G_{M1} Gangliosidosis and Galactosialidosis

Pathology and Therapy

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Proefschrift

Ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus
Prof. dr. ir. J.H. van Bemmel
en volgens besluit van het College voor Promoties

De openbare verdediging zal plaats vinden op
June 11, 2003

door

Maria del Pilar Martin

geboren te Puerto Rico

PROMOTIECOMMISSIE:

Promotor: Prof. Dr H. Galjaard

Overige leden: Prof. Dr. R.J.A. Wanders
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The studies described in this thesis were performed from 1995-2000 in the Department of Genetics, St. Jude Children's Research Hospital, Memphis, Tennessee, USA, as a continuation of the long term collaboration in lysosomal storage disorders research initiated at Erasmus University in Rotterdam, The Netherlands. This research is supported in part by the National Institutes of Health grant RO1-GM60950, the Assisi foundation of Memphis, the Cancer Center Support Grant (CA 21765), and the American Lebanese Syrian Associated Charities (ALSAC) of St. Jude Children's Research Hospital.

CONTENTS

Scope of this thesis

GM_1 gangliosidosis and galactosialidosis belong to the large group of metabolic disorders collectively known as lysosomal storage diseases (LSDs). LSDs are caused by a single or combined deficiency of specific hydrolytic enzymes resulting in progressive accumulation of undigested substrates in lysosomes of many cells, and ultimately in cellular and organ dysfunction. In general, the patients exhibit multi-systemic symptoms mostly involving severe neurological manifestations. The severity and onset of the symptoms range from neonatal to adulthood and often correlate with the level of residual enzyme activity. Phenotypic variations are particularly evident in the broad range of neurological symptoms that include mental retardation, motor dysfunction, sensory deficits, and seizures.

Although some clinical manifestations are common in different LSDs it is becoming increasingly clear that at the cellular level each individual disease displays a distinct and characteristic pattern of morphological changes and extent of cellular dysfunction. The pathogenesis of each disease is, therefore, determined by the enzyme function in different cell types and in turn by the type and amount of accumulated products.

The main objective of the present study was to gain a better understanding of the molecular bases of GM_1-gangliosidosis and galactosialidosis and to use genetic approaches as curative therapy for murine galactosialidosis. The detailed characterization of the two mouse models of these two diseases has provided new insights into the molecular and cellular mechanisms that underlie neuronal degeneration in GM_1 gangliosidosis and has given the bases for implementing efficient strategies for the cure of galactosialidosis.

General Introduction

GENERAL INTRODUCTION

Lysosomal Biogenesis

The major site of compartmentalized degradation of macromolecules is the lysosomal system, a heterogeneous population of organelles surrounded by a single membrane and present in virtually all animal cells, with the exception of mature erythrocytes (Lloyd, 1996). The intralysosomal pH is acidic and is maintained as such by an H^+-ATPase pump, which is essential for the organelle function. With their content of hydrolytic enzymes lysosomes maintain cellular homeostasis by regulating the turnover of many cellular constituents: proteins, glycoproteins, nucleic acids, polysaccharides and glyco-, phospho- and neutral lipids are their natural substrates (Mortimore, 1996). Extracellular macromolecules destined for degradation reach the lysosomes via either selective, receptor-mediated transport or non-selective phagocytosis. Intracellularly, micro- and macroautophagy account for the majority of the breakdown of cytosolic and membrane components. However, approximately 30% of cytosolic proteins can be selectively degraded in lysosomes by chaperon-mediated autophagy or CMA (Cuervo and Dice, 1998; Cuervo *et al.*, 2000). CMA is mainly activated in response to stresses such as nutrient deprivation (Auteri *et al.*, 1983) or exposure to some toxic compounds (Cuervo *et al.*, 1999). Finally, it has been demonstrated in both yeast and mammalian cells that mono-ubiquitination of integral membrane proteins target them for degradation by the lysosome (del Pozo and Estelle, 2000). These findings link the ubiquitin-pathway of proteasome-mediated degradation to the lysosomal system.

