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ULLA DUNDER

The Application of Enzyme Replacement Therapy in Vitro and in a Mouse Model in Aspartylglycosaminuria

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ABSTRACT

Aspartylglycosaminuria (AGU) is a recessively inherited lysosomal storage disease caused by the deficient activity of lysosomal enzyme, glycosylasparaginase (AGA). The enzyme normally functions in lysosomes in degradation of carbohydrate-protein linkage in glycoproteins. The deficient AGA activity leads to the accumulation of glycoasparagines, mainly aspartylglucosamine (GlcNAc-Asn), in cells resulting in this multisystem disease characterized by slowly progressing psychomotor retardation with shortened life span. There is no disease-specific treatment for AGU, but some other lysosomal storage disorders with similar cause have been treated with enzyme replacement therapy (ERT) using exogenous recombinant enzymes. In this study the applicability of ERT was evaluated in the treatment of AGU. Therefore, a human recombinant glycosylasparaginase was produced and the mechanism of enzyme transfer and capability of an exogenous enzyme to treat AGU in a cell culture and in a mouse model were studied.

The human recombinant AGA was transported from cell culture media into the AGU fibroblasts and lymphocytes through mannose 6-phosphate (M6P) receptor-mediated endocytosis. The GlcNAc-Asn storage in AGU lymphocytes was cleared and further accumulation was prevented when intracellular glycosylasparaginase activity in these cells reached 3-4% of the activity in normal lymphocytes. Cell-to-cell contact was needed for the M6P-receptor independent transfer of the enzyme from normal to AGU cells. The recombinant enzyme was biologically active also in the AGU mouse model. The lysosomal storage was cleared in many somatic tissues of adult AGU mice when 70% of normal glycosylasparaginase activity was reached in these tissues. The response to the therapy was dose-dependent. The recombinant enzyme crossed blood-brain barrier (BBB) of adult AGU mice and decrease in GlcNac-Asn storage was detected also in brain. The overall therapeutic effect in new-born animals was similar to the effect in the adult animals, but the therapeutic effect in brain was better when the therapy was initiated before the full development of the BBB.

This study sheds light on details involved in ERT of AGU. The results show that ERT might be efficient in treating the somatic tissues of AGU, but more efficient ways to treat the disease in neuronal tissues are needed. The results form a basis for further development of treatment protocols in AGU, but also contribute to research in other diseases with similar cause.

National Library of Medicine Classification: QU 136, QU 350, QU 55, QY 60.R6, WD 205

Medical Subject Headings (MeSH): Aspartylglycosylaminase; Disease models, Animal; Lysosomal Storage Diseases; Recombinant Proteins; Lysosomes

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TIIVISTELMÄ

Aspartyyliglykosamiiniuria (AGU-tauti) on peittyvästi periytyvä aineenvaihduntasairaus, joka johtuu glykosyyliasparaginaasi -entsyymin (AGA) aktiivisuuden puutoksesta. Normaalisti entsyymi toimii lysosomeissa glykoproteiinien hajotuksessa pilkkoen proteiinin ja sokeriketjun välisen sidoksen. Aktiivisen entsyymin puuttuessa elimistön soluihin kertyy hajoamattomia glykoasparagiineja, pääasiassa aspartyyliglukosamiinia (GlcNAc-Asn). Tämä johtaa taudin kliinisiin oireisiin, joita ovat mm. hitaasti etenevä psykomotorinen jälkeenjääneisyys ja lyhentynyt elinikä. AGU-tautiin ei ole spesifistä hoitoa, mutta samankaltaisia lysosomaalisia kertymäsairauksia on hoidettu entsyymikorvaushoidolla (ERT) käyttäen rekombinanttia entsyymiä. Tässä työssä arvioitiin ERT:n soveltuvuutta AGU-taudin hoitoon tuottamalla ihmisen rekombinanttia glykosyyliasparaginaasia, tutkimalla mekanismia, joilla entsyymi siirtyy solusta soluun sekä tutkimalla tuotetun entsyymin kykyä hoitaa AGU tauti soluviljelmässä ja taudin hiirimallissa.

Soluviljelymediumiin lisätty ihmisen rekombinantti AGA otettiin sisään lymfosyytteihin ja fibroblasteihin mannoosi 6-fosfaatti (M6P) –reseptorivälitteisellä endosytoosilla. Kertymätuote (GlcNAc-Asn) hävisi lymfosyyteistä ja sen lisäkertyminen estyi, kun entsyymiaktiivisuus tautisolun sisällä oli 3-4 % normaalin solun entsyymiaktiivisuudesta. Entsyymi siirtyi terveestä solusta sairaaseen solu-solu-kontaktissa ilman M6P-reseptorivälitteistä kuljetusmekanismia. Tuotettu entsyymi oli biologisesti aktiivinen myös AGU-taudin hiirimallissa. Lysosomaalinen kertymä hävisi monessa AGU-tautia sairastavan aikuisen hiiren somaattisessa kudoksessa, kun entsyymiaktiivisuus kudoksessa oli 70 % normaalista aktiivisuudesta. Hoidon teho riippui annetusta annoksesta. Rekombinantti entsyymi läpäisi aikuisen AGU-hiiren veri-aivoesteen ja kertymä pieneni aivokudoksessa. Vastasyntyneellä AGU-hiirellä hoidon kokonaisteho oli samankaltainen kuin aikuisilla hiirillä, mutta teho aivoissa oli parempi, kun hoito aloitettiin ennen veri-aivoesteen täydellistä kehittymistä.

Tämän tutkimuksen tulokset antavat lisätietoa entsyymikorvaushoidon soveltamisesta AGU-taudin hoitoon. Tulokset osoittavat, että ERT voisi olla mahdollinen somaattisten kudosten hoitomuoto, mutta hermokudoksen hoitamiseen tarvitaan tehokkaampia keinoja. Tulokset luovat pohjaa AGU-taudin hoitomuotojen kehittämiselle ja sen lisäksi täydentävät muiden samakaltaisten tautien parissa tehtyä tutkimusta.

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Yleinen suomalainen asiasanasto (YSA): entsyymit, koe-eläimet, lysosomaalinen kertymäsairaus, lysosomi, rekombinantti proteiini

To my family with love

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Kuopio, February 2010

Ulla Dunder

List of original publications

This dissertation is based on the following articles which are referred to in the text by Roman numerals:

I **Mononen I, Heisterkamp N, Dunder U, Romppanen E-L, Noronkoski T, Kuronen I, Groffen J.** Recombinant glycosylasparaginase and in vitro correction of aspartylglycosaminuria. FASEB J. 1995; 9: 428-433.

II **Dunder U, Mononen I**. Human leukocyte glycosylasparaginase: cell-to-cell transfer and properties in correction of aspartylglycosaminuria. FEBS Lett. 2001; 499: 77-81

III **Dunder U, Kaartinen V, Valtonen P, Väänänen E, Kosma V-M, Heisterkamp N, Groffen J, Mononen, I.** Enzyme replacement therapy in a mouse model of aspartylglycosaminuria. FASEB J. 2000; 14: 361-367.

IV **Dunder U, Valtonen P, Kelo E, Mononen I**. Early initiation of enzyme replacement therapy improves metabolic correction in the brain tissue of aspartylglycosaminuria mice. Submitted.

The publishers of the original publications have kindly granted permission to reprint the articles in this dissertation.

Contents

TABLES

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ABBREVIATIONS

AAV	Adeno virus vector
AGA	GA; glycosylasparaginase; aspartylglucosaminidase; N^4-(β-N-acetyl-D-glucosaminyl)-L-asparagine amidohydrolase; EC 3.5.1.26
AGU	Aspartylglycosaminuria; aspartylglucosaminuria; McKusick 208400
Bb	Base pair
BBB	Blood-brain barrier
BMT	Bone marrow transplantation
BSA	Bovine serum albumin
CmCys	Carboxymethyl cysteine
CNS	Central nervous system
Dabs-Cl	Dabsylchloride, dimethylaminoazobenzene-4'-sulphonyl chloride
DNA	Deoxyribonucleic acid
EBV	Ebstein-Barr virus
ER	Endoplasmic reticulum
ERT	Enzyme replacement therapy
FBS	Foetal bovine serum
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GA	AGA; glycosylasparaginase; aspartylglucosaminidase; N^4-(β-N-acetyl-D-glucosaminyl)-L-asparagine amidohydrolase; EC 3.5.1.26
GLC	Gas-liquid chromatography
GlcNAc-Asn	Aspartylglucosamine; aspartyl-N-acetylglucosamine; 2-acetamido-1-(L-β-aspartamido)-1,2-dideoxy-β-D-glucose; 2-acetamido-1-N-(β-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine
HCT	Hematopoetic stem cell transplantation
HE	Heterozygous
HPLC	High-performance liquid chromatography
HSC	Hematopoetic stem cells
Km	Michaelis constant
KO	Knock-out
LSD	Lysosomal storage disroder
M6P	Mannose 6-phosphate
MRI	Magnetic resonance imaging
MSC	Mesenchymal stromal cells
NtN	N-terminal nucleophile
NSC	Neural stem cells
PAGE	Polyacrylamide gel electrophoresis
pI	Isoelectric point
PITC	Phenylisothiocyanate
PPNP	Paranitrophenylphosphate
RNA	Ribonucleic acid
SDS-PAGE	Sodiumdodecylsulphate polyacrylamide gel electrophoresis
WT	Wild-type

1 Introduction

Lysosomes are cellular compartments in the endosomal-lysosomal system where the degradation of macromolecules of the human body occurs (Walkley 1998; Vellodi 2005). They contain numerous enzymes which in cascade degrade endocytosed or phagocytosed molecules into smaller components for re-use or elimination (Vellodi 2005; Winchester 2005). In lysosomal storage disorders (LSD), the degradation is disturbed due mainly to the defective action of a particular lysosomal enzyme or a transport protein. The deficient enzyme activity is usually caused by an inherited genetic defect in a single gene coding the enzyme, leading to the accumulation of undigested storage material in the lysosomes, which results in a disease (Reuser et al. 1994; Futerman and van Meer 2004; Vellodi 2005). However, the exact pathogenesis and mechanisms behind the cellular dysfunction in these diseases are currently not known. The large phenotypic heterogeneity of LSDs is explained by the nature of the enzyme defect, the accumulating substrate and the target tissues of the storage. Some of the diseases are restricted to somatic tissues, whereas some also affect the brain. Over 40 disorders have been described since the first identified LSD, Pompe disease in 1963 (Hers 1963), and they are classified on the basis of the affected lysosomal function or the main accumulating substrate (*Table 1*) (Reuser et al. 1994; Wilcox 2004; Vellodi 2005).

Glycoproteinoses, also called olicosaccharidoses, are lysosomal storage disorders where the stepwise degradation of glycoproteins (*Figure 1*) is disturbed, leading to the accumulation of different glycopeptides (Aronson 1999; Winchester 2005). Aspartylglycosaminuria (AGU, McKusick 208400) is a glycoproteinose where a deficiency of an enzyme called glycosylasparaginase (aspartylglucosaminidase, GA, AGA) leads to the accumulation of glycoasparagines, mainly aspartylglucosamine (GlcNAc-Asn), in tissues and body fluids (Jenner and Pollit 1967; Mononen and Mononen 1997; Winchester 2005). The accumulation starts already in the fetus, but the first sign of the disease is usually delayed speech development, which is detected in early childhood. The constant accumulation of the undegraded glycopeptides finally leads to a multisystem disease with a wide range of symptoms characterized by a slowly progressive psychomotor retardation and coarsening of the habitus (Arvio et al. 1997; Aula et al. 2001). Patients affected by AGU have a shortened

life span, typically not more than 40-50 years. Currently, there is no cure for AGU or any other glycoproteinose.

The possible strategies to treat the lysosomal storage disorders have been widely studied. In addition to non-specific treatment of the symptoms, the object is to find disease-specific therapies addressing the biochemical cause of the diseases. Here, the replacement of the malfunctioning enzyme with a correctly functioning one appears as a natural choice, as most of these disorders result from the malfunction of a single enzyme in the degradation cascade (Winchester et al. 2000; Vellodi 2005). The possible strategies could be the replacement of the deficient enzyme with an endogenous one produced in the patient's body either after bone marrow transplantation (BMT), cell transplantation or gene therapy, or the replacement by an exogenous enzyme injected into the body (enzyme replacement therapy, ERT). Bone marrow and cell transplantations have been carried out in many LSDs, but the host versus craft reaction in bone marrow transplantations and the failure to affect the skeletal and central nervous system have stalled the use of these applications (Malatack et al. 2003). However, a successful transplantation provides for a continuous supply of the correctly functioning enzyme and might therefore be helpful in the early stage of the disease prior to other therapies. The continuous supply of the enzyme might also be achieved by gene therapy, but the lack of safe vectors providing for the stable expression of the enzyme has thus far been the major obstacle for utilizing this approach (Sands and Davidson 2006). Currently, safer and more stable viral and non-viral vectors with different injection routes are being developed, and there is considerable interest in this therapeutic appreach (Sands and Davidson 2006; Zhang et al. 2008). Moreover, during the past few years stem cell research has been extensive and stem cells might provide a continuous source of endogenous enzyme in ERT of LSD in the future.

Table 1. Lysosomal storage disorders.

Disease		Enzyme deficiency	Storage products
Mucopolysaccharidoses (MPS)			
I	Hurler/Scheie	α-L-Iduronidase	Heparan sulfate, dermatan sulfate
II	Hunter	Iduronidase sulfatase	Heparan sulfate, dermatan sulfate
IIIA	Sanfilippo A	Heparan N-sulfamidase	Heparan sulfate
IIIB	Sanfilippo B	α-N-acetylglucosaminidase	Heparan sulfate
IIIC	Sanfilippo C	Acetyl-CoA:alpha-glucosamide	Heparan sulfate
IIID	Sanfilippo D	N-acetylglucosamine 6-sulfatase	Heparan sulfate
IVA	Morquio A	Galactose 6-sulfatase	Keratan sulfate
IVB	Morquio B	β-Galactosidase	Keratan sulfate, chondroitin sulfate
VI	Maroteaux-Lamy	N-Acetylgalactosamine-4-sulfatase	Dermatan sulfate
VII	Sly	β-Glucuronidase	Dermatan sulfate, heparan sulfate, keratan sulfate
Glycoproteinoses			
Aspartylglycosaminuria		**Glycosylasparaginase**	**Glycoasparagines**
α-Fucosidosis		α-Fucosidase	Fucosyl-oligosaccharides/-glycolipids
α-Mannosidosis		α-Mannosidase	α-Mannosyl-oligosaccharides
β-Mannosidosis		β-Mannosidase	β-Mannosyl-oligosaccharides
Sialidosis (Mucolipidosis I)		α-Neuraminidase	Sialylololigosaccharides/gangliosides
Schindler disease		α-N-acetylgalactosaminidase	Oligosaccharides/glycosaminoglycans/glycosphingo-lipids
Sphingolipidoses			
Fabry disease		α-Galactosidase	Globotriaosyl-digalactosylceramide/blood group B Trisaccharide
Farber disease (lipogranulomatosis)		Ceramidase	Ceramide
Gaucher disease		Glucocerebrosidase	Glucosylceramide
GM1 gangliosidosis		β-Galactosidase	GM1 ganglioside/galactosyloligosacharides/keratan sulfate
Tay-Sachs disease (GM2		α-Subunit of β-hexosaminidase	GM2 ganglioside/asialo-GM2
Sandhoff disease		β-Subunit of β-hexosaminidase	GM2 ganglioside/asialo-GM2/globoside
Globoid cell leukodystrohpy (Krabbe disease)		Galactosylceramidase	Galactosylceramide, galactosylsphingosine
Metachromatic leukodystrophy		Arylsulfatase A	Sulfatides
Niemann-Pick A and B		Sphingomyelinase	Sphingomyelin
Other lipidoses			
Neuronal ceroid lipofuscinosis		?	ATP synthase
Niemann-Pick C		?	Cholesterol, phospholipids, glycolipids
Wolman disease (choresteryl-ester-storage disease)		Acid lipase	Choresterylesters, triglyserides
Glycogen storage disease			
Glycogen storage disease type II (Pompe disease)		α-Glucosidase	Glycogen
Multiple enzyme deficiency			
Galactosialidosis		Cathepsin A/protective protein	Sialyloligosaccharides
I-Cell disease (pseudo-Hurler polydystrohpy) Mucolipidosis II/III		UDP-N-acetylglucosamine:lysosomal enzyme N-Acetyl-glucosaminyl-1-?	Oligosaccharides/lipids/glycosaminoglycans
Mucolipidoses IV		?	Gangliosides, phospholipids
Multiple sulphatase deficiency		Various sulfatases	Sulfated glycosaminoglycans/sulfated glycolipids
Lysosomal transport defects			
Cystinosis		Cystine transporter	Cystine
Salla Disease (sialic acid storage)		Sialic acid/acid sugar transporter	Sialic acid
Vitamin B12 transport defect		B12 transport system	Cobalamin
Activator deficiencies			
Complex lipidosis		Prosaposin	(Glucosyl)ceramide, sulfatides, ceramide
Gaucher variant		SAP C	(Glucosyl)ceramide
Metachromatic leukodystrophy		SAP B	Sulfatides/digalactosylceramide/globotriaosylceramide
Tay-Sachs AB variant		GM2 activator deficiency	GM2 ganglioside
Other			
Dannon disease			
Hyaluronidase deficiency			

