijn grote hoeveelheden gezuiverd enzym

nodig. Recent hebben we kunnen aantonen dat menselijk zure α-glucosidase met de juiste kenmerken kan worden gesynthetiseerd met behulp van het gekloneerde zure α-glucosidase gen.
De belangrijkste conclusie die we uit het werk gepresenteerd in dit proefschrift kunnen trekken is, dat enzymtherapie voor glycogenose type II in principe haalbaar is. Vermoedelijk is deze vorm van therapie het meest succesvol voor patienten met de volwassen vorm van glycogenose type II. Bij deze patienten is namelijk de deficientie het minst geprononceerd.
Thans worden methoden om grote hoeveelheden enzym te produceren uitgetest.

CURRICULUM VITAE

De schrijfster van dit proefschrift werd op 8 juni 1959 geboren te Tzum. In 1977 behaalde zij haar eindexamen gymnasium β aan het Chr. Gymnasium te Leeuwarden. Vervolgens startte zij met de studie scheikunde aan de Rijks Universiteit te Groningen. Zij behaalde haar propedeuse chemie in 1978. Na alsnog ingeloot te zijn voor de studie geneeskunde begon zij met deze opleiding in januari 1979 aan de Erasmus Universiteit te Rotterdam. In april 1985 behaalde zij cum laude haar artsexamen.
Van 1 april 1985 tot 31 maart 1989 werkte zij als wetenschappelijk onderzoeker op de afdeling celbiologie en genetica van de Erasmus Universiteit te Rotterdam.

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1. P.M. Hoogerbrugge, G. Wagemaker, D.W. van Bekkum, A.J.J. Reuser, and A.T. van der Ploeg. 1986. Bone marrow transplantation for Pompe's disease. N. Engl. J. Med. 315:65-66.

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clinical heterogeneity of glycogenosis type II. Ann. Hum. Genet., in press.

10. L.H. Hoefsloot, A.T. van der Ploeg, M.A. Kroos, M. Hoogeveen-Westerveld, B.A. Oostra, and A.J.J. Reuser. Adult and infantile glycogenosis type II in one family explained by allelic diversity, submitted.

11. R. Willemsen, H. Wisselaar, and A.T. van der Ploeg. Transendothelial transport of lysosomal enzymes and mannose 6-phosphate receptor fragments, submitted.

12. A.T. van der Ploeg, A.M.M. van der Kraaij, R. Willemsen, M.C.B. Loonen, A.J.J. Reuser, and J.F. Koster. Acid α-glucosidase perfusion in rat heart: a model system for enzyme replacement therapy in glycogenosis type II, submitted.

13. A.T. van der Ploeg, R. Willemsen, R. Brons, and A.J.J. Reuser. Intravenous administration of phosphorylated acid α-glucosidase leads to uptake of enzyme in heart and skeletal muscle of mice, submitted.

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ABBREVIATIONS

MPS	mucopolysaccharidosis
SRP	signal recognition particle
RER	rough endoplasmic reticulum
Asn	asparagine
Ser	serine
Thr	threonine
TGN	trans Golgi network
M6P	mannose 6-phosphate
SDS	sodium dodecyl sulphate
kD	kilo Dalton
IGF II	insulin like growth factor II
cDNA	complementary DNA
LDL	low density lipoprotein
ML	mucolipidosis
ADA	adenosine deaminase
PEG	polyethylene glycol
CNS	central nervous system
BBB	blood brain barrier
GVDH	graft versus host disease
HLA	major histocompatibility antigen
MLC	mixed lymphocyte culture
MLD	metachromatic leukodystrophy
GSD II	glycogenosis type II
mRNA	messenger RNA