Biosynthesis of lysosomal enzymes begins like that of secretory proteins. They are synthesized on membrane bound polysomes and translocated into the lumen of the endoplasmic reticulum (ER) by a conventional signal sequence. Inside the ER, the enzyme precursor is glycosylated co- or posttranslationally at specific asparagine residues; processing of the oligosaccharide side chains begins at this site and ensures exit of the precursor from the ER (Braukle, 1996; Sabatini, 2001). These combined events, occurring during early biosynthesis, are accompanied and /or followed by the folding of the precursor polypeptide and assembly of multicomponent or multisubunit proteins, both processes are likely to be mediated by specialized proteins, called molecular chaperones.

These constitutively expressed ER resident proteins recognize and bind to partially unfolded polypeptides, thereby promoting a proper conformation and preventing their aggregation or premature release from the ER. The ER stress-signaling cascade is also designated the Unfolded Protein Response (UPR) because it was first identified in association to the accumulation of misfolded proteins in the ER (Kaufman, 1999; Ma and Hendershot, 2001). It is now established that a variety of stress-stimuli, e.g. oxidative stress, chemical toxicity, calcium imbalance and inhibitors of glycosylation, can activate this pathway.

It has been further demonstrated that promoters of genes encoding molecular chaperones and several transcription factors contain consensus sequences, named ER stress elements (ERSE), that contribute to their transcriptional regulation under stress conditions (Kaufman, 1999; Ma and Hendershot, 2001). The first characterized molecular chaperone was the 78-kD glucose-regulated protein GRP78, also referred to as BiP because its ability to bind non secreted immunoglobulin heavy chains and incompletely assembled immunoglobulin intermediates. It was subsequently demonstrated that BiP binds to many different unfolded proteins in the ER, retaining them in this compartment. This ER 'quality control' prevents unassembled, denatured, mutated or misfolded proteins to move further down the secretory pathway. In turn, any alterations of the ER environment caused

by a variety of pathological or physiological conditions may affect protein folding and lead to the induction of BiP and other molecular chaperones at both transcriptional and translational level (Gottesman *et al.*, 1997; Kaufman, 1999; Travers *et al.*, 2000). The cascade of events that follows the response of the cell to different insults is presented in Chapters 2 and 4.

Once transported out of the ER, enzyme precursors reach the lysosome by going across the secretory pathway to the trans Golgi network (TGN) and the endosomal compartment. This tightly regulated process entails further processing of the sugar chains, and the synthesis of the mannose-6-phosphate (M6P) recognition marker, a sorting signal, which allows lysosomal precursors to be segregated from the bulk of secretory proteins (Braukle, 1996; Kornfeld, 1992) (Figure 1). This post-translational modification is carried out in two sequential reactions catalyzed by a phosphotransferase (N-acetylglucosamine-1-phosphotransferase) and a phosphodiesterase (Sabatini, 2001). At the TGN lysosomal precursors with a fully modified M6P recognition marker are recognized by and bind to the M6P receptor and are selectively transported to the endosome/intermediate compartment and eventually the lysosome.

This general mechanism of lysosomal compartmentalization is applicable to most soluble lysosomal precursors, although some lysosomal proteins can apparently reach the lysosome in an M6P-independent manner (Braukle, 1996). In addition, integral membrane proteins, which have a transmembrane domain, use a specific lysosomal targeting motif contained within their cytoplasmic tail (Hunziker and Geuze, 1996). There are also lysosomal enzymes that are unable to efficiently reach the lysosome without the aid of auxiliary (transport) proteins with which they associate. Examples of the latter proteins are lysosomal neuraminidase (sialidase) and β-galactosidase that are routed to the lysosome via their interaction with the carboxypeptidase protective protein/cathepsin A (PPCA). The complex of these three proteins and its involvement in the LSD galactosialidosis are presented in Chapter 2.

In the endosomal/lysosomal compartment the pH is low enough for dissociation of the ligand from the M6P receptor, which recycles back to the Golgi complex. The next step is the formation of a fully active enzyme. Many lysosomal proteins undergo discrete proteolytic processing or maturation prior to or upon arrival in lysosomes. This final process may serve different functions: it may activate a catalytically inactive precursor, zymogen, like in the case of lysosomal proteases; it may render an already active precursor stable in the lysosomal acidic environment; it may alter the kinetics of the enzyme and its affinity to the substrates (Braukle, 1996).