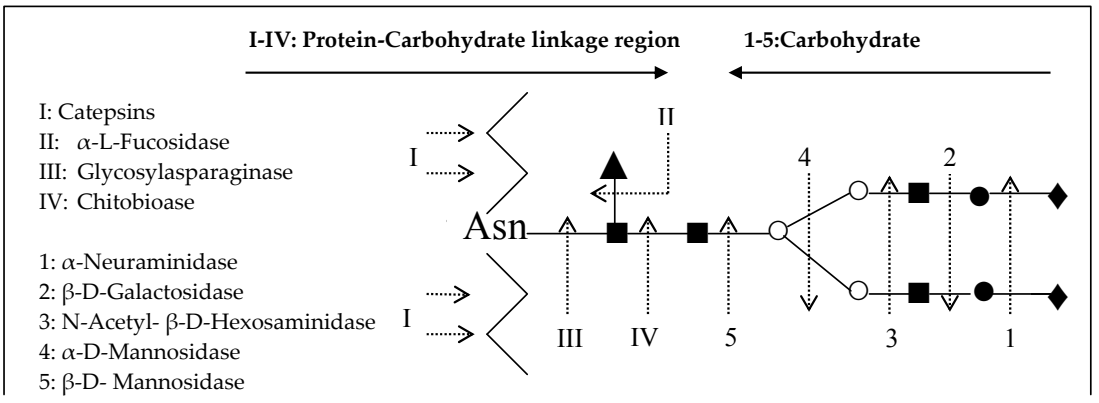

Figure 1. The bidirectional pathway of degradation of human complex type N-linked glycoprotein (Aronson 1999; Winchester 2005). Enzymes I-IV degrade the protein-carbohydrate linkage region with simultaneous degradation of the carbohydrate by enzymes 1-5. Specific glycoproteinoses are caused by a defect in these proteolytic enzymes as seen in Table 1. ▲ *= fucose;* ■ *= N-acetylglucosamine;* ○ *= mannose;* ● *= galactose;* ◆ *= N-acetylneuraminic acid.*

ERT in the treatment of lysosomal storage disorders has been the focus of much research since the 1970s, when it was found that a lysosomal enzyme was transferred from the cell culture media to certain cells by mannose 6-phosphate (M6P) receptor-mediated endocytosis (Kaplan et al. 1977). Thereafter, the nature of N-linked glycoproteins, which many lysosomal enzymes are, and the receptor-mediated transfer of proteins into lysosomes have been under active research, creating a basis for the development of ERT for lysosomal storage disorders (Kornfeld 1990; Hawkes and Kar 2004; Helenius and Aebi 2004; Hebert et al. 2005; Ohtsubo and Marth 2006). As a result, the disease- specific factors in Gaucher, Fabry and Pompe diseases and in MPS I, MPS II, and MPS VI with no central nervous system involvement have been characterized in adequate animal models, and ERT is in clinical use in these diseases (Brady 2006; Lidove et al. 2007; Harmatz et al. 2008; Katzin and Amato 2008; Wraith 2008). Once begun, the ERT lasts for a life-time and the exogenous enzyme is injected regularly into the body. Therefore, recombinant proteins with a suitable targeting signal have been developed for the therapy, to overcome the problem of the shortage of naturally occurring enzyme and to achieve the maximal targeting of the enzyme to the affected cell type. The succesfull treatment of these diseases by ERT has boosted hope for the development of ERT in other lysosomal storage diseases as well. However, the transfer of enzyme through the blood-brain-barrier (BBB) in diseases that affect the brain needs to be efficient to ameliorate the involvement in central nervous system.

When this Ph.D. thesis project was started in 1993, no results had been reported on therapeutic approaches in AGU or in any other disorder of glycoprotein degradation (*Table 2*). The enzyme defect behind the disease was known and the enzyme had been characterized. The main accumulating compounds had also been identified and methods to measure the enzyme activity as well as the accumulating compounds had been developed. The disease was quite well characterized clinically and it could be diagnosed, but only supportive medical care was avaliable. By that time, many studies on the treatment of other lysosomal storage diseases had been published. Therefore, in the continuum of research on AGU it appeared natural to aim towards disease-specific therapy. Thus, encouraged by the results obtained especially in Gaucher disease (Barton et al. 1991), we developed a recombinant glycosylasparaginase and further explored the mechanism of AGA transfer and the applicability of ERT in the treatment of AGU in cell culture and in a mouse model. This thesis outlines these studies, summarizes the data and applies it to the current knowledge of AGU and to the research in the treatment of lysosomal storage disorders in general.

Table 2. Milestones related to aspartylglycosaminuria research from patients', families' and clinicians' point of view when the present study was started. The data were collected from the studies referred to in Chapter 2 (Review of literature).

	Milestones in research related to AGU	**AGU patient and family**	**Clinician**
1962	- The first diagnostic picture of an AGU patient is drawn.	- No knowledge of the reason for the abnormal state and mental retardation of the child.	- Diagnosis cannot be made and the patient cannot be helped.
1965-1967	- GlcNAc-Asn linkage is characterized. - AGA and the reaction mechanism of the enzyme are discovered. - AGA is found to be a lysyomal enzyme.		- Hint that new findings may be related to mental retardation in some patients.
1968	- AGU is discovered; missing AGA activity and GlcNAc-Asn in urine is related.	- The disease is named and information about the causes is given, but otherwise families cannot be helped.	- AGU disease can be diagnosed and named, but patients and families cannot be helped otherwise.
1970-1975	- A high prevalence of AGU is found in Finland. - The clinical picture, mode of inheritance and epidemiology of AGU are established. - Morphological and histochemical characteristics are established. - Diagnostic method using urinary GlcNAc-Asn is developed.	- Families can get information about the clinical outcome and can plan the future of the AGU child. - The AGU child can get better supportive care, which can be planned and organized.	- AGU can be more easily suspected and detected. - Speculative genetic counseling as well as plans and instructions for supportive care can be given.
1976	- AGA is purified from human liver. - AGU patients and carriers can be diagnosed using blood AGA activity.	- Possible anxiety in relatives of an AGU patient when planning a family may occur.	- Genetic counseling can be given.
1984-1989	- Automated HPLC and GLC methods to detect GlcNAc-Asn are developed. - Prenatal diagnosis (AGA activity) from chronic villus samples, cultured amniotic cells or amniotic fluid is established. - Subunit composition of AGA is found.	- Prenatal diagnosis is available. - Parents are allowed to terminate the pregnancy, which can also cause a lot of distress and mixed feelings.	- As AGU can be detected more easily than before and the information of the status of the fetus can be given more rapidly, the role of genetic counseling becomes more important.
1990-1992	- cDNA for AGA is cloned: one gene, two subunits. - AGU_{Fin} mutations and AGA gene is characterized. - Human leukocyte AGA is characterized. - Series of studies on the activation of AGA are initiated - DNA-based detection of AGU or AGU mutation (carrier) is available - Chromosomal location of AGA gene is established.	- Gene test is available. - Families and children can be tested and knowledge of carrier status can be obtained. - An area of difficult decisions is opened.	- AGU and carrier status can be diagnosed by gene tests. - Informing the families becomes more important and may be difficult.
1993-1994	- Automated HPLC methods to detect AGA is developed.	- The diagnosis can be reached faster than earlier.	- Different choices for laboratory testing are available to make the diagnosis.

2 Review of literature

2.1 ASPARTYLGLUCOSAMINURIA

Aspartylglycosaminuria (AGU; MIM 208400) is a recessively inherited lysosomal storage disease due to a deficiency in glycoprotein degradation (Mononen and Mononen 1997; Aula et al. 2001). It is the most common disorder in glycoprotein degradation in humans (Mononen et al. 1993a). AGU is part of the Finnish disease heritage, with an estimated prevalence from 1:3600 to 1:18500, thus being the most common lysosomal and the fifth most common recessively inherited disease in the Finnish population (Mononen et al. 1991; Arvio et al. 1997; Norio 2003). In Finland there are over 200 AGU patients, compared with approximately 30 patients reported in the rest of the world (Norio 2003).

2.1.1 Discovery of AGU

AGU was discovered in England in the late 1960s through the detecting of an unknown polar compound in the urine of a 31-year-old mentally retarded woman (Pollit 1997). The compound was defined as an amino acid asparagine attached to N-Acetylglucosamine (AADG; 2-acetamido-1[β-L-β'-aspartamido]- 1,2-dideoxyglucose; aspartylglucosamine; GlcNAc-Asn) (Jenner and Pollit 1967). The lack of a previously identified enzyme cleaving the asparagine-carbohydrate linkage (Murakami and Eylar 1965; Makino et al. 1966), later called glycosylasparaginase (AGA, GA), was now connected to AGU disease (Pollit et al. 1968). The enzyme was not found in the seminal fluid of AGU patients, while secretion of GlcNAc-Asn was detected (Murakami and Eylar 1965; Makino et al. 1966; Pollit et al. 1968). At the same time, AGU was also identified in Finland: a previously unknown glycopeptide was found in the urine of eleven mentally retarded Finnish patients (Palo and Mattsson 1970; Palo 1997). A new disease had been found and extensive research on its pathophysiology started.

2.1.2 Molecular biology behind the disease

The lack of the glycosylasparaginase activity leading to the secretion of glycopeptides into tissues and body fluids of an AGU patient is due to the recessively inherited mutations in the gene coding for this enzyme (Murakami and Eylar 1965; Makino et al. 1966; Ikonen et al. 1991; Mononen et al. 1991; Aula et al. 2001). Altogether 98% of Finnish AGU patients carry the so-called AGU$_{FIN}$ major mutation, consisting of two point mutations in the *AGA* gene (Fisher and Aronson 1991; Ikonen et al. 1991; Mononen et al. 1991; Syvanen et al. 1992). These mutations result in two amino acid substitutions in the polypeptide chain of the protein, Arg161Gln and Cys163Ser. The first substitution is a neutral polymorphism but the second one is responsible for the misfolding of the protein, leading to the inactivation of the enzyme. This mutation is termed a founder mutation in the Finnish population. The AGU$_{FIN}$ minor mutation is a 2-bp deletion which creates a frameshift and premature translational termination codon, and is found in 1.5% of Finnish AGU patients (Isoniemi et al. 1995; Aula et al. 2001). In addition to these so-called Finnish mutations, 25 other mutations causing the disease have been found, including missense, splicing and nonsense mutations as well as insertions, deletions and genomic rearrangements (Park and Aronson 1997; Aronson 1999; Aula et al. 2001). Almost all of them lead to incorrect maturation and complete or partial inactivation of the enzyme.

2.1.3 Lysosomal storage in AGU

An inactive glycosylasparaginase cannot hydrolyze the protein-oligosaccharide linkage, which is the final step in the lysosomal catabolism of Asn-linked glycoproteins (*Figure 1*) (Aronson 1999). These undigested glycoasparagines are subsequently secreted into body fluids and accumulate in lysosomal vacuoles in the tissues (Arstila et al. 1972; Isenberg and Sharp 1976; Rapola 1994; Aula et al. 2001). The lysosomal storage in turn causes morphological changes in the cells, such as enlarged lysosomes filled with amorphous granular material which can be seen in histological studies. In the neuronal cells of the adult brain, these storage vacuoles contain electron-dense granular bodies, as shown by histology and magnetic resonance imaging (MRI) (Arstila et al. 1972; Haltia et al. 1975). The cellular vacuoles in some tissues also seem to include lipid droplets. The exact content of the storage vacuoles is difficult to detect by staining methods, probably because of the small size of the

storage compounds and their high solubility in water and solvents, and it is therefore poorly characterized (Mononen and Mononen 1997; Aula et al. 2001;). In contrast to somatic tissues, where lysosomal storage has been detected already during fetal life in several organs and cell types, fetal brain tissue has been found to be minimally affected (Aula et al. 1984b).

Although our knowledge of the gene defects underlying the AGU has expanded and is currently quite good, the exact pathogenesis of the disease is not known. In various lysosomal storage disorders it is currently thought that the accumulation of storage material may lead to the specific disease by enlargement of the organs and by secondary biochemical and structural events (Mononen and Mononen 1997; Vellodi 2005). These secondary events might include deregulation of apoptosis due to the disturbed lysosomal function with altered inflammatory response (Wada et al. 2000; Tardy et al. 2004; Wu et al. 2005) or cytokine release followed by macrophage activation (Vellodi 2005), and most likely occur in AGU as well.

2.1.3.1 Aspartylglucosamine

In AGU, the main accumulating compound is aspartylglucosamine (GlcNAc-Asn; 2-acetamido-1-(L-β-aspartamido)-1,2-dideoxy-β-D-glucose; N-acetylglucosaminyl-asparagine; AADG; GlcNAc-Asn) (*Figure 2*)(Maury 1980; Mononen and Mononen 1997; Aula et al. 2001). It is practically absent in tissues of healthy individuals, but it has been identified in many organs of adult AGU patients, including the liver, spleen and thyroid gland, kidneys, skeletal muscle, spinal cord, sciatic nerve, lymphocytes and brain tissue (Maury 1980; Maury and Palo 1980a; Maury and Palo 1980b; Maury et al. 1981). GlcNAc-Asn is also secreted into the body fluids and urine of AGU patients at concentrations many hundred-fold those in healthy individuals or heterozygous carriers with only trace amounts of storage material detected in their urine (Kaartinen and Mononen 1989). However, the urinary concentration of GlcNAc-Asn is not linked to the severity of the disease (Aula et al. 1980).

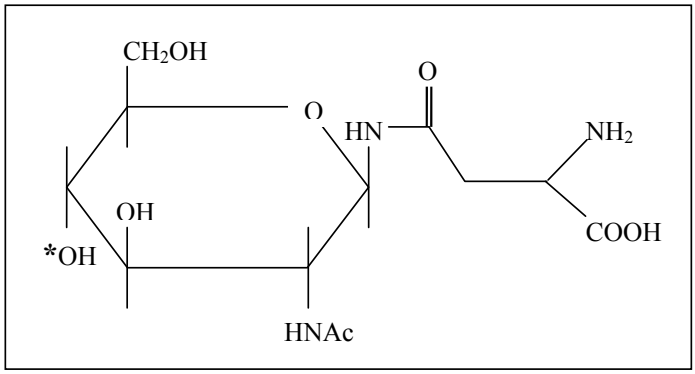

*Figure 2. The structure of an aspartylglucosamine (GlcNAc-Asn). *The hydroxyl group (OH) is replaced by a specific carbohydrate moiety in higher order glycoasparagines.*

2.1.3.2. Other accumulating glycoasparagines

In all accumulating substrates in AGU, L-asparagine is attached to the N-acetylglucosamine (*Figure 2*). Various amounts of higher order glycoasparagines with different carbohydrate moieties including mannose, fucose, sialic acid and galactose attached to the N-acetylglucosamine (marked with * in *Figure 2*) are also found in the urine and tissues of AGU patients (Maury 1980; Mononen and Mononen 1997; Aula et al. 2001; Kelo et al. 2005). These have been concluded to be partial degradation products of N-linked oligosaccharides.