By default, a small percentage of the precursor proteins can also be recovered in the extracellular milieu, but these secreted forms retain the capacity to be re-internalized via receptor-mediated endocytosis, and to be efficiently targeted to the lysosome through the endocytic pathway (Fratantoni *et al.*, 1968a; Fratantoni *et al.*, 1968b; Neufeld, 1991; Neufeld and Fratantoni, 1970; Sabatini, 2001). This unique feature of lysosomal enzyme precursors is at the basis of many of the therapeutic approaches that have been and are sought for LSDs (see Chapter 7).

In general, lysosomal enzymes are specific with respect to the chemical linkage and structure of the monomeric unit that they hydrolyze. This specificity is reflected in the wide range of glycosidases that represent, together with the proteases, one of the largest classes of lysosomal enzymes. Glycosidases bring about the catabolism of polysaccharides, like glycogen, as well as sugar moieties on glycoconjugates, such as glycoproteins, proteoglycans and glycoshingolipids, in a step-wise and concerted fashion. For the hydrolysis of carbohydrate moieties that are linked to glycosphingolipids the action of auxiliary non-enzymatic factors (activators) is required. Four Sphingolipid Activator Proteins (Sap or saposin A, B, C, D) and the GM2-activator act as natural detergents on

water insoluble substrates, such as glycosphingolipids. Sap A-D are encoded by a single gene and are generated from a common precursor (prosaposin) by proteolytivc processing. These activator proteins interact with either sphingolipid substrates or phospholipid-containing membranes, and thereby render the substrates accessible to the enzymes. GM2-activator also binds to the β-hexosaminidase A, the enzyme that hydrolyzes GM2-ganglioside (Gravel, 2001; Schuette *et al.*, 2001)

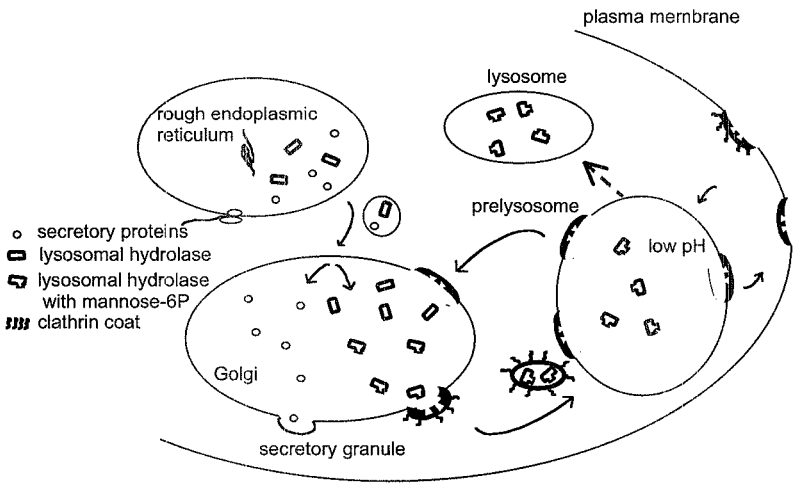

Fig. 1. Biogenesis of the lysosomes. Lysosomal hydrolases are synthesized by membrane-bound ribosomes in the endoplasmic reticulum (ER). Similarly, in the ER, secretory glycoproteins and lysosomal proteins acquired mannose oligosaccharides chains and processed cleavage of their signal sequence. Subsequently, lysosomal polypeptides undergo modifications that confer a sorting signal, mannose 6-phosphate (M6P) residue. The M6P distinguishes lysosomal proteins from proteins with other subcellular destinations and it is responsible for addressing the former to the lysosomal compartement. Transport through the endosomal compartment is accomplished by the presence of Man-6-P receptors in this compartement. The Man-6-P receptor is concentrated in clathrin coated pits, which bud off into the cytoplasm to form a coated vesicle. This vesicle then fuses with the endosomal membrane and in acidic conditions the ligand is release from the receptor into the lumen. The final step in the conversion of a prelysosome to a mature lysosome is the return of the Man-6-P receptor.

Lysosomal Storage Disorders

If any one of the lysosomal enzymes is deficient or defective, the process of substrate degradation is halted at the level of the missing enzyme. This block leads first to the progressive intralysosomal accumulation of partially degraded metabolites, and subsequently to the cellular and organ dysfunction associated with a lysosomal disorder (Neufeld, 1991; Sabatini, 2001). Most of the genetic lesions associated with LSDs lead to faulty catalytic activity of the enzyme in lysosomes or hamper the posttranslational processing and lysosomal compartmentalization of the enzyme precursor. In addition,

defects in proteins that are essential for lysosomal biogenesis, such as enzyme-activator or substrate-activator proteins, protective protein, and enzyme modifiers (Hopwood, 2001; Sandhoff, 2001; von Figura *et al.*, 1998), will also result in impaired lysosomal function and will clinically manifest as a lysosomal disorder.

LSDs comprise a group of over 40 monogenic disorders of metabolism most of which are autosomal recessive with the exception of Fabry disease and Mucopolisaccharidosis (MPS) type II (MPS II or Hunter disease), which are X-linked (Meikle *et al.*, 1999a). Individually LSDs are considered as rare genetic disorders. However, as a group their estimated incidence is about 1 in 5000 live births (Hopwood, 2001; Meikle *et al.*, 1999a; Meikle *et al.*, 1999b; Sandhoff, 2001; von Figura *et al.*, 1998). Examples of a 'founder effect' are also found among LSDs: Gaucher disease and Tay-Sachs disease have an incidence of 1 in 600 and 1 in 3900, respectively, among Ashkenazi Jews (Beutler, 2001; Gravel, 2001; Meikle *et al.*, 1999a; Meikle *et al.*, 1999b). Aspartilglucosaminuria occurs primarily in the Finish population, where 95% of the patients carry the same point mutation (Aula, 2001). In general, the characterization of the genes encoding lysosomal enzymes, and the more thorough understanding of enzyme function(s) and mechanism(s) of pathology have improved the laboratory diagnosis and increased the number of reported cases with LSDs.

With few exceptions, these disorders are caused by either single or multiple deficiency of glycosidases, and are classified, according to the type and site of the primary accumulated products, as mucopolysaccharidoses (MPS), mucolipidoses, glycoproteinoses, sphingolipidoses and others. In general, LSDs are complex, systemic diseases that affect to different extent, depending on the missing enzyme, the visceral organs, the musculature, the bones and cartilage and most importantly the nervous systems (Neufeld, 1991; Scriver *et al.*, 2001). Common features include severe psychomotor delay, visceromegaly, growth retardation and early death. Variations in disease penetrance have been documented, that might reflect differences in the metabolic needs of individual cell types and lysosomal enzyme cell type specific function. Both severe, early onset forms and milder late onset variants are distinguished in most of the LSDs. This clinical heterogeneity often correlates with the residual amount of functional enzyme in lysosomes, implying that even modest increases in enzyme activity, if they occur early in life and at the correct cellular site, might prevent/cure the disease.

Although the pathological hallmark of LSDs in tissues is the presence of vacuolated cells, the cellular and molecular consequences of the intralysosomal accumulation of various metabolites are largely unknown. The large number of genetically engineered mouse models of LSDs that are currently available combined with spontaneously occurring animal models, are instrumental in understanding these aspects. In fact, studies have now began to emerge that will help to relate storage of potentially toxic metabolites to cell dysfunction and cell death (Im *et al.*, 2001; Kanazawa *et al.*, 2000).

Upregulated expression of markers known to be involved in inflammatory responses has been observed in both G_{M1}- and G_{M2}-gangliosidoses animal (Myerowitz *et al.*, 2002; Wada *et al.*, 2000). These authors have postulated that an inflammatory response may precede the development of neurological symptoms, suggesting either a direct or indirect involvement of this process in neurodegeneration of these diseases. The important contribution of factors secondary to the lysosomal deficiency to the pathogenesis is clearly exemplified in globoid cell leukodystrophy. The interaction of psychosine, a by-product of glycosphingolipid metabolism with its recently found ligand, the G protein–coupled receptor named T cell death associated gene 8, revealed an unexpected connection between lipid mediators and the formation of globoid cells due to impaired cytokinesis (Im *et al.*, 2001; Kanazawa *et al.*, 2000). In Chapter 2 we introduce another example of a possible mechanism(s) underlying neurodegeneration in LSDs: the activation of the ER stress

response. The involvement of this pathway in G_{M1}-gangliosidosis mice provides a surprising link between abnormal lysosomal accumulation and the 'quality control' in the ER. Our results suggest the existence of biological mechanism at the level of the ER that sense the improper function of the lysosomal degradative machinery and activate a cellular response that will lead to either cell survival or cell death. The elucidation of the molecular mechanism underlying cell degeneration will not only provide new insights in lysosomal biological properties and functions, but will also have impact on the development of efficient therapeutic strategies.