2.1.4 Clinical manifestations of AGU

In spite of the accumulation of GlcNAc-Asn, the development of a fetus with AGU usually proceeds normally and the newborn shows no signs of the disease (Arvio et al. 1993; Arvio et al. 1997; Arvio and Arvio 2002). In Finland, AGU is diagnosed on average at the age of 5.5 years, when delayed speech development together with concentration difficulties, with clumsiness and restlessness in some patients, attract attention. Most children with AGU also have recurrent respiratory infections. The nature of the disease is progressive and the developmental lifespan of the patients has been divided into three phases (Arvio and Arvio 2002). During the first phase, from childhood up to the age of 15, AGU children learn new skills although their development is delayed. The skills peak at the age of 13-16 years, but the children are mildly mentally retarded and their developmental age corresponds to that of healthy 5-6–year-old children. In the second phase, from puberty up to the age of 25-28 years, the patients are in a stable phase but they may lose some skills. Finally, in the third

phase, a rapid mental and physical retardation occurs, leading to death at approximately the age of 35-45 years. Patients with AGU carrying a non-Finnish mutations have similar phenotypic outcome as patients with the Finnish mutations (Aula et al. 2001).

Thus, progressive mental retardation is the most characteristic feature of AGU. Adult patients may also be stubborn and aggressive when disturbed, and females in particular may also have psychotic periods of varying lengths. Additional findings in adult AGU patients include epileptic seizures, coarse facial features with puffy eyelids, thick eyebrows, round nostrils, skeletal changes, skin abnormalities, hepatospelenomegaly and joint disease (Arvio et al. 1997; Arvio et al. 2002; Arvio et al. 2004). Moreover, alterations on T2- and T1-weighted MRI images have recently been related to AGU (Autti et al. 2008). In addition, carriers of AGU with the mutation only in one allele, resulting in 50% of the normal AGA activity, may have a slightly altered facial appearance (Arvio et al. 2004) and a risk of developing chronic arthritis (Arvio et al. 2002).

2.1.5 Biochemical diagnosis of AGU

When a child is suspected of having AGU, the exact diagnosis can be made via quantitative determination of GlcNAc-Asn in urine or leukocytes by liquid chromatography (Mononen et al. 1986; Kaartinen and Mononen 1989; Kaartinen and Mononen 1990b). These HPLC methods can be automated and are thus feasible in clinical laboratories. Other methods for the determination of GlcNAc-Asn from urine include thin-layer chromatography (Humbel and Collart 1975; Sewell 1979), gas-liquid chromatography (Maury 1979) or an enzymatic treatment of GlcNAc-Asn (Sugahara et al. 1976).

Since the AGA activity in the tissues or urine of an AGU patient is less than 5% of that in a healthy person (Mononen and Mononen 1997), reliable detection of the AGU can also be made by measuring the AGA activity in the urine, leukocytes, plasma, serum or cultured fibroblasts of the patient. The lack of enzyme activity can be detected by colorimetric (Makino et al. 1966; Aula et al. 1976) or fluorometric methods (Mononen et al. 1993b; Voznyi et al. 1993; Mononen et al. 1994), or by liquid chromatography (Kaartinen and Mononen 1990a).

For prenatal diagnosis of AGU, the practically absent AGA activity detected in cultured amniotic fluid cells can be used (Aula et al. 1984b; Mononen et al. 1988b; Aula et al. 1989).

However, due to the low fetal excretion of GlcNAc-Asn into the amniotic fluid, diagnosis based on the measurement of the storage material from the amniotic fluid is not reliable for prenatal diagnosis (Mononen et al. 1988a).

Today, diagnosis and carrier detection of AGU can be achieved by DNA analysis if the specific mutation in the *AGA* gene causing the disease is known, which often is the case in Finland (Mononen et al. 1991; Syvanen et al. 1992; Delahunty et al. 1995). However, the cost of DNA analyses is high: in carrier screening performed in 1995-1996, the cost of detecting one adult AGU carrier was $434 (€334), which would result in a theoretical cost of $107,632 (€82,790) to detect one affected fetus among 2912 screened pregnancies (Kallinen et al. 2001). Nevertheless, when the study was done, the attitudes of the parents tested and their close relatives towards genetic testing were positive (Hietala et al. 1995).

2.2 HUMAN GLYCOSYLASPARAGINASE

In the 1960s, the carbohydrate-to-protein linkage containing asparagine and N-acetylglucosamine was precisely characterized (Yamashina et al. 1965) and glycosylasparaginase (N^4-(β-N-acetyl-glucosaminyl)-L-asparagine aminohydrolase; asparagine-N-acetylglucosamine; aspartylglucosaminidase; AGA; GA; EC 3.5.1.26) was discovered, when it was found that sweet-almond emulsion and sheep epididymis extract played a role in the degradation of this linkage region in glycoproteins (Lee et al. 1964; Murakami and Eylar 1965). It was also found that the same enzyme extracted from a guinea pig serum was an aminidase and not a glycosidase as was initially thought (Makino et al. 1966). The enzyme was also soon connected to the AGU disease, as mentioned in Chapter 2.1 (Discovery of AGU) (Jenner and Pollit 1967). In humans, the enzyme was first purified from the liver (Savolainen 1976). It has been found that the biochemical properties of the enzymes varies slightly from species to species (Noronkoski et al. 1997b), and this literature review concentrates on the human enzyme.

To date, several names describing the enzyme's action in the catabolism of glycoproteins have been used (Noronkoski et al. 1997b). The name glycosylasparaginase was eventually introduced to differentiate the enzyme from the β-N-acetylglucosaminidases, which are endoglycosidases cleaving the N-acetylglucosaminyl moiety from the carbohydrates

(Tarentino et al. 1975) and to address the similarity of its reaction mechanism to that of L-asparaginase (Kaartinen et al. 1992). The enzyme has been found to have aminase activity and the name glycosylasparaginase/β-aspartyltransferase has also been proposed, but is not yet used (Noronkoski et al. 1998).

2.2.1 Synthesis and protein structure of AGA

Glycosylasparaginase is synthesized in the endoplasmic reticulum (ER) as a single inactive 346-amino-acid-long precursor protein with two N-glycosylation sites and 9 cysteine residues (Fisher et al. 1990; Ikonen et al. 1991; Mononen et al. 1991; Mononen et al. 1992; Oinonen et al. 1995). The gene encoding AGA is localized on a long arm of chromosome 4 (region q32-q33) (Aula et al. 1984a; Morris et al. 1992).

The precursor protein of the active enzyme goes through several post-translational processing steps in the ER, starting with the removal of a 23-amino-acid N-terminal signal peptide. A properly folded precursor protein is then autocatalytically cleaved between the amino acids Asp205 (corresponding to the Asp182, if the 23-amino-acid signal peptide is not included in the numbering) and Thr206 (Thr183) into two subunits (pro-α and pro-β) (Fisher et al. 1990; Kaartinen et al. 1992; Ikonen et al. 1993; Riikonen et al. 1996; Guan et al. 1996; Saarela et al. 1998). These subunits are held together by non-covalent forces and are subsequently associated into a heterotetramer ($\alpha_2\beta_2$) structure which forms an active enzyme (Fisher et al. 1990; Kaartinen et al. 1991; Oinonen et al. 1995). Although the enzyme is a heterotetramer in the active form, it has also been postulated to exist as a heterodimer in solutions (Wang and Guo 2003).

The active precursor enzyme is next transported into the lysosomes, where the processing proceeds by the cleavage of short sequences from the C-terminal ends of both subunits (Mononen et al. 1991; Tollersrud and Aronson 1992; Ikonen et al. 1993). This trimming has no effect on the catalytic activity of the enzyme. Instead, the critical posttranslational processing steps affecting the proper function of AGA include the formation of disulfide bridges between eight out of nine cysteine residues in the protein. These four intramolecular disulfide bridges between Cys64/Cys69 and Cys163/Cys179 in the α-subunit and Cys285/Cys306 and Cys317/Cys345 in the β-subunit are crucial for the stabilization, correct folding and subunit processing, and thus for the activation of the

enzyme (Enomaa et al. 1992; McCormack et al. 1995; Oinonen et al. 1995; Park et al. 1996; Riikonen et al. 1996). The $AGU_{FIN,}$ mutation prevents the formation of one of the disulphide bridges due to the substitution of the cysteine 163 by serine, which leads to the inactivation of the enzyme.

Another important step in the posttranslational processing of the active AGA in the lysosomes is the attachment of carbohydrate chains to the glycosylation sites located in the α- and β-subunits. One glycosylation site in both α-subunits is located in Asn38, and one in Asn308 in both β-subunits. These four glycosylation sites in the the mature heterotetrameric enzyme contain both complex and high-mannose type oligosaccharide chains (Kaartinen et al. 1991). The glycosylation does not affect the folding or the processing of the enzyme, but the phosphorylation of these oligosaccharides is important for the stability of the enzyme inside the cells, and for the normal transport of the AGA from the ER into the lysosomes. The importance of the phosphorylation for the correct trafficking of the AGA indicates that the enzyme utilizes the mannose 6-reseptor mediated pathway in the transport process into lysosomes (Tikkanen et al. 1995; Park et al. 1996; Tikkanen et al. 1997).

As a result of the activation process of the AGA, the sizes of subunits in the fully processed enzyme are 19-25 kDa for the heavy (α) subunit and 16-19 kDa for the light (β) subunit (Baumann et al. 1989; Halila et al. 1991; Kaartinen 1991; Kaartinen et al. 1991; Enomaa et al. 1992; Rip et al. 1992; Tollersrud et al. 1994). The small heterogeneity in the reported molecular masses probably results from the heterogeneity of the attached carbohydrate chains in enzymes purified from different sources, and from the diversity in the purification procedures of the enzyme and in the determination of the molecular weights in different studies.

AGA is widely expressed in human tissues, but the highest activities are found in leukocytes and the strongest immunoreactivity in hepatocytes, renal tubule cells of kidneys and in pyramidal cells of the cerebral cortex (Enomaa et al. 1993). Immunostained AGA is also present in the Purkinje cells, in the soma and extensions of other neurons, and in the myelinated fibers in infant brains (Uusitalo et al. 1999).

2.2.2 Chemical properties of AGA

The glycosylasparaginase in the human liver (Savolainen 1976; Dugal and Stromme 1977; McGovern et al. 1983; Baumann et al. 1989; Tollersrud and Aronson 1992), leukocytes (Kaartinen et al. 1991), urine (Kaartinen 1991) and chorionic villus cells (Kaartinen and Mononen 1990a) has similar chemical properties, having a broad pH optimum at pH 6-9. Although the enzyme is located in acidic lysosomes, the neutral or alkaline pH optimum suggests that the AGA could also have some function outside the lysosomes. The isoeletric point (pI) of the enzyme varies slightly, being at pH 5.7 for liver enzyme (McGovern et al. 1983; Baumann et al. 1989; Enomaa et al. 1992;), pH 4.6-5.2 for leukocyte enzyme (Baumann et al. 1989; Kaartinen et al. 1991; Enomaa et al. 1992) and pH 4.9-5.0 for urinary enzyme (Baumann et al. 1989; Kaartinen 1991; Enomaa et al. 1992), indicating the charge heterogeneity of the enzymes purified from different sources. The enzyme is also very heat stable, remaining active in temperatures up to 60 °C (Baumann et al. 1989; Kaartinen et al. 1991; Enomaa et al. 1992).

2.2.3 Substrates, reaction mechanism and inhibitors of AGA

The natural substrates of the enzyme include aspartylglucosamine (GlcNAc-Asn) (Makino et al. 1966) and larger glycoasparagines (Kaartinen et al. 1992; Risley et al. 2001; Kelo et al. 2005). A prerequisite for the hydrolysis of these glycoasparagines is the existence of a free α-amino group in the substrate, which anchors it to the binding site of the enzyme, and α-carboxyl groups, which are crucial for the action of the enzyme in the asparagine moiety of the substrate (Lee et al. 1964; Kaartinen et al. 1991; Kaartinen et al. 1992; Risley et al. 2001; Kelo et al. 2005). Furthermore, a L-fucose attached to the N-acetylglucosamine prevents the action of glycosylasparaginase, indicating that the α-fucosidase acts prior to AGA in the degradation of glycoproteins (Noronkoski and Mononen 1997).

In the hydrolysis of L-Asparagine and N-Acetylglucosamine in the protein-carbohydrate-linkage in glycoproteins, the aspartic acid (Asp) and 1-amino-N-acetylglucosamine (GlcNAc-Asn-NH2) are formed through the β-aspartyl enzyme intermediate. GlcNAc-Asn-NH2 is further non-enzymatically hydrolyzed into N-acetylglucosamine (GlcNAc) and ammonia (NH_3) (Makino et al. 1966; Kaartinen et al. 1992;). The Michaelis constant (Km) for the reaction varies from 0.09 to 1.25 mM for leukocyte, liver

or urinary AGA (Dugal and Stromme 1977; McGovern et al. 1983; Baumann et al. 1989; Kaartinen 1991; Kaartinen et al. 1991; Enomaa et al. 1992).

Precise reaction mechanism of AGA has been reavealed by using crystal structures and functional analyses of the enzyme (Brannigan et al. 1995; Oinonen et al. 1995; Tikkanen et al. 1996). According to these studies, AGA uses the same catalytic mechanism as the enzymes in the enzyme class termed N-terminal nucleophile (Ntn) hydrolases. The requirements for this mechanism include the existence of a free α-amino group in the N-terminal residue of the substrate that is situated at the active site of the enzyme, and the existence of catalytic groups in the substrate binding pocket of the enzyme that act as a proton donor. In addition, the polar side-chain of the N-terminal amino acid residue is used as a nucleophile during the substrate hydrolysis. In AGA, a funnel-shaped active site is situated in a deep pocket between the αβ-heterodimers (βααβ sandwich core structure), where Thr206 is the N-terminal residue in the β-chain acting as a base, enhancing the nucleophility of the hydroxyl group of its own side chain (Kaartinen et al. 1991; Fisher et al. 1993). In the high pH optimum (pH 7-9) of the enzyme, significant amounts of α-amino groups of Thr206 are uncharged and are thus able to act as a base in the acidic environment of the lysosomes. The amino acids Thr257 and Gly258 in the active site form an oxyanion hole which stabilizes the transition state of the enzyme-substrate complex (Oinonen et al. 1995; Riikonen et al. 1995; Tikkanen et al. 1996). The asparagine moiety of a substrate binds to the active site amino acids Arg234, Asp237, Thr257 and Gly258 mainly by hydrogen-bonding (Oinonen et al. 1995; Riikonen et al. 1995; Tikkanen et al. 1996). By serving as a binding site for the substrate, these amino acids stabilize the correct conformation of the active site and modify the properties of Thr206, leading to the breakage of an amine bond. Recently, Wang and Guo have proposed in the model of *Flavobacterium meningosepticum* that in addition to serving as a base in the activation of a nucleophile, Thr206 serves as an acid in the polarization of the leaving group during the catalytic process (Wang and Guo 2007). The authors also postulate that no water is included in the catalytic process. Instead, the complex is shielded from water and the free α-amino group of Thr206 and its own side chain nucleophile are hydrogen-bonded to the sidechain hydroxyl group of Thr224, mediating the polarization process necessary for the activation. Based on this precise reaction mechanism and on the data of the autocatalytic activation process of AGA, it has been proposed that it belongs to the Ntn hydrolases

(Brannigan et al. 1995; Oinonen and Rouvinen 2000; Qian et al. 2003; Wang and Guo 2003; Saarela et al. 2004).

In addition to the glycoasparagines, AGA is also capable of hydrolyzing β-aspartyl peptides and derivatives as well as L-asparagine (Kaartinen et al. 1992; Mononen et al. 1993a; Noronkoski et al. 1997a; Noronkoski et al. 1998), thus demonstrating that glycosylasparaginase does not need carbohydrate in its substrates and may play a role in the metabolism of β-aspartyl peptides.

The 5-diazo-4-oxo-L-norvaline (DONV) is a specific inhibitor to AGA, abolishing the activity by irreversible binding to Thr206 in the active site of the enzyme (Kaartinen et al. 1991).