Because this thesis focuses on the mouse models of galactosialidosis (GS) and G_{M1}-gangliosidosis (GM1) I will briefly introduce these two lysosomal disorders. GS is a glycoproteinosis caused by mutations in the serine carboxypeptidase protective protein/cathepsin A (PPCA) leading to a combined, secondary deficiency of β-galactosidase (β-gal) and neuraminidase (Neur). PPCA has at least two distinct functions: as protective protein, it associates with the two glycosidases to ensure their correct intralysosomal targeting, catalytic activation, and stability; as a carboxypeptidase, it mediates the in vitro catabolism of a selected number of bioactive neuropeptides, including endothelin I, substance P, and oxytocin (Jackman *et al.*, 1992; Jackman *et al.*, 1990). Although increased levels of endothelin I in specific areas of the brain of patients with GS have been reported, the physiologic significance of the carboxypeptidase activity remains elusive (Itoh *et al.*, 2000). Despite the triple enzyme deficiency in GS, most of the clinical abnormalities seem to be associated primarily with the severe Neur deficiency. Sialylated oligosaccharides and glycopeptides accumulate in affected tissues and body fluids (d'Azzo *et al.*, 2001). Pathologic changes occur in some cells of most organs, including brain, kidney, retina, and skin, and in the hematopoietic system (Claeys *et al.*, 1999; Nordborg *et al.*, 1997; Tekinalp *et al.*, 1999; Usui *et al.*, 1991). Clinical phenotypes range from severe, neurodegenerative disease, which is fatal in early infancy because of accompanying renal and cardiac failure, to mild disease that is nonneuropathic and has a late onset (d'Azzo *et al*, 2001).

GM1 is a glycolipidosis caused by lysosomal β-gal deficiency. This severe neurosomatic disease occurs mainly in infants, but a milder form of the disease, which is associated with a longer survival time, is also diagnosed in adolescents and adults (Callahan, 1999; Chen *et al.*, 1998; Folkerth *et al.*, 2000; Hirayama *et al.*, 1997). The severe form of GM1 is characterized primarily by growth retardation, progressive neurologic deterioration due to extensive brain atrophy, visceromegaly, and skeletal dysplasia. Abnormal amounts of G_{M1}-ganglioside and, to a lesser extent, its asialo-derivative (G_{A1}) accumulate in the brain, and oligosaccharides derived from glycoproteins and keratan sulfate are excreted in the urine (Susuki, 2001). The identification of several mutations underlying different clinical phenotypes of GS and GM1 has enabled us and others to establish a reasonably close correlation between genotype and phenotype (d'Azzo *et al.*, 2001; Richard *et al.*, 1998; Silva *et al.*, 1999; Susuki Y., 2001).

Mouse Models as Experimental System

Animal models represent a powerful tool to assess in vivo the biological function of a lysosomal protein in normal and disease conditions. Spontaneously occurring mutations that closely resemble LSDs have been reported in several other species (Scriver *et al.*, 2001). However, the limitation of the use of naturally occurring animal models has been their limited availability, difficult and costly maintenance, and in some cases insufficient resemblance to the corresponding human disease.

In contrast, the genetically modified laboratory mouse offers multiple advantages as an experimental system. They are small and relatively inexpensive to maintain, have a short life span and gestation period, and produce abundant offspring, allowing the timely generation of large experimental groups for analysis. In addition, creation of inbreed strains of mice is feasible eliminating the variability of a spurious genetic background, thereby facilitating the interpretation of the results. In general, the mouse shares biochemical pathways and developmental stages with larger mammals including humans, and its genomic organization is relatively conserved compared to humans (Sabatini, 2001). Because of this similarity, the generation of mutant mouse strains by gene targeting technology in embryonic stem (ES) cells has contributed greatly to the understanding of biochemical pathways, protein function, and pathological mechanisms of disorders observed in humans (Elsea and Lucas, 2002; Sabatini, 2001).