2.3 TREATMENT OF LYSOSOMAL STORAGE DISEASES

The main goal in the treatment of lysosomal storage diseases is to decrease or eliminate substrate accumulation in order to prevent symptoms of the disease. The choice of treatment strategy is disease-specific and is based on the pathophysiology and severity of the disease, on the target organ and on the availability of tools and techniques for carrying out the therapy (Winchester et al. 2000; Treacy and others 2001; Wenger et al. 2003). One treatment strategy might be diminishing the substrate accumulation by restricting the lysosomal substrate flow or by dispersing the accumulated substrate by substrate deprivation therapy. Furthermore, the residual enzyme activity found in some diseases can be enhanced, and this, in turn, results in diminished accumulation of the substrate. When the disease is caused by one malfunctioning enzyme, a feasible approach is to replace this enzyme either by direct administration of an exogenous enzyme or by creating an endogenous enzyme pool by transplanting cells or genes into a body or a specific tissue. Today, these different treatment strategies are being intensively researched in animal models (Winchester et al. 2000; Haskins et al. 2002; Suzuki et al. 2003; Wenger et al. 2003; Zhu et al. 2006). However, to date only bone marrow and stem cell transplantation (BMT), which creates an endogenous enzyme pool, enzyme replacement (ERT) by exogenous enzyme administration, and substrate reduction therapy are in clinical use in in some LSDs (Beck 2010). However, there is no curative treatment for the diseases with symptoms in the central nervous system.

2.3.1 Enzyme replacement therapy

The term enzyme replacement therapy (ERT) is commonly used for a therapeutic approach where the malfunctioning enzyme is replaced and thus the lysosomal storage is corrected by direct injection of an exogenous enzyme into a patient. Usually, the enzyme is injected into the blood stream. The prerequisite for ERT is the availability of a sufficient quantity of stable, safe and non-immunogenic exogenous enzyme, which is correctly transported from an extracellular environment into the lysosomes of the cells. Moreover, the enzyme has to be biologically active, i.e. capable of degrading the lysosomal storage and prohibiting further accumulation of the substrate once it has reached the target cells. Beyond this, many disease-specific factors such as optimal dosage and injection intervals and the pharmacologic effects of the enzyme need to be clarified for the successful treatment of a specific disease. To fulfill these requirements, the nature and function of the enzyme inside the cells and pathophysiological events in the disease need to be characterized.

To date, ERT with exogenous enzymes has been approved for clinical use in the non-neurological variant of Gaucher disease (Barton et al. 1991; Grabowski et al. 1995; Davies et al. 2007), Fabry disease (Eng et al. 2001; Schiffmann et al. 2001; Lidove et al. 2007; Morel and Clarke 2009), MPS I (Kakkis et al. 1994; Wraith et al. 2004; Lidove et al. 2007; Sifuentes et al. 2007), MPS II (Wraith et al. 2008), MPS VI (Anson et al. 1992; Harmatz et al. 2008), and Pompe disease (Katzin and Amato 2008; Strothotte et al. 2010) (*Table 3*). None of these diseases involve serious neurological symptoms which need to be treated. In addition, ERT has been studied in animal models, in addition to AGU, in MPS VII (Vogler et al. 1993), GM1 gangliosidosis (Samoylova et al. 2008), MPS IIIA (Bielicki et al. 1998; Urayama et al. 2008), MPS IIIB (Yu et al. 2000), Nieman-Pick disease (Miranda et al. 2000), α-mannosidosis (Roces et al. 2004; Crawley et al. 2006; Samoylova et al. 2008;), metachromatic leukodystrophy (Matzner et al. 2005), sialidosis (Wang et al. 2005), and Krabbe disease (Lee et al. 2005) (*Table 3*). Most of these diseases affect the central nervous system.

Table 3. Lysosomal storage disorders in which ERT has been studied (excluding AGU).

Disease	CNS involvement	Recombinant enzyme [a)]	Animal model	In clinical use	References
Mucopolysaccharidoses					
MPS I		X	X	X	Kakkis et al, 1994; Wraith et al, 2004; Lidove et al, 2007; Sifuentes et al, 2007
MPS II		X	X	X	Muenzer et al, 2002, Muenzer et al, 2006, Wraith, 2008
MPS IIIA	X	X	X		Bielicki et al, 1998; Urayama et al, 2008
MPS IIIB	X	X	X		Yu et al, 2000
MPS VI		X	X	X	Anson et al, 1992; Harmatz et al, 2008
MPS VII	X	X	X		Vogler et al, 1993
Glycoproteinoses					
α-Fucosidosis	X	X			Bielicki et al, 2000
α-Mannosidosis	X	X	X		Berg et al, 2001; Roces et al, 2004; Crawley et al, 2006; Samoylova et al, 2008
Sialidosis	X	X (murine)	X		Wang et al, 2005
Sphingolipidoses					
Fabry disease		X	X	X	Ioannou et al, 1995; Eng et al, 2001; Schiffmann et al, 2001; Lidove et al, 2007; Morel & Clarke, 2009
Gaucher disease	X [b)]	X	X	X [c)]	Barton et al, 1991; Grabowski et al, 1995; Davies et al, 2007;
GM1 gangliosidosis	X	X (feline)	X		Samoylova et al, 2008
Tay-Sachs disease	X	X			Akeboshi et al, 2007
Sandhoff disease	X	X			Akeboshi et al, 2007
Krabbe disease	X	X (murine)			Lee et al, 2005
Metachromatic leukodystrophy	X	X	X		Matzner et al, 2005
Niemann-Pick A and B	X	X	X		Miranda et al, 2000; He et al, 1999
Other lipidoses					
Neuronal ceroid lipofuscinosis (Batten)	X	X			Lin & Lobel, 2001
Glycogen storage disease					
Pompe disease		X	X	X	van Hove et al, 1996; Bijoet et al, 1998, Bijvoet et al, 1999; Katzin & Amato, 2008; Strothotte et al, 2010

[a)] Human recombinant enzyme unless otherwise stated, [b)] In neuronopathic variant, [c)] In non-neuronopathic variant

2.3.1.1 Receptor-mediated endocytosis of exogenous enzymes

In ERT, an exogenous enzyme injected into the patient is transported and targeted to cells and lysosomes by the lysosomal/endosomal system. Although in some cell types a newly synthesized lysosomal enzyme can be sorted by a non-receptor mediated transfer, the soluble enzyme protein injected into the extracellular environment of the patient is mainly targeted by receptor-mediated endocytosis (Kornfeld and Mellman 1989; Dittmer et al. 1999; Grabowski and Hopkin 2003; Vellodi 2005;). The key factor in correct targeting is that the

enzyme bears an appropriate signal which is recognized by the receptor in the desired cell type. These targeting signals include mannose/N-acetylglucosamine, recognized by monocytes and macrophages, sialic acid, recognized by glial cells, asialogalactose, recognized by hepatocytes, fucose, recognized by fibroblasts, and mannose 6-phosphate (M6P), recognized by most of the cell types, including brain cells (Bou-Gharios et al. 1993a; Dittmer et al. 1999; Sly 2000; Grabowski and Hopkin 2003; Urayama et al. 2004; Sleat et al. 2005; Vellodi 2005; Beck 2007).

The M6P receptor, which is the most abundant, has been investigated extensively. Indeed, two receptors belonging to the P-type lecitin family have been identified (Dahms and Hancock 2002; Ghosh et al. 2003). They are termed the cation-dependent (CD-MPR) and the cation independent (CI-MPR) M6P receptors. The latter are also called cation-independent insulin-like growth factor II/MPR receptors (IGF-II/MPR), based on their ability to bind IGF-II in the cell surface, and thus playing a role also in other important physiological processes such as fetal development and apoptosis (Ghosh et al. 2003; Hawkes and Kar 2004). Although both of these receptors function in the sorting and trafficking of the newly synthesized lysosomal enzymes, they have distinct functions *in vivo* (Pohlmann et al. 1995; Sohar et al. 1998; Schellens et al. 2003). Importantly, only CI-MPR is capable of binding soluble extracellular lysosomal enzymes which have M6P recognition markers (Stein et al. 1987; Dahms and Hancock 2002; Olson et al 2004;). Interestingly, the M6P recognition marker has also been identified in a set of lysosomal enzymes found in the brain, and the receptor has been demonstrated to mediate the transport of lysosomal enzyme across the blood-brain barrier (Urayama et al. 2004; Sleat et al. 2005; Urayama et al. 2008).

In receptor-mediated targeting, attention should be paid to whether the enzyme is delivered and distributed correctly also in the diseased state. It may not be enough that the enzyme has an appropriate targeting signal, since the longlasting lysosomal storage may disturb the endosomal/lysosomal system and the cellular uptake of exogenous enzymes (Dhami and Schuchman 2004; Keslová-Veseliková et al. 2008; Ohashi et al. 2008).

2.3.1.2 Recombinant enzymes

When the concept of ERT was introduced in the 1970s (Brot et al. 1974), one limiting step was the availability of pathogen-free corrective enzyme in the large quantities needed for the therapy. However, the development of DNA techniques in the 1990s enabled the mass-

production of the recombinant human enzymes by expressing them in bacterial or animal cells. The prerequisites in the production are the stable expression of the recombinant protein and the correct post-translational processing, stability and safety of the produced enzyme preparate, none of which can be taken for granted. Although the recombinant enzymes can be quite easily and cheaply produced in insect cells or in yeast, to date mammal cells have been preferred as an expression system due to their ability to carry out the complex post-translational processing steps of the recombinant protein, in a similar way to natural processes (Chen et al. 2000a; Chen et al. 2000b; Du et al. 2001; Bonten et al. 2004; Akeboshi et al. 2007). In addition, the recombinant enzymes produced in mammalian cells have a glycan structure similar to the natural ones, making them less immunogenic when introduced into a mammalian body.

The use of recombinant enzymes for therapeutic purposes gives an opportunity to modify the oligosaccharide targeting signals of these enzymes in a controlled way (Kornfeld and Mellman 1989; Ginsel and Fransen 1991; Sands et al. 2001; Dhami and Schuchman 2004; Du et al. 2005; Zhu et al. 2005). By this means, the therapeutic effect can be enhanced and made more wide compared with those of natural enzymes. Indeed, during the last decade, the modified glucocerebrosidase has been used in the treatment of non-neuronopathic Gaucher disease, although the advantage of modification in this enzyme preparation has lately been questioned (Grabowski et al. 1995; Van Patten et al. 2007).

To date, many different recombinant human lysosomal enzymes in addition to AGA have been produced (*Table 4*). Only a few of them are in clinical use, the majority being used only in exploring the possibilities of ERT (*Table 3*). Among these enzymes is a recombinant α-N-acetylglucosaminidase produced for the treatment of MPS IIIB, which turned out be of limited value due to deficient mannose 6-phosphorylation, resulting in incomplete targeting and uptake of the enzyme in the affected cells, which emphasizes the importance of the correct posttranslational processing of the recombinant enzymes (Zhao and Neufeld 2000; Weber et al. 2001). In addition to the production of human enzymes, a feline β-galactosidase has been produced to study ERT in a feline model of GM1 (Samoylova et al. 2008), and a murine sulfaminidase, neuraminidase (Neu1) and galactosylceramidase to study ERT in mouse models of MPS IIIA, sialidosis and Krabbe disease, respectively (Gliddon and Hopwood 2004; Lee et al. 2005; Wang et al. 2005).

Table 4. The human recombinant enzymes produced for ERT (excuding AGA).

Disease	Recombinant enzyme	References
Mucopolysaccharidoses		
MPS I	α-L-Iduronidase	Kakkis et al, 1994
MPS II	Iduronate-2-sulphatase	Muenzer et al, 2002; Muenzer et al, 2006; Wraith, 2008
MPS IIIA	Sulphamidase	Bielicki et al, 1998
MPS IIIB	α-N-Acetylglucosaminidase	Yu et al, 2000
MPS VI	Arylsulphatase B	Anson et al, 1992
MPS VII	β-Glucuronidase	Vogler et al, 1993
Glycoproteinoses		
α-Fucosidosis	a-L-Fucosidase	Bielicki et al, 2000
α-Mannosidosis	α-Mannosidase	Berg et al, 2001
Sphingolipidoses		
Fabry disease	α-Galactosidase A	Ioannou et al, 1992
Gaucher disease	Glucocerebrosidase	Barton et al, 1991; Grabowski et al, 1995; Davies et al, 2007
Tay-Sachs disease	β-Hexosaminidase A	Akeboshi et al, 2007
Sandhoff disease	β-Hexosaminidase A	Akeboshi et al, 2007
Metachromatic leukodystrophy	Arylsulphatase A	Matzner et al, 2005
Niemann-Pick A and B	Acidic sphingomyelinase	He et al, 1999; Miranda et al, 2000
Other lipidoses		
Neuronal ceroid lipofuscinosis (Batten)	Neuronal 2 protein (CNL2p)	Lin & Lobel, 2001
Glycogen storage disease		
Pompe disease	Acid α-glucosidase	Van Hove et al, 1996; Bijvoet et al, 1998; Bijvoet et al 1999

2.3.1.3 Enzyme dosage

In every clinically approved ERT, there are recommendations for the dosage and the injection intervals. However, these recommendations are not necessarily optimal for every patient, or even for every organ or for the different tissues of the patient. For example, a low amount of enzyme may cure some symptoms, and changing the dose in the course of the therapy may sometimes be beneficial (Garcia et al. 2007; Wilson et al. 2007; de Fost et al. 2008). Furthermore, although a sufficient amount of the enzyme should always exist in the body to prevent the accumulation of the substrate, increasing the dose into supraphysiological amounts can be harmful for the function of the cell and will not benefit the therapy. In other words, the enzyme dose should be optimal for the function of the cell and the lysosome, and the current scientific view emphasizes the fact that for an optimal

therapy, the enzyme dose should also be individualized (Hollak et al. 1995; Pastores et al. 2004; Andersson et al. 2005). This raises the need for knowledge of the pathophysiology of the disease, the stage of the disease in the individual patient and detailed knowledge of the enzyme preparation that is used.

2.3.1.5 ERT and the brain

The major limitation and future challenge in ERT is the targeting of the exogenous enzymes to the brain. The enzyme replacement therapies that are now in clinical use do not affect the CNS involvement of the disease. The challenge is to get the corrective enzyme to traverse the blood-brain barrier (BBB) and thereby achieve the therapeutic effect also in the brain.

The development of the BBB has been shown to occur gradually and the full tightening is achieved if not during the fetal development then during the first few days or weeks after birth (Stewart and Hayakawa 1987; Rubin and Staddon 1999; Engelhardt 2003). The solutes of the blood cannot passively diffuse across the fully developed barrier, but there are various transport mechanisms for e.g. macromolecules (Begley 2003). In particular, it was recently discovered that the recombinant human β-glucuronidase and sulfaminidase were able to cross the BBB in newborn mice via MPR/IGFII receptor-mediated endocytosis (Urayama et al. 2004; Urayama et al. 2008). Furthermore, this active transport capacity was lost in adult animals, suggesting that in addition to the BBB, the transport mechanisms are also developmentally downregulated. However, it has also been shown that following intravenous injection of the enzyme, some recombinant enzymes can be transported into the adult brain by some so far unclear mechanisms (Lee et al. 2005; Matzner et al. 2005; Vogler et al. 2005; Crawley et al. 2006). Interestingly, it has been shown that the elimination of the mannose 6-phosphate and mannose residues in recombinant β-glucuronidase dramatically enhance the delivery of this enzyme cross the adult mouse BBB and consequently the correction of lysosomal storage in brain (Grubb et al. 2008). In addition, a less phosphorylated α-mannosidase has been shown to pass the BBB of adult α-mannosidosis mice further proposing an alternative delivery system different from the M6P mediated endocytosis for therapeutic enzyme to enter to adult brain (Blanz et al. 2008).

One way to overcome the BBB is to inject the enzyme straight into the cerebrospinal fluid (CSF) by intrathecal injection (Kakkis et al. 2004), or into the brain by intrastriatal (Lonser et al. 2005) or intraventricular injection (Chang et al. 2008). Importantly, in all of

these studies, the recombinant enzymes were distributed into different areas of the brain where they were able to diminish the lysosomal storage. This indicates that the different cells in the brain are not rigid but are capable of internalizing the exogenous enzyme and of correcting the lysosomal storage in vivo. However, in the efficient treatment of the disease requiring life-lasting repeated injections, this might not be a suitable approach.

2.3.2 Bone marrow or stem cell transplantation

Bone marrow transplantation (BMT) or the transplantation of cord blood, fetal liver or brain-derived stem cells is a therapeutic approach in which a constant supply of endogenous corrective enzyme is created by transplanted donor cells (Olsen et al. 1988; Krivit et al. 1992; Malatack et al. 2003; Wenger et al. 2003; Vellodi 2005; Givogri et al. 2006; Martin et al. 2006; Ringden et al. 2006; Terskikh et al. 2006; Malm et al. 2008). Therefore, these are also referred to as endogenous enzyme replacement therapies. BMT or hematopoetic stem cell transplantation (HCT) are currently clinically available in more than 20 lysosomal storage disorders. The best results have been obtained in individuals with mild soft-tissue involvement or in cases where the therapy has been started before the symptoms arise.