This methodology allows the direct manipulation of the genes of interest by the introduction of all types of allelic mutations, including loss of function, dominant negative, or gain of function (Ramirez-Solis *et al.*, 1995; Zheng *et al.*, 2001) via homologous recombination in ES cells. These cells are screened in vitro by the combined use of positive and negative selection systems that allow the discrimination between random integration and target recombination (Figure 2). Homologous recombinant ES clones are microinjected into recipient blastocysts, which are subsequently transferred into pseudopregnant female mice. The resulting embryo is chimeric, containing cell lineages derived from injected and blastocyst derived cells. Chimeras that carry the mutant allele in the germ line will transmit it to the offspring in a Mendelian ratio. Coat color of the offspring is usually indicative of germ line transmission. For example, a commonly used strain of ES cells is 129/sv with an agouti coat color. The recipient blastocysts are usually derived from the C57BL/6 strain, which carries recessive black coat color. As a result of the microinjection half of the agouti pups will be mutants (Sabatini, 2001).

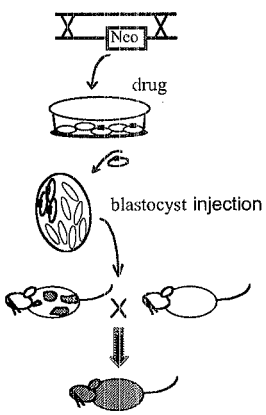

Fig. 2. Generation of knockout mice. Cultured embryonic stem cells are modified by homologous recombination. Target gene transcription is interrupted by the insertion of an antibiotic resistant cassette in the DNA coding sequence. Modified cells are selected and injected into a mouse blastocyst to produce a mouse chimera. Mice that exhibit germ line transmission are subsequently breed to establish a mutant mouse strain line.

A modification of this technology allows investigators to overcome cases of embryonal lethality, or to inactivate a gene only in specific cell populations. The Cre-Lox system permits the conditional deletion of a gene of interest by the combined use of the Cre DNA recombinase of bacteriophage P1 and Lox P sites, short DNA sequences that are selectively and efficiently recognized by the recombinase. This method is based on the generation of two independently manipulated mouse lines: one transgenic line expressing a

Cre recombinase transgene under the control of a tissue- or cell-specific promoter, and the other in which the gene that should be selectively disrupted carry two Lox P sites in strategic positions. Interbreeding of these mouse lines will eventually results in the targeted deletion of the sequences flanked by the Lox P sites only in cells or tissues where the Cre recombinase is expressed (Sauer, 1998). This method has infinite applications since the expression of a gene can in principle be modified or altered at a specific developmental stage or in a tissue-specific manner.

In the past decade mouse models for human LSDs have become commonplace in the quest for understanding the molecular basis of the diseases and for the development of therapeutic strategies. Table 1 summarizes the general phenotype of the mouse models of LSDs currently available and how they compare to the corresponding human conditions. In general, the mouse models mimic the severe forms of the corresponding human disease by genetic, biochemical and pathological criteria; but milder clinical course, later age of onset, and time of death that are characteristic of the mild forms of the disease. A few of these models reproduce only specific aspects of the disease with a less severe phenotype or no clinical phenotype at all. Curiously, however, the unexpected results obtained with some of these latter models have helped to sort out differences in metabolic pathways between mouse and human and to give new insights into the pathogenesis of the disease (Elsea and Lucas, 2002; Raben *et al.*, 2000; Sidransky and Ginns, 1997; Sidransky *et al.*, 1992; Zhou *et al.*, 1995),(Chapter 3).