Bone marrow or stem cell transplantation arose from the realization that many mammalian tissues can self-renew and differentiate throughout life. Indeed, it has been found that stem cells, e.g. hematopoetic stem cells (HSC) of the bone marrow or fetal liver, mesenchymal stormal cells (MSC) of the bone marrow or neural stem cells (NSC) of the brain are capable of differentiating into many types of cells depending on the origin of the cells and the microenvironment of the transplant (Bjorklund and Svendsen 1999; Kim et al. 2004; Nakano et al. 2001; Simard and Rivest 2004; Terskikh et al. 2006; Pelagiadis et al. 2008). The donor-derived normal cells produce the missing enzyme, which is then transferred into the surrounding cells, where it clears the accumulation of the substrate. In somatic tissues, the transfer of the enzyme has been shown to occur directly in cell-to-cell contact from the normal to the diseased cells or by a receptor-mediated endocytosis after the enzyme has been released from normal cells into the circulation (Olsen et al. 1981; Olsen et al. 1983; Bou-Gharios et al. 1993b; Olsen et al. 1993; Wilcox 2004). The direct transfer is restricted to certain soluble enzymes, but when it occurs it has been been demonstrated to induce the synthesis of new lysosomal enzyme precursors (Olsen et al. 1988).

Although the renewal of bone or CNS by stem cell therapy is more limited, a restraining of the neurocognitive decline has been shown in humans after BMT (Krivit et al. 1999; Simard and Rivest 2004) or HCT (Olsen et al. 1988; Grewal et al. 2004; Malm et al. 2008;). In addition, the neuronal stem cells or progenitor cells have been shown to cross the BBB and to generate neurons and microglia and also to decrease the lysosomal storage in the brain in animal models (Snyder et al. 1995; Bjorklund and Svendsen 1999; Shihabuddin et al. 2004). Stem cells have also been shown to cross the BBB in humans, even though the engraftment of microglia after bone marrow transplantation is a very slow process (Simard and Rivest 2004; Vellodi 2005).

The drawbacks of BMT are high mortality and morbidity and difficulties in finding an HLA-matching donor. On the other hand, it is suggested that by using HLA-matched unrelated donor stem cells or mesenchymal stormal cells in the transplantation, immunological reactions and the graft-versus-host rejection could be avoided (Pelagiadis et al. 2008). In addition, partially HLA-matched unrelated donor cord blood has been used with good outcome when a suitable bone marrow donor cannot be found (Martin et al. 2006). But before establishing the clinical value of stem cells or neural progenitor cells, many transplantation-related issues need to be solved. These include how to expand the stem cell amount *in vitro* and what is the optimal administration route. Transplantation is also currently a high risk procedure and might not be suitable for a newborn child.

2.3.3 Gene therapy

Gene therapy of lysosomal storage diseases caused by the defect of a single gene involves introducing a gene of a malfunctioning protein into the body or tissue where it is then expressed, generating a long-term pool of a normally functioning corrective protein (Verma and Weitzman 2005; O'Connor and Crystal 2006). Preclinical experiments of gene therapy in animal models of more than 25 LSDs have been reviewed recently (Ellinwood et al. 2004; Sands and Davidson 2006; Geel et al. 2007; Sevin et al. 2007a) and reported by several authors (Cardone et al. 2006; McIntyre et al. 2008; Ramsubir et al. 2008).

To accomplish gene transfer, the gene has to be introduced into the recipient genome for expression. This can be achieved by attaching the gene to a vector capable of transferring the gene. To date, the most commonly used vectors in studies with LSDs are RNA or DNA viral

vectors, including retro- and lentiviruses and adeno- and adeno-associated viruses (Ellinwood et al. 2004; Sands and Davidson 2006). In addition to these viral vectors, non-viral vectors have recently been studied and are already used in cancer treatment, for example (Nishikawa et al. 2008). In LSDs, the non-viral vectors that have been most researched include a plasmid DNA with or without chemical capsulation (Hodges and Cheng 2006; Nishikawa et al. 2008; Zhang et al. 2008), and most recently a plasmid DNA encapsulated in Trojan horse liposomes (Schlachetzki et al. 2004; Zhang et al. 2008).

The gene transfer can be attained *in vivo* or *ex vivo* (Sands and Davidson 2006). In *in vivo* transfer the gene is introduced into the body by an intravenous injection (Cardone et al. 2006; Traas et al. 2007; Herati et al. 2008; McIntyre et al. 2008; Tessitore et al. 2008) or by injecting it into a specific organ (Geel et al. 2007; Tessitore et al. 2008). Although the therapeutic enzyme itself or the vector with the corrective gene has been shown to cross the BBB and to evoke a therapeutic effect at least to a certain extent, site-directed injection of the gene product has been used to enhance the therapeutic effect of the transfer in the brain (Eto et al. 2004; Schlachetzki et al. 2004; Sands and Davidson 2006; Broekman et al. 2007; Cearley and Wolfe 2007; Chung et al. 2007; Liu et al. 2007; Sevin et al. 2007b; Sands and Haskins 2008). In addition, a neonatal injection into the brain has been investigated in this therapeutic approach as well (Ramsubir et al. 2008). In *ex vivo* therapy, the gene transfer and genetic altering is done in specific cells *in vitro* prior to the transplantation of these cells into the affected body (Sands and Davidson 2006). *Ex vivo* gene therapy has been performed by transplanting modified fibroblasts (Moullier et al. 1993), but currently the research has turned to transplanting genetically modified bone marrow or stem cells(Biffi et al. 2004; Enquist et al. 2006; Sands and Davidson 2006; Ramsubir et al. 2008). This approach of modifying multipotent cells has been shown to have potential for more efficient treatment than just modifying the already differentiated cells.

Although the results obtained in the studies mentioned above are promising, larger animal models have been developed to better mimic the size of the human newborn (Casal and Haskins 2006; Zhang et al. 2008). In fact, in the late-infantile form of neuronal ceroid lipofuscinosis, a clinical trial using intracranial injection and recombinant AVV vector has already been started (Enns and Huhn 2008). However, there are still problems to overcome before this approach can be in wider use: stable expression of the gene in the transfected cell type is not guaranteed with current techniques, and safety issues including immunogenity

and tissue-specific expression of the vectors needs to be addressed in detail (Sands and Davidson 2006). In addition, prior to widely used clinical applications, targeted integration techniques have to be studied and developed to prevent unwanted events such as leukemia (Kohn et al. 2003; Ramsubir et al. 2007). In the distant future, novel strategies such as the substitution of an affected gene by a wild type one using targeted restriction endonucleases and homologous recombination might also be available for lysosomal storage disorders with a single gene defect.

2.3.4 Other therapeutic approaches

In addition to creating an exogenous or endogenous enzyme pool for the correction of lysosomal storage, other strategies involve enzyme enhancement therapy and substrate reduction therapy. These so-called small-molecule therapies introduced in this chapter might be an option for the treatment either alone in certain diseases or in combination with other therapies.

In enzyme enhancement therapy, also called chaperone therapy, the residual activity of the mutated enzyme is increased by small-molecular-weight ligands (Desnick 2004; Beck 2007; Enns and Huhn 2008; Parenti 2009). These molecules, e.g. substrate analogues, active-site inhibitors, enzyme cofactors or effector molecules, bind reversibly to the misfolded proteins, stabilizing or reshaping them, leading to correct targeting and thus functioning in the lysosomes. This therapy approach has been studied in animal models in Pompe disease, Gaucher disease, MPS I, GM1–gangliosidosis and Tay-Sachs disease (Desnick 2004; Geel et al. 2007; Sidransky et al. 2007; Enns and Huhn 2008). The one advantage of this approach is that these small molecules can cross the BBB, thus eliciting the therapeutic effect also in neuronal tissues. The studies have been promising, but because these molecules are mutation-specific their use is also disease or even individual specific.

Substrate reduction therapy, also called substrate deprivation therapy, using an iminosugar inhibitor, is in clinical use in non-neurological Gaucher disease (Cox et al. 2003). A small inhibitor molecule reduces the biosynthesis of accumulating macromolecules, which in turn leads to a reduced toxic effect of the substrate in the lysosomes (Beck 2007). As in chaperone therapy, the small inhibitor molecules used in substrate deprivation therapy can cross the BBB and have been thought to be good candidates for treating the diseases with

CNS symptoms (Jakóbkiewicz-Banecka et al. 2007; Enns and Huhn 2008). The therapy has been shown to reduce the level of the substrate accumulating in the brain in Nieman-Pick disease type C (Patterson et al. 2007), and to have a possible anti-inflammatory effect in the brain of newborns affected by Tay-Sachs disease (Bembi et al. 2006). On the other hand, this therapeutic approach did not arrest the neurological deterioration related to these diseases. It might have a beneficial effect on neurological symptoms, when used in combination with some other therapy, as shown in Gaucher disease type III in combination with ERT (Capablo et al. 2007). However, more research is needed in this area.

2.3.5 Application of therapeutic approaches to AGU

Antibiotics, psychoactive drugs and surgery have been used as supportive medical care in AGU disease, but to date there is no cure for the disease (Arvio and Arvio 2002). Since AGU is caused by the lack of activity of one lysosomal enzyme, and there is no considerable amount of residual enzyme activity in the tissues of AGU patients, the approaches to consider for the treatment are enzyme-based therapies: ERT, bone marrow or cell transplantation, and gene therapy. These approaches have been studied in cell cultures, and the generation of mouse models mimicking human AGU disease has opened the possibilities for *in vivo* studies (Kaartinen et al. 1996; Jalanko et al. 1998; Kaartinen et al. 1998). Some patients have also been treated by bone marrow transplantation, but so far there is no recommendation for the disease-specific treatment in addition to supportive medical care.

2.3.5.1 BMT or stem cell transplantation and gene therapy

It has been shown that bone marrow transplantation in adult AGU mice corrects the tissue pathology in the liver and the spleen (Laine et al. 1999). Six months after the transplantation, 3% of the normal enzyme activity was detected in the brain, but no effect on the lysosomal accumulation of the substrate was seen. When the transplantation was performed on 3-week-old AGU mice, a clear reduction of vacuolization of neurons was detected eight and ten months after BMT, indicating the importance of early transplantation (Laine et al. 2004). To date, an allogenic BMT has been performed on seven AGU patients aged 1 to 10 years (Autti et al. 1997; Arvio et al. 2001; Malm et al. 2004). The BMT was shown to slowly normalize some pathological, biochemical and MRI findings or at least to halt the

progression of AGU-specific health problems, e.g. neurological deterioration in some transplanted AGU patients (Autti et al. 1999; Malm et al. 2004). Despite these results, it has been stated that the benefits of the transplantation are somewhat difficult to estimate due to a short follow-up time, and currently BMT is not recommended as a treatment at least after infancy (Arvio et al. 2001). Indeed, it has been suggested that the follow up should be extended to at least 10 years and the transplantation should be performed as early in life as possible (Malm et al. 2004; Ringden et al. 2006).

Retrovirus-mediated gene transfer has been performed *in vitro* in human AGU fibroblasts and lymphocytes as well as in mouse neuroblastoma and rat glioma cells (Enomaa et al. 1995). Gene transfer was successful in all of these cells, and in the AGU fibroblasts the AGA activity was nearly normalized. In addition, transduced AGU lymphocytes secreted AGA into cell culture medium and corrected the enzyme deficiency in AGU fibroblasts in co-culture. *In vivo* gene transfer has been performed in the AGU mouse using adenovirus vectors with either a sarcoma virus promoter or a tissue-specific non-viral promoter (Enomaa et al. 1995; Peltola et al. 1998; Virta et al. 2006). An expression of AGA was demonstrated in the liver after intravenous injection of gene product. Interestingly, when the transgene was injected intraventricularly or intrastriatally, diffusion of the AGA activity into neighboring cells was detected. The use of non-viral tissue-specific promoters lead to more stable expression than the use of a viral promoter, which emphasizes the significance of the choice of promoter (Peltola et al. 1998; Virta et al. 2006). These results may lead future research towards therapy of distinct cell types, which in turn may open new possibilities in the area of gene therapy.

2.3. 5..2 Enzyme replacement therapy

In addition to this study, enzyme replacement has been studied in vitro using a recombinant glycosylasparaginase purified or secreted from CHO cells (Enomaa et al. 1995; Kyttala et al. 1998). In these studies, mouse primary fibroblasts and neural cells and rat glioma cells endocytosed the recombinant AGA. The internalization of the AGA was shown to be M6P dependent, though these cell lines were shown to express cation-independent M6P receptors (Kyttala et al. 1998). Also, tumor-derived mouse or rat neuronal cell lines internalize the AGA, but to a much lower extent (Kyttala et al. 1998). Interestingly, these cell lines did not express CI-M6P receptors. The observation that glial cells have a better capacity than

neurons for uptaking AGA has been verified in later studies (Harkke et al. 2003). Although internalization of the enzyme was demonstrated by different cell types, the biological activity, i.e. the capability of endocytosed enzyme to degrade the accumulated substrate in the AGU cells, has not been shown.

ERT with suitable recombinant enzyme might be benefical in AGU, but to confirm this further research is needed on the enzyme and its biological activity and on disease-related factors in the ERT of AGU.

3 Aims of the study

When this study started, the mechanism of enzyme transfer and ERT as a treatment strategy for many mucopolysaccharidoses and mucolipidoses had been studied but no studies of the treatment of AGU had been published.

The aims of the present work were:

1. To produce a recombinant glycosylasparaginase and to characterize its biological activity in AGU cell culture.

2. To study the mechanism of cell-to-cell transfer of glycosylasparaginase from normal to diseased cells in a cell culture model.

3. To study the efficacy of recombinant glycosylasparaginase in the correction of aspartylglycosaminuria in a mouse model with different enzyme dosages and treatment schedules.

4. To evaluate the effect of the early initiation of ERT in the AGU mouse model.

4 Materials and methods

4.1 CHEMICALS AND REAGENTS

All commercial chemicals and reagents used in the study were purchased from suppliers as described in the original papers (I-IV). The affinity-purified AGA antibodies from hen egg-yolk were produced by immunization of hens with a synthetic peptide representing amino acids S(156)-R(169) of the human glycosylasparaginase (Mononen et al. 1991; Kokko and Karenlampi 1992). The rabbit immune serum was obtained by immunization of rabbits with the human glycosylasparaginase.

4.2 HUMAN RECOMBINANT GLYCOSYLASPARAGINASE (I, III, IV)

4.2.1 Expression and purification (I, III, IV)

The cDNA for the coding region of the glycosylasparaginase was isolated as described elsewhere (Ikonen et al. 1991; Mononen et al. 1991). The 2.1 kb cDNA was isolated from the human placental cDNA library and inserted into a modified mammalian expression vector pCDX under the control of SV40 promoter sequences. The DNA from the pCDX-GA was transfected into NIH-3T3 mouse fibroblasts by the calcium phosphate precipitation method. DNA from pSV-2 was used in cotransfection to attain the neomycin (G418)-resistance for the selection of stable transformants.

The recombinant human glycosylasparaginase was purified from NIH-3T3 fibroblasts based on the method described by Kaartinen and co-workers using DEAE-Sepharose and Mono-Q anion exchange columns, and gel filtration as a chromatographic purification steps (Kaartinen et al. 1991). The purity of the enzyme preparation was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12 % gels in which proteins were localized by silver or Coomassie blue staining. Protein markers with known molecular weight were used to confirm the molecular weights of the protein subunits.

4.2.2 Endoglycosidase and phosphatase digestion (I)

For endoglycosidase digestion, 10 mU of the recombinant glycosylasparaginase was incubated with 20 mU of endoglycosidase H in 100 mM citrate/phosphate buffer, pH 5.5 at 37°C for 17 hours. Another 20 mU of endoglycosidase H was added and incubation was continued at 37°C for 17 hours more. Liberation of carbohydrates was confirmed by SDS-PAGE using 12% gels, protein markers of known molecular weight and Coomassie blue staining. For dephosphorylation, 4.25 mU of the recombinant glycosylasparaginase was incubated with 1.4 U of Escherichia coli alkaline phosphatase in 260 µl of 50 mM Tris-HCl buffer, pH 8.0 at 37°C for 3.5 hours. The glycosylasparaginase activity of the treated enzyme preparations was confirmed by fluorometric assay (4.8.1) after both treatment procedures.

4.3 HUMAN LEUKOCYTE GLYCOSYLASPARAGINASE (II)

The leukocyte glycosylasparaginase was purified from leukocytes isolated form human blood as described by Kaartinen and coworkers (Kaartinen et al. 1991). The purification procedure involved precipitation, gelfiltration and chromatographic steps, the last step being anion exchange chromatography on Mono-Q column. The purity and subunit composition of the enzyme preparation was studied on SDS-PAGE with 12% gel. The specific activity of the enzyme preparation was 96 mU/mg protein measured by fluorometric assay (4.8.1).

4.4. CELL LINES (I, II)

Epstein Barr virus (EBV) transformed B-cell lines were developed from peripheral blood lymphocytes obtained from a Finnish AGU patient carrying the AGU_{FIN}-mutation (Cys163Ser) or from healthy persons by a method described by Volkman et al. (Volkman et al. 1984). The transformed cells were grown in RPMI medium supplemented with 10%-15% FCS. The AGU fibroblasts were obtained from a Finnish AGU patient carrying the AGU_{FIN}-mutation, and the normal fibroblasts were obtained from a healthy person. The fibroblasts were grown in DMED containing 10% fetal calf serum (FCS) or RPMI/15% FCS.

4.5. ANIMALS (III, IV)

The AGU mouse model was generated through targeted disruption of a mouse glycosylasparaginase gene in embryonic stem cells as described elsewhere (Kaartinen et al. 1996). The mice completely lacked glycosylasparaginase activity and accumulated the aspartylglucosamine in tissues and urine, resembling the clinical, biochemical and histochemical characteristics of the human disease (Kaartinen et al. 1996; Kaartinen et al. 1998). The genotype of the mice were confirmed by southern blot analysis of the tail DNA samples (wild-type, WT: $Aga^{+/+}$, heterozygous, HE: $Aga^{+/-}$, homozygous, KO: $Aga^{-/-}$).

4.6 CELL CULTURE EXPERIMENTS (I, II)

AGU fibroblasts or lymphocytes and normal fibroblasts or lymphocytes treated with the experimental protocols described below without enzyme addition were used as negative and positive controls, respectively, in the experiments described below. In these cell culture experiments, biochemical analyses of the cell homogenates included determination of the glycosylasparaginase activity by fluorometric assay (4.8.1) and the aspartylglucosamine concentration by the HPLC method (4.8.2), and determination of the protein concentration (4.8.3) unless otherwise stated. Prior to biochemical analyses the cells were washed two to six times with ice-cold PBS and lysed in 0.5 ml of 1% sodium deoxycholate in 50 mM Tris-HCl, pH 7.4 by freezing and thawing three times, followed by sonication and centrifugation.

4.6.1 Endocytosis of recombinant glycosylasparaginase in fibroblasts and lymphocytes (I)

To study the endosytosis of human recombinant AGA to EBV-transformed lymphocytes, 8 x 10^7 cells grown in RPMI/10% FCS were rinsed twice with PBS and incubated in 6 ml of PBS/10% FCS containing recombinant glycosylasparaginase at concentrations of 0, 0.1, 1, 3, 10, 30, 100 and 300 mU/l up to 14 days. 3 ml of cell suspension was withdrawn from the culture for biochemical analyses and 3 ml of fresh AGA containing PBS-FCS media was added.

To study the effect of M6P or sugar residues of AGA in endosytosis, human AGU fibroblasts were grown to confluency in DMEM/10% FCS on 35 mm tissue culture dishes.

The cells were rinsed twice with 3 ml of PBS and incubated with 2 mU of endoglycosidase H or alkaline phosphatase-treated glycosylasparaginase or 2 mU of untreated glycosylasparaginase with mannose 6-phosphate addition at concentrations of 0.05 mM, 0.2 mM, 0.5 mM, 2 mM and 4 mM in duplicates in 1 ml of PBS/10% FCS up to 3 hours. After the incubation, the cells were washed and lysed for biochemical analyses as described above.

For immunofluorescence microscopy (4.8.5), 4.6 x 10^6 EBV-transformed lymphocytes grown in RPMI/10% FCS media were rinsed two times with PBS, after which they were cultured in 6 ml of media containing 100 mU of AGA/ml. 3 ml of cell suspension was replaced with fresh AGA- containing media after one day of culture. 1 ml of cell suspension was withdrawn from the culture for analysis on day 2. The cells were washed twice with PBS and suspended to the final concentration of 0.2 x 10^6 cells/ml of PBS for cytocentrifugation.

4.6.2 Endocytosis of human leukocyte glycosylasparaginase in lymphocytes (II)

To study the endosytosis of human leukocyte AGA to EBV-transformed lymphocytes, 5-6 x 10^6 cells were cultured in 25 cm^2 tissue culture flasks in 6 ml of RPMI/15% FCS up to 10 days in the presence of the leukocyte glycosylasparaginase at concentrations of 0, 0.1, 1, 3, 10, 30, 100 and 300 mU/l. For biochemical analyses, 3-5 x 10^6 cells (3 ml) were withdrawn from the culture at each time point up to 14 days. 3 ml of fresh AGA-containing media was added to the culture. The cells were washed 2 times with PBS and lysed for biochemical analyses.

For determination of the duration of the therapeutic effect, 25 x 10^6 EBV-transformed lymphocytes were cultured in the presence of 1000 mU/l of leukocyte AGA in 30 ml of RPMI/15% FCS medium for 9 hours, after which the cells were washed and the culture (5 x 10^6 cells) was continued in a medium without AGA supplementation. For biochemical analyses, 5 x 10^6 cells (3 ml of medium) were withdrawn from the culture 2, 3, 6 and 9 hours and 1, 2, 4, 7, 10 and 13 days after the start of the experiment. Equal amounts of fresh medium were added at each time point.

4.6.3 Co-cultivation of normal and AGU cells (II)

To study the enzyme transfer in co-cultivation, a total of 10 x10^6 normal (female) and AGU (male) EBV-transformed lymphocytes were co-cultured in 80 cm^2 tissue culture flasks in a mixture containing 20-100% of normal lymphocytes in 20 ml of RPMI/15%FCS up to 10 days.

5 ml of the cell suspension containing 2.5 to 10 x 10^6 cells was withdrawn for biochemical analyses, and another 5 ml for determination of the number of Y-chromosome-containing cells at each time point. For the biochemical analysis the cells were washed twice with PBS and lysed as described above. For in situ Y-chromosome hybridization assay (4.8.4) the cells were treated with colcemid solution and KCl, fixed with methanol-acetic acid solution and attached to ethanol-treated slides.

To study the transfer of the enzyme without cell-to-cell contact, 2 x10^6 AGU lymphocytes and 2 x10^6 normal lymphocytes in 8 ml of RPMI/15% FCS medium were cultured up to 10 days in a 6-well dish separated by 0.4 μm semi-permeable polycarbonate membrane (Nunc) to prevent cell-to-cell contact. During the culture 1-4 x10^6 cells in 2 ml of medium from both sides of the membrane were collected for biochemical analyses and an equivalent volume of the fresh medium was added.

For immunofluoresence microscopy (4.8.5), human AGU fibroblasts were cultured to near confluency in a 9.2 cm Petri dish. 2 x10^6 EBV-transformed AGU lymphocytes were added to the culture in 20 ml of RPMI/15% FCS. The cultivation was continued for six days. The lymphocytes were washed from the fibroblast culture and the fibroblasts were analyzed by immunofluorescence.

Mannose 6-phosphate inhibition was studied by culturing 1 x10^6 EBV-transformed normal lymphocytes with 4 x10^6 EBV-transformed AGU lymphocytes in 10 ml of RPMI/FCS containing 5 mM mannose 6-phosphate for 10 days. 5 ml of suspension was withdrawn from the culture and an equal amount of fresh mannose 6-phosphate-containing media was added every second day.

4.7 ENZYME REPLACEMENT IN AGU MOUSE MODEL (III, IV)

4.7.1 Preparation of recombinant glycosylasparaginase (III, IV)

The recombinant human glycosylasparaginase (isoform GA-1) was dialyzed into injection buffer containing 150 mM NaCl - 10 mM Tris-HCl, pH 7.5 - 1 mM β-glycerophosphate. The specific activity of the enzyme preparations were 336 mU/mg of protein (III) or 167 mU/mg and 339 mU/mg of protein (IV), determined by fluorometric assay (4.8.1).

4.7.2 Treatment of animals (III, IV)

The animals were handled according to institutional guidelines. The adult AGU mice, from 9 to 19 months of age, received 1 x 1 mg/kg (0.34 U/kg), 8 x 1 mg/kg (0.34 U/kg) or 8 x 10 mg/kg (3.4 U/kg) AGA injections into the tail vein in 100 µl of injection buffer every second day for two weeks (III). One-week-old AGU mice received either 8 x 10 mg/kg (3.4 U/kg) ip injections of AGA every second day for two weeks, or 5 x 10 mg/kg (1.7 U/kg) ip/iv injections every second day followed by an injection once a week (10 mg/kg; 1.7 U/kg) for 4 weeks (IV). Three AGU mice received an intraperitoneal AGA injection (50 mg/kg, 17 U/kg) at the age of seven and nine days (IV). For determination of the tissue-half life of the enzyme, the heterozygous adult mouse (III) or one-week-old mouse (IV) received a single AGA injection of 1 mg/kg (0.34 U/kg). The untreated wild-type (WT) or AGU animals at the same age were used as controls. Body weight and general appearance of all the treated animals were followed daily (III) or at the time of the injection (IV).

4.7.3 Collection of samples (III, IV)

Urine samples for determination of the GlcNAc-Asn concentration (4.8.2) were collected on filter paper daily (III), or at the time of injection/tissue collection (IV). The blood samples were collected to heparinized capillars and plasma was separated for determination of AGA activity by fluorometric method (III, 4.8.1). For the biochemical analyses the animals were anesthetized and intracardically perfused with 0.9% NaCl (III, IV). The tissues were collected and stored at –70°C prior to homogenization and handled in ice after thawing. Tissues were homogenized in 50 mM Na-K-phosphate buffer, pH 7.2, containing 0.1% Triton X-100. AGA activity from tissue homogenates was determined by HPLC (4.8.1). The AGA activity from liver homogenates for determination of tissue half-life of the enzyme was measured by a fluorometric method (4.8.1). For electron microscopy (III, 4.9.1) the mice were intracardically perfused with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The tissues were removed and stored in 2.5% glutaraldehyde prior to analyses. For immunohistochemical studies (4.9.2) the animals were intacardically perfused with 0.9% NaCl, then the tissues were removed and immersed in formalin.

4.8 BIOCHEMICAL AND OTHER ASSAYS

4.8.1. Assay for glycosylasparaginase activity

The enzyme activity of the recombinant human glycosylasparaginase (I-IV), the glycosylasparaginase activity of cell homogenates from the cell culture experiments (I-II), the AGA activity in liver homogenates for tissue half-life (III) and the AGA activity from plasma (III) were determined by fluorometric assay using β-(7-amido-4-methylcoumarin)-L-aspartic acid as a substrate (Mononen et al. 1993b). One unit of the enzyme causes the loss of 1 μmol of substrate/minute under standard conditions.

The glycosylasparaginase activity from tissue homogenates was measured by a high-performance liquid chromatography (HPLC) method as described previously (Kaartinen and Mononen 1990a; Kelo et al. 2005). The carboxymethyl cysteine (CmCys) was used as an internal standard. The samples were derivatized before they were analyzed on C_{18} reverse-phase column.

4.8.2 Aspartylglucosamine assay (I-IV)

The HPLC method using precolumn derivatization and CmCys as an internal standard were used for determination of aspartylglucosamine (GlcNAc-Asn) concentration in the cell (I, II) or tissue (III, IV) homogenates (Kaartinen and Mononen 1989; Kaartinen and Mononen 1990a; Kelo et al. 2005). The GlcNAc-Asn concentrations in urine samples collected on filter paper (III, IV) were determined as described previously (Kaartinen and Mononen 1990b).

4.8.3 Protein concentration (I-IV) and creatinine analysis (III, IV)

Protein concentrations from the purified enzyme preparations, the cell homogenates and the tissue homogenates were determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, California) according to the manufacturer's instructions. The creatinine analysis from the urine samples was performed in a Konelab 60i clinical chemistry analyzer (Thermo Electron Corporation) according to the kinetic Jaffe method.

4.8.4. In situ Y-chromosome hybridization assay (II)

The Onchor Rapid Chromosome *In Situ* Hybridization kit (Onchor, Inc., Gaithersburg, MD) with a fluorescein-labeled Y-chromosome specific DNA-probe was used according to the manufacturer's instructions for identifying the male AGU lymphocytes. 3 times 100 cells were counted on the fluorescence microscope to determine the percentage of the Y-chromosome- containing cells.

4.8.5. Immunofluorescence microscopy (I-II)

Lymphocytes attached to objective slides or fibroblasts grown in coverslips were fixed with methanol and permeabilized with Triton X-100 in PBS. The cells were blocked with FBS or FCS and incubated with hen S(156)-R(169) antibodies prepared as described by Kokko and Kärenlampi (Kokko and Kärenlampi, 1992). The rabbit anti-hen antibodies were used as a secondary antibody, and FITC-conjugated anti-rabbit antibodies were used for detection. The preparations were mounted with 50% glycerol in PBS containing 1% n-propylgallate.

4.9 HISTOLOGY

4.9.1 Electron microscopy (III)

The tissues were postfixed with 1% osmium tertoxide, dehydrated and embedded in LX-112 resin. Ultra-thin sections of the tissues were cut and double-stained with uranyl-asetate and lead citrate for transmission electron microscopy (Joel JEM-1200EX, Joel, Chicago, Ill).

4.9.2 Immunohistochemistry (III)

The tissues were postfixed in formalin for 48 hours, after which they were embedded in paraffin. 4 μm sections were immunostained with a polyclonal hen glycosylasparaginase S(156)-R(169) antibody (1:300) or a rabbit immunoserum (1:1000) using the avidin-biotin complex method according to the manufacturer's instructions (Vectastain Elite ABC Kit, Vector, Burlingame, California). Polyclonal anti-chicken IgG (Zymed, South San Francisco,

California) at a dilution of 1:200 was used as a secondary antibody, and sections were counterstained with hematoxylin.

5 Results

5.1 HUMAN RECOMBINANT OR LEUKOCYTE GLYCOSYLASPARAGINASE AND *in vitro* CORRECTION OF ASPARTYLGLUCOSAMINURIA (I, II)

The human recombinant glycosylasparaginase was purified from the cultured NIH-3T3 fibroblasts derived from the clone expressing the highest AGA (GA) activity (I). In this cell clone, the AGA activity was 120-fold higher than in the non-transfected normal NIH-3T3 fibroblasts, and the cells did not secrete measurable enzyme activity into the medium during the cell culture. In the final purification step of the enzyme by Mono-Q anion exchange chromatography, the enzyme eluted in two separate peaks based on charge heterogeneity (I). The pooled fractions in the first eluted peak were designated GA-1, and the second eluted fractions in a wider peak GA-2. The GA-1 preparation had a higher specific activity than GA-2 (I). The recombinant AGA consisted of a 24 kDa heavy chain and a 16 kDa light chain with a minor portion of a 19 kDa light chain, based on the SDS-PAGE analysis (I). The GA-2 preparation had a slightly larger portion of the 19 kDa chain than GA-1. Human leukocyte AGA eluted in one peak in the last purification step. In this leukocyte enzyme, the light chain band appeared at the size of 19 kDa and the minor band at 16 kDa on SDS-PAGE, suggesting the difference in the post-translational processing of the light chain of the human recombinant and leukocyte AGA.

The recombinant AGA was taken up by the AGU fibroblasts and lymphocytes (I). The endocytosis of GA-2 was more effective than that of GA-1. The normal glycosylasparaginase activity (average 20 μU/mg; range 17.5-24.5 μU/mg, n=8) was reached in EBV-transformed AGU lymphocytes within 24 hours from the beginning of the culture when the cell culture medium was supplemented of either with 300 mU/l of GA-1 or 10 mU/l of GA-2. The endocytosed AGA was localized in the lysosomes of treated cells, according immunofluorescence staining of the treated cells. The GlcNAc-Asn storage in AGU lymphocytes was on average 5.8 nmol/mg of protein. This storage was cleared under the detection limit (less than 0.3 nmol/mg of protein) after the cells were cultured with 1 mU of GA-1/l medium for 14 days or with 0.1 mU of GA-2/l medium for 3 days (*Table 5*). At these

time points glycosylasparaginase activity in the cells treated with the GA-1 preparation were 0.59 μU/mg of protein and 0.72 μU/mg of protein in the cells treated with the GA-2 preparation. These activities correspond to approximately 3-4% of the activity in normal transformed lymphocytes.

When the AGU lymphocytes were cultured with AGA purified from human leukocytes, the normal AGA activity was reached, and GlcNAc-Asn storage fell below the detection limit (0.3 nmol/mg cell protein) in the cells when they were cultured with 300 mU of human leukocyte AGA/l of culture medium for one day (II, *Table 5*). When the culture medium was supplemented with 10 mU of AGA/l of culture medium, the GlcNAc-Asn storage was cleared on day 7 and further accumulation was prevented in ten-day culture. The activity in the cells at that time point was 0.8 μU/mg protein, which corresponds to 2-7% of the mean activity in the normal lymphocytes (19.4 μU/mg; 8.1 -32 μU/mg). This effect of treatment is in the same range as with recombinant AGA (I). Based on immunofluorescence microscopy, leukocyte AGA has been suggested to localize in the lysosomes of the cells, like recombinant AGA (II).

Table 5. AGA activity in AGU lymphocytes during the treatment with recombinant GA-1 or GA-2, or the leukocyte AGA on the day when the GlcNAc-Asn concentration in the cells had dropped below the detection limit (0.3 nmol/mg cell protein). Bold text indicates the point where the normal AGA activity (the average of 20 μU/mg of protein) was reached in the cells with various enzyme preparations.

	AGA activity in the cell homogenate (μU/mg)/Day		
AGA supplementation (mU/ml)	**GA-1**	**GA-2**	**Leukocyte AGA**
0.1	-	0.72 on day 3	-
1	0.59 on day 14	1.49 on day 2	-
3	0.15 on day7	4.39 on day1	-
10	0.6 on day 2	**12.3 on day 1**	0.8 on day 7
100	3.2 on day 1	59.5 on day 1	8 on day 1
300	**15.6 on day 1**	nd	**17.7 on day 1**

The half-life of leukocyte enzyme in the cells was approximately one day and reaccumulation of the storage was detected four days after the end of the enzyme supplementation (1000 U/l for 9 hours) (II). At that time, the AGA activity in the cells had fallen below 0.5 μU /mg of protein.

Mannose 6-phosphate was demonstrated to be a competitive inhibitor in the uptake of AGA into fibroblasts (I). The inhibition constant K_i was 2×10^{-4} for GA-1 and 6×10^{-4} for GA-

2. When M6P residues of the enzyme were digested with alkaline phosphatase, or the sugar chains were removed by Endoglycosidase H digestion, the transfer of the enzyme was completely abolished or at least markedly decreased (*Table 6*).

Table 6. Effect of alkaline phosphatase (AFOS) or endoglycosidase H (Endo H) treatment on the uptake of recombinant glycosylasparaginase (AGA) into AGU fibroblasts. GA-1 and GA-2 preparations were studied independently and results are expressed as percentage of untreated GA preparations.

	Enzyme activity after treatment (%)		Uptake (%)/mg/h	
Treatment	**GA-1**	**GA-2**	**GA-1**	**GA-2**
None	100	100	100	100
AFOS	80	60	11	0
Endo H	88	84	0	4

5.2 CELL-TO-CELL TRANSFER OF GLYCOSYLASPARAGINASE (II)

When the normal and AGU lymphocytes were co-cultured, we found that the normal cells grew faster than the AGU cells. However, AGA was transported from the normal to the diseased cells in cell-to-cell contact and the transported enzyme was biologically active. The lysosomal storage was cleared below the detection limit (0.3 nmol/mg cell protein) when the proportion of normal cells was more than 60% in the cell culture containing normal and AGU lymphocytes (*Table 7*).

Table 7. *Co-cultivation of normal and AGU lymphocytes. The concentration of normal lymphocytes in the cell pool was detected by Y-chromosome in situ hybridization (n=1).*

Day	% of normal cells in cell culture on the day stated	GlcNAc-Asn concentration (% of concentration in untreated AGU cells)
1	80	0
2	65	0
8	65	0
10	60	16
1-10	0	100

In addition, the direct cell-to-cell transport of AGA was not inhibited by adding mannose 6-phospate into the cell culture medium (*Table 8*). If the cell-to-cell contact was prevented by a semipermeable membrane, no degradation of accumulated storage material was detected in the AGU cells (II).

Table 8. AGA activity in the cell pool during the co-cultivation of normal and AGU lymphocytes with or without addition of mannose-6-phosphate.

	AGA activity (% of activity in normal lymphocytes)	
Day	**Without M-6-P (n=1)**	**With M-6-P (n=1)**
0	23	23
2	39	28
4	48	46
6	119	107
8	90	105
10	102	105

5.3 ENZYME REPLACEMENT THERAPY OF ASPARTYLGLUCOSAMINURIA IN A MOUSE MODEL (III, IV)

Intravenously injected AGA was cleared from the systemic circulation of an adult mouse in two phases. Within the first phase at half-life of 4 min the enzyme was probably distributed into the vascular space or the extracellular fluids, whereas during the second phase at half-life of 39 min the enzyme was taken up by the tissues (III).

The main destinations of the AGA enzyme in a mouse body regardless of the age of the animal were liver and spleen tissues. The half-life of the enzyme in the liver of adult mice was approximately 1.9 days (III) and in newborn mice 1.2 days (IV). After one low-dose (0.34 U/kg) injection, the AGA activity in the liver of AGU (KO) mice was almost half of that in the liver of wild-type (WT) mice (*Table 9*). When the adult AGU animals had received eight injections at this dose, AGA activity comparable to the activity in the tissues of normal animals was detected in the liver and spleen (*Table 9*). When the dose was increased 10-fold to 3.4 U/kg, AGA activity in the adult animals increased above the normal activity not only in the liver and spleen but also in the heart and jejunum, and was close to normal in the kidneys and lung (*Table 9*). After a two-week therapy with this high enzyme dose (3.4 U/kg) in newborn AGU mice, AGA activities were 10-fold higher in the liver and 3-fold higher in the spleen than in the tissues of WT animals (*Table 9*). In the tissues of these animals, the kidneys were the only tissue besides the liver and spleen where enzyme activity was detected. When the dose was halved to 1.7 U/kg in terms of enzyme activity and the therapy was continued for four more weeks with a weekly injection, AGA activity was at the same level in the liver and spleen, and double in the kidneys, compared with newborn mice treated with a high dose enzyme (3.4 U/kg). Surprisingly, the activity was also detected in lung and heart tissues with the lower enzyme dose.

The endocytosed exogenous AGA in the tissues of the treated animals degraded the accumulated GlcNAc-Asn. The GlcNAc-Asn concentration was cleared from the liver in all treated AGU animals of all ages that received eight injections or more (*Table 9*). When the animals were treated with a high enzyme dose (3.4 U/kg), the GlcNAc-Asn concentration fell below the detection limit also in spleen, heart, jejunum and kidney tissues in adult animals, but not in newborns (*Table 9*). The spleen was the only tissue besides the liver in newborn animals where the GlcNAc-Asn accumulation was terminated by the therapy. The reduction of GlcNAc-Asn was more pronounced in the newborn animals treated with the short-term high-dose (3.4 U/kg) therapy than in those treated with half the dose for a longer time, even though the enzyme activities in the tissues of the animals in this group were higher. In the newborn animals, the GlcNAc-Asn concentration decreased more than 80% in all the somatic tissues examined in the group of animals treated with the high-dose, compared with a 55-80% decrease in the other group (*Table 9*). Interestingly, marked clearance of storage material was detected in all tissues in all groups even when no or only a trace amount of enzyme activity was detected. The results demonstrate complete clearance of the GlcNAc-Asn in the studied tissues of adult mice when AGA activity was above 66% of that in normal tissue. This might apply to newborn animals as well.

The intracellular vacuolization of the tissues of adult AGU animals was seen using electron microscopy (III). When these animals were treated with an enzyme injection of 3.4 U/kg for two weeks, the electron microscopic evaluation showed the clearance of intracellular storage vacuoles from the meseangial cells of renal glomeruli, the proximal and distal tubules and the collecting tubules of the kidneys, and from the sinusoidal lining cells and macrophages of spleen tissue (III). In the liver, which is the most affected organ in AGU, the treatment diminished the level of storage material in the storage vacuoles of the hepatocytes, the sinusoidal lining cells and the Kupffer cells, but did not completely normalize the histological appearance of the tissue (III). In addition, immunohistochemical staining of the treated tissues showed extensive and wide staining of the AGA enzyme in the sinusoidal lining cells and in the lymphoid cells in the spleen and in the macrophages of red pulp in the spleen (III).

Table 9. *The glycosylasparaginase and GlcNAc-Asn concentrations in tissues of adult and young mice after different therapy protocols presented as a percentage of AGA level in tissues of normal mice (WT) or GlcNAc-Asn in tissues of untreated AGU mice (KO).*

	ADULT						**NEWBORN**			
Dose	**1 x 0.34 U/kg (n=2)**		**8 x 0.34 U/kg (n=2)**		**8 x 3.4 U/kg (n=2)**		**8 x 3.4 U/kg (n=2)**		**5+4 x 1.7 U/kg (n=4)**	
Protocol	**1 injection**		**1 x every second day for 2 weeks**		**1 x every second day for 2 weeks**		**1 x every second day for 2 weeks**		**1 x every second day for 1 week + 4 x once a week**	
Injection route	**iv**		**iv**		**iv**		**ip**		**ip/iv**	
Tissue	**[GlcNAc-Asn] % of KO**	**[AGA] % of WT**	**[GlcNAc-Asn] % of KO**	**[AGA] % of WT**	**[GlcNAc-Asn] % of KO**	**[AGA] % of WT**	**[GlcNAc-Asn] % of KO**	**[AGA] % of WT**	**[GlcNAc-Asn] % of KO**	**[AGA] % of WT**
Liver	5.7	45	0	103	0	8548	0	999	0	730
Spleen	18	21	9.8	107	0	6647	0	279	2	366
Heart	84	1.4	35	0.3	0	249	15	0	30	1.9
Jejunum	46	1.9	20	0	0	200	-	-	-	-
Kidney	83	0	14	0	0	79	7	18	45	6.5
Lung	71	1.6	37	0.5	1	66	6	0	24	12
Brain	104	0	106	0	79	10	66	0	99	0

In addition to somatic tissues, the enzyme was internalized into the brain of the treated adult AGU mice. One tenth of the AGA activity in normal adult mice was achieved by eight 3.4 U/kg injections given every second day (*Table 9*). This enzyme activity yielded a 21% decrease in the GlcNAc-Asn concentration compared with the level of the substrate in untreated AGU mice. No enzyme activity was detected in the brains of three-week-old AGU mice treated with the same protocol, but a 34% reduction in the storage material was detected. Although enzyme activity was detected in the adult brain, it could not be localized in any particular cell type or structure by immunohistochemistry.

In AGU, the large amount of storage material is secreted into urine. The excretion of the GlcNAc-Asn into urine during the therapy of adult animals showed that the maximal therapeutic effect was achieved within the first 4-5 enzyme injections and the effect was sustained at the same level by further injections. The determination of the urinary GlcNAc-Asn showed dose dependence of the reduction of the excretion (*Figure 3*). The treatment of the adult AGU animals with eight 0.34 U/kg enzyme injections decreased the GlcNAc-Asn in urine by 58%. When the enzyme dose was increased 10-fold (8 x 3.4 U/kg), the amount of storage was reduced in adult animals by 91% and in newborn animals by 93% compared with the urinary concentrations in the untreated adult or newborn mice (*Figure 3*). After the newborn mice had been treated with a lower enzyme dose but for a longer period of time, the concentration of GlcNAc-Asn in the urine had decreased approximately to the same level as in adult animals treated with a low-enzyme dose (*Figure 3*).

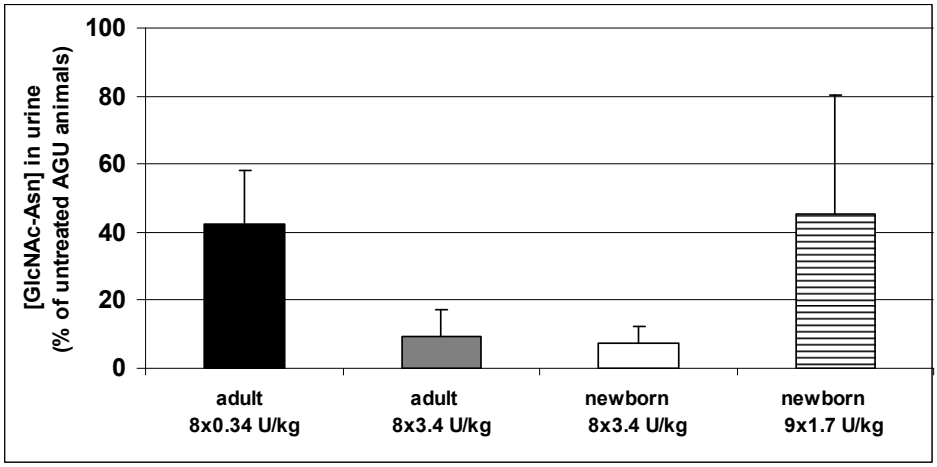

Figure 3. Urinary GlcNAc-Asn of treated AGU mice. The columns represent the concentration of GlcNAc-Asn in the urine of adult AGU mice treated with 8 x 0.34 U/kg (n=15) (■) and 8 x 3.4 U/kg (n=12)(■), and newborn mice treated with 8 x 3.4 U/kg (n=2) (□) or 9 x 1.7 U/kg (n=4) (≡). The bars represent the mean ±range from mean values.

5.4 EFFICACY OF EARLY INITATION OF ERT IN AGU (IV)

The GlcNAc-Asn concentration in urine as a proportion of creatinine or in most of the somatic tissues as a proportion of protein was higher in newborn animals than in adults. However, early initiation of the therapy made no difference to the effect of the therapy on lysosomal storage in somatic tissues (*Table 9*) nor to the overall therapeutic effect, based on the urinary excretion of accumulating storage material (*Figure 3*). In both adult and newborn mice, the effect of the therapy with the same treatment protocol was similar.

The efficacy of the therapy in the brains of newborn animals was more pronounced. Although the effect of the therapy was surprisingly high in the adult brain, the GlcNAc-Asn concentration decreased 14 % more in the brain of newborn animals with the same treatment protocol. In addition, when one-week-old AGU mice received two very high enzyme injections (17 U/kg) every second day, an 8-17 % increase in AGA activity and a 41% decrease in the GlcNAc-Asn concentration was detected in the brain tissue 12 hours after the second injection (*Figure 4*).

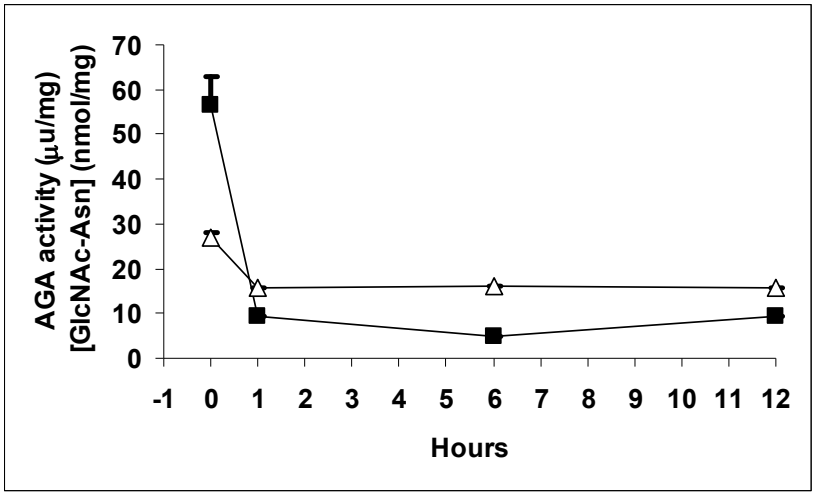

***Figure 5**. AGA activity (■) and concentration of GlcNAc-Asn (△) in the 9-day-old mouse brain treated with AGA (17 U/kg) 1, 6 and 12 hours after the second injection (n=1). The values at 0 hours represent the AGA activity in the brains of untreated WT mice (n=2, ± 1SD) and the concentration of GlcNAc-Asn in untreated AGU mice (n=2, ± 1SD).*

6 Discussion

Finding a treatment for the lysosomal storage diseases has become a goal of many research teams when the pathophysiology of the disease has been discovered. Although the diseases in the same subgroup share many common characteristics, they all have disease-specific factors that need to be understood and taken into consideration when searching for the therapy. When this study was launched, the basic knowledge of the cause of AGU disease and the technology to quantitatively measure the compounds of interest were already available. Encouraging results related to enzyme replacement therapy in some mucopolysacchoridoses and sphingolipidoses channeled the research interest towards ERT also in AGU. Finally, the development of DNA techniques provided a tool to produce a recombinant protein fundamental to the ERT. In the course of this study the development of a mouse model for AGU (Kaartinen et al. 1996; Gonzalez-Gomez et al. 1998) made it possible to study the ERT and the enzyme's efficacy in an animal model.

When producing recombinant proteins for ERT, a basic but challenging task is to achieve the correct post-translational processing of the recombinant enzyme that is crucial for its biological activity, for stability and for endocytosis into deficient cells and organs. This goal was reached with recombinant glycosylasparaginase in this work. The enzyme was biologically active and its properties resembled those of the enzyme purified from human leukocytes (Kaartinen et al. 1991). The occurrence of the recombinant AGA in two distinguishable peaks in the final purification step, and the difference in the uptake of these peaks into the cells, were presumably due to the differences in the post-translational processing of polypeptides (e.g. the degree of phosphorylation, position of M6P residues or sialylation of the oligosaccharide chains), leading to the slight charge difference between these two peaks. However, this processing should not affect the activity of AGA, as seen with the glycoasparagines purified from different human or animal sources (Tollersrud and Aronson, Jr. 1992). The slightly more charged preparation (GA-2) was more efficiently taken up by the cells than the less charged preparation (GA-1), but they both seemed to

represent high-uptake forms as the inhibition constants were 10-fold higher than reported in the high-uptake hydrolases (Kaplan et al. 1977; Sando and Neufeld 1977; Fischer et al. 1983). Both of these AGA preparations, like many other lysosomal enzymes, were transported into the cells by mannose 6-phosphate receptor-mediated endocytosis. The less charged peak, GA-1, was used in *in vivo* studies due to its higher specific activity. Being less charged, it was also hypothesized to be less immunogenic.

The intravenously injected AGA was cleared from the systemic circulation in two phases, like the enzyme in Hurler syndrome (Shull et al. 1994), and the half-life in the adults corresponded to the tissue half-lives reported with recombinant N-acetyl-galactosamine-4-sulfatase in Maroteaux-Lamy syndrome cats (Crawley et al. 1996) and β-D-glucuronidase in MPS VII mice (Vogler et al. 1993). In the cell culture experiments, approximately 5% of the normal AGA activity reached in the AGU cells was enough to reverse the pathology. Obviously, a mouse and a human are more complicated systems and this might not be enough for the whole organism. Indeed, 66% of the normal activity was needed in the lung tissue of adult animal to completely clear the GlcNAc-Asn storage. On the other hand, a 94% decrease in storage was demonstrated in the lung of 2-week-old mice even when no AGA activity was detected. Moreover, a 12% increase in AGA activity in the lung of 7-week-old AGU mice led to a 76% decrease in GlcNAc-Asn storage. Despite the clearance of the storage in the lysosomes of different tissues, histological changes occur more slowly than these biochemical changes. Although the storage was cleared, the empty storage vacuoles were still present to a certain extent in many of the tissues studied. This emphasizes the fact that therapy should be initiated as early as possible to minimize the irreversible changes in tissue pathology prior to the therapy. However, the AGA activities in the tissues of the treated adult and newborn AGU mice were not perfectly comparable, due to differences in the half-life of the enzyme and to the same timing of the sample collection used in the studies. To get comparable results, the samples should probably have been collected from the newborn animals sooner after the last injection. These age- related differences in the catabolism of the enzyme should have been more carefully addressed before the planning of the experiments.

Resnick et al. have shown that urinary excretion of glycosaminoglycans in different mucopolysaccharidoses correlated with cellular levels of this enzyme (Resnick et al. 1992). In AGU, a large amount of storage material is secreted into the urine, and this is used to

diagnose the disease. Hence, the urinary level of GlcNAc-Asn could be used as a convenient and reliable means for the estimation of the overall therapeutic effect. Indeed, GlcNAc-Asn secretion into urine was at the same level with the same treatment protocols regardless of the age of the animal, based on the decrease in the GlcNAc-Asn in the urine of treated animals compared with untreated mice at the same age, and it also showed clear dose dependence.

The transport of lysosomal enzymes through the adult BBB is controversial. In many disorders no enzyme activity, or a decrease in the storage material, have been detected in adult brains after ERT (Van der Ploeg et al. 1991; Shull et al. 1994; Crawley et al. 1996), but transport has been shown to occur in some disorders such as α-mannosidosis, metachromatic leukodystrophy and MPS VII (Roces et al. 2004; Matzner et al. 2005; Vogler et al. 2005). Interestingly, the 10% increase in AGA activity and 21% decrease in the storage in the brain of adult AGU mice are surprisingly high compared with the results of MPS VII or MDL. Moreover, AGA was presumably distributed quite diversely into the adult brain, since it was not detected by immunohistochemistry. The therapeutic effect in the brain of α-mannosidosis mice was comparable to that of AGU. Both α-mannosidosis and aspartylglycosaminuria are glycoproteinoses, and the higher therapeutic effect in the brains of mice suffering from these diseases might be related to the type of basic metabolic defect. On the other hand, Enns and Huhn have suggested that different recombinant enzymes might have a different capacity to diffuse into the brain (Enns and Huhn 2008), and Blanz and coworkers has proposed an alternative transfer route for a-mannosidase (Blanz et al. 2008). However, the recombinant AGA used in our study might be one of the enzyme preparations that are able to cross the BBB more readily than others.

In the brains of newborn AGU mice treated with the same protocol and enzyme dose as adult animals, and which received a few enzyme injections prior to the full developmental maturation of the BBB, an over 30% decrease in lysosomal storage was found. The studies done with newborn mice with MPS VII, MPS III or globoid cell leukodystrophy have demonstrated similar results (Sands et al. 1994; Gliddon and Hopwood 2004; Lee et al. 2005) suggesting the importance of starting the therapy early. The changes in the skeleton and in mental function are irreversible and should be prevented by starting the therapy early before the symptoms appear. In addition, autophagosytosis might be higher in advanced diseases, diminishing the effect of ERT, as shown in mouse

Pompe disease (Fukuda et al. 2006). The effect of ERT in the brains of newborn AGU mice was marked, but a longer study is needed to determine the effect of the therapy on the mental function of the treated mice. Furthermore, to enhance the effect of the therapy in the brain, the enzyme should be transported into the brain to an even greater extent. The current research in ERT of LSDs has already moved in this direction, and the uptake of exogenous enzymes has been improved by adding M6P residues to the enzyme (Zhu et al. 2004) or by trimming them, for example (Grubb et al. 2008). Moreover, a new approach for the targeted delivery of the exogenous enzyme is to link it to a fusion protein domain or even the BBB receptor-specific monoclonal antibody, which may then act as an aid in penetrating cell membranes (Wadia and Dowdy 2005; Zhang and Pardridge 2005; Cai et al. 2006; Eavri and Lorberboum-Galski 2007). Finally, growth factors have been used for opening the newborn BBB prior to ERT (Young et al. 2004). This step looks promising and would be worth exploring in AGU as well.

The transport of certain lysosomal enzymes from normal lymphocytes into enzyme-deficient cells has been shown to occur in cell-to-cell contact (Olsen et al. 1981; Olsen et al. 1983; Abraham et al. 1985). These transported enzymes have been biologically active in recipient cells. Enomaa and co-workers showed that GlcNAc-Asn was secreted into cell culture medium, from where it was taken up by cells (Enomaa et al. 1995). Different cell types might act differently in terms of enzyme secretion. We found that in addition to the M6P-mediated uptake of exogenously supplied AGA, the enzyme was transported from normal to diseased cells in cell-to-cell contact which did not utilize M6P receptor mediated endocytosis. The precise transfer mechanism of this is not clear, but it has been suggested that cell-to-cell contact might induce the formation of new lysosomal precursors, which are then transported directly into the recipient cells (Abraham et al. 1985; Olsen et al. 1988; Bou-Gharios et al. 1993b; Olsen et al.1993). This combined evidence shows that AGA may utilize several different transport systems in endocytosis into cells and lysosomes. These may play a role in the ERT in terms of breaking the BBB, as in the M6P-independent transfer of modified β-glucuronidase in the murine brain (Grubb et al. 2008). Furthermore, both delivery systems might be utilized in ERT or even in stem cell transplantation, where normal stem cells might theoretically differentiate into various cell types depending on the microenvironment of the transplant. These cells could then either release corrective enzyme into circulation for M6P- dependent uptake or they could correct neighboring cells by

contact-mediated transfer of the enzyme. Our study was too short to evaluate the possible effect of this contact-mediated correction after ERT.

The findings related to the treatment of AGU and other lysosomal storage disorders give affected patients and their families hope. However, there are also many practical and ethical questions to be considered and solved in parallel with scientific research. For example, is ERT in its present form suitable for life-long therapy? It is quite cumbersome with regular injections, with no common recommendation concerning dosing schedules or doses, and with individual-specific differences in the response to the therapy (Kishnani et al. 2007). Moreover, it lasts for a lifetime, so the cost of the therapy may become huge. The annual cost of treatment of one Gaucher, Fabry, MPS I or MPS VI patient weighing 50 kg varies between $145,000 and $377,000 (€112,000-290,000) (Beutler 2006). Therefore, even if the recombinant AGA produced in this study proved to be excellent for the therapy, it might not be optimal for largescale production in terms of costs of the purification. Moreover, what are the costs versus the advantages in terms of the society and the patients, and would the therapy be available to every patient who would benefit from it? The high costs might even have effects on the social security and health insurance systems in many countries in the long run. Health-care systems are not yet ready for expenditure on items such as this. Furthermore, what are the opinions of the families and patients towards the therapy? Opinions might vary depending on the severity of the disease, which also has to be taken into consideration (Coman et al. 2008). However, disease-specific treatment may improve the quality of life of the patients dramatically even if not all symptoms are cured (Sifuentes et al. 2007). It should be remembered that there is a long way to go from preliminary studies to the point where actual treatment can be offered, and that so far ERT does not provide a complete cure of any lysosomal disorder. The issues raised above need to be considered in relation to AGU as well.

The milestones in AGU research during the past decade are gathered up in *Table 10*. Moreover, the results described in this study illuminate the disease-specific mechanisms related to ERT in AGU in more detail. In addition, these results also contribute to research in other glycoproteinosis and lysosomal storage disorders. However, there is still a lot to be done in developing a treatment for AGU. For example, the right dose, the timing of the injections and the mechanism of enzyme transfer into the brain have to be explored more thoughly. Furthermore, ERT should also be followed up for several years to establish its

effectiveness in AGU. The long experience of ERT in Gaucher disease has shown this therapeutic approach to be safe and efficacous in the therapy of non-neuronal tissues (Starzyk et al. 2007), and we might speculate a similar outcome in AGU in terms of safety.

Table 10. Milestones in AGU research from AGU patients', their families' and clinicians' point of view from the time this work was initiated to the present day. The data were collected from the references mentioned in Chapter 2 and from the publications presented in this thesis. The bold text refers to results of the present study.

	Milestones in research related to AGU	**AGU patient and family**	**Clinician**
1995	- Screening of the AGU_{Fin} mutation is offered to pregnant women in Finland. - Three-dimensional structure of AGA is generated. - **Recombinant AGA is generated.** - **M6P-mediated transfer of AGA is detected.** - ***In vitro* ERT of AGU by recombinant AGA is investigated.** - *In vitro* gene therapy of AGU is studied.	- Parents will have to consider ethical questions, and knowledge can cause distress. - Parents may have better choice for family planning.	- Professional skills for meeting couples planning a family are emphasized. - Many ethical questions need to be considered also in terms of cost of testing. - Hope for specific treatment is raised.
1996-1999	- AGA is found to be Ntn hydrolase. - Activation of AGA is presented. - A mouse model for AGU is generated. - BMT in human AGU is done. - M6P-independent transport of AGA is detected. - Murine brain cells are found to internalize AGA by M6P. - Early initiation of murine BMT is shown to reduce vacuolization in the brain.	- Hopes for treatment are raised.	- A new treatment option emerges.
2000	- **ERT in mouse model of AGU is studied.**	- Hopes for getting a similar therapy as in Gaucher disease are raised.	
2001	- BMT is not recommended as a treatment if it is performed after infancy. - **M6P-mediated transfer of AGA in cell-to-cell contact is demonstrated.**	- Disappointment with BMT as a treating option may occur.	- Genetic counseling and carrier detection becomes more important. - Possibility of BMTneeds to be estimated as a treatment option.
2003	- Glial cell are shown to be important in gene therapy.		
2004	- BMT is applied to young AGU mice.		
2006	- Non-viral promoters in gene therapy of murine AGU are studied.		
2007	- A more precise mechanism of AGA activation is presented.		
2008	- T2 signal of thalami is detected in AGU patient.		
2009-2010	- **ERT of young AGU mice is demonstrated to be effective in treatment of storage in the brain.**	- Frustration with limited therapeutic options in spite of hope can occur.	

7 Conclusions

The main aim of the present work was to evaluate the applicability of enzyme replacement therapy in aspartylglycosaminuria. This included the production of recombinant glycosylasparaginase, and the determination of its efficacy, properties and transfer mechanisms *in vitro*. The efficacy of the recombinant enzyme in correcting the disease in a mouse model of AGU was studied. The following goals were achieved:

1. Human recombinant AGA was stably over-expressed and correctly post-translationally processed in NIH-3T3 fibroblasts. This recombinant enzyme resembled human leukocyte AGA, was unlimited in availability, and efficiently corrected the enzyme deficiency and lysosomal storage in AGU lymphocytes and fibroblasts, where it was taken up by the mannose 6-phosphate receptor-mediated pathway.

2. Human leukocyte AGA had the same corrective properties as human recombinant AGA. The transfer of the AGA enzyme from normal to diseased cells occurred through cell-to-cell contact, which did not utilize M6P receptors. Thus human AGA can be transferred either with or without M6P-mediated transfer.

3. The recombinant AGA was biologically active in the animal model. The response to the therapy was dose-dependent. 10 mg of AGA /kg of animal weight (3.4U/kg) was enough to inhibit the substrate accumulation in many somatic tissues including the liver and spleen, which were the main targets for the exogenous enzyme. When AGA activity was 70% of normal activity in the tissues, the substrate storage fell below the detection limit. In addition to the disappearance of storage material, the number of storage vacuoles decreased, even though they did not vanish, in many somatic tissues. The quantitative analysis of urinary GlcNAc-Asn could be used for the follow-up of the treatment effect. The recombinant AGA crossed the adult BBB, and a 10% increase in AGA activity and 21% decrease in GlcNAc-Asn storage in brain tissue was detected.

4. With the same protocol, the overall therapeutic effect was in the same range in newborn animals and in adults. When the therapy was initiated before the full development of the BBB, the effect was more marked, with a 30-40% decrease in GlcNAc-Asn storage in brain. The results with newborn mice emphasize the importance of early initiation of the therapy.

The thesis presents results of the first studies concerning ERT in AGU. The results illuminate the disease-specific factors involved in the ERT therapy for AGU, and will form a basis for further development of treatment protocols alone or in combination with other therapy approaches for this disease. The findings also contribute to research in other LSDs with a similar cause.

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Ulla Dunder

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Aspartylglucosaminuria (AGU) is an inherited lysosomal storage disease. Deficiency of enzyme called glycosylasparaginase results in an accumulation of glycopeptides in cells leading to a multisystem disease. There is no disease-specific treatment for AGU. In this study, a treatment approach called enzyme replacement therapy (ERT) was applied to AGU. The results reveal disease-specific details involved in ERT of AGU and form a basis for further development of treatment protocols.

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