Striking examples of mouse models that do not mimic all aspects of the corresponding human disease are represented in the animal models of Gaucher disease and G_{M2}-gangliosidosis (Beutler, 2001; Susuki, 2001). Mouse models of Gaucher disease carrying null or nonsense mutations at the glucocerebrosidase locus died prematurely, manifesting clinical features substantially more severe that any of the human forms of the disease previously described (Sidransky and Ginns, 1997). However, the characterization of the first mouse model of Gaucher disease (Sidransky and Ginns, 1997; Stephens *et al.*, 1981) provided the basis for the identification of a subgroup of Gaucher patients who die soon after birth with a fulminant disease. This phenotypic variant was previously misdiagnosed.

The mouse models of the two G_{M2}-gangliosidoses, Tay-Sachs and Sandhoff diseases, presented with phenotypic characteristics that differed greatly compared from the corresponding human disorders. In particular, mice with an isolated deficiency of the β-hexosaminidase A (Hex A) isoenzyme did not manifest the progressive mental and motor deterioration present in the human disease. It was found that these differences in glycolipid storage are the result of distinctive ganglioside degradation pathways in mice and humans. In humans, degradation of G_{M2} ganglioside is exclusively executed by the Hex A isoenzyme with the aid of the G_{M2} activator protein. In mice, an alternative pathway involves the conversion of G_{M2}–ganglioside (primary metabolite accumulated in lysosomes) into its asialo derivative G_{A2} by a murine sialidase that is more active in mice than in humans. In turn, the converted glycolipid becomes a substrate for the Hex B isoenzyme and the G_{M2}-activator protein. In Hex A -/- mice this alternative pathway is overactive, explaining the modest storage of G_{M2}-ganglioside and the absence of a severe neurological phenotype (Sandhoff and Kolter, 1998). Similar differences in ganglioside catabolism were observed in the mouse model of G_{M1}-gangliosidosis (GM1), which is discussed in Chapter 3 of this thesis.

In conclusion, when comparing the findings between the two species issues to be taken into account are alternative metabolic pathways, time of development of the pathology, environmental factors and genetic background that might influence disease penetrance. Murine models still remain to be investigated beyond characterization of the basic phenotypic features to fully understand the molecular bases of disease pathogenesis.

Therapy for Lysosomal Storage Disorders

A unique feature of lysosomal enzyme precursors is their ability to be secreted in small quantities into the extracellular millieu, reinternalized via mannose or mannose-6-phosphate cell surface receptors (Burudi *et al.*, 1999; Kornfeld, 1992), and routed to lysosomes where they function (d'Azzo *et al.*, 1982; Galjart *et al.*, 1988; Ioannou *et al.*, 1992; Morreau *et al.*, 1992). In principle, the enzyme deficiency of cells can be corrected by uptake of the missing enzyme from exogenous sources. This correction forms the basis of various therapies for LSDs, such as enzyme replacement therapy (ERT), bone marrow transplantation (BMT), and gene therapy.

The results of ERT in Gaucher disease (Barton *et al.*, 1991; Beutler *et al.*, 1991), Pompe disease (Kikuchi *et al.*, 1998; Van den Hout *et al.*, 2000), and mucopolysaccharidoses (O'Connor *et al.*, 1998; Sands *et al.*, 1997; Shull *et al.*, 1994; Vogler *et al.*, 1999) have demonstrated that this procedure may be effective in patients with some non-neuropathic LSDs. However, ERT alone is unlikely to ameliorate neuropathic forms, because of the inability of proteins to cross the blood brain barrier (Vogler *et al.*, 1999).

BMT could provide a permanent source of normal enzyme, since BM progenitor cells can differentiate and repopulate target organs, including the CNS, and function as donors of the corrective enzyme to deficient cells. The overall outcome of allogeneic and syngeneic BMTs in both patients and animal models has indicated that this procedure is relatively effective in alleviating visceral symptoms and in stabilizing bone lesions, especially if BMT is performed early in life (Freeman *et al.*, 1999; Hsu *et al.*, 1999; Sands *et al.*, 1993; Sands *et al.*, 1997; Vellodi *et al.*, 1995; Vellodi *et al.*, 1999; Vellodi *et al.*, 1997; Zhou *et al.*, 1995), (Chapter 6 of this thesis). However, diseases that have an early onset and involve predominantly the CNS respond poorly or do not respond to BMT, albeit some variations in outcome have been observed among disease subtypes (Krivit *et al.*, 1999; Walkley *et al.*, 1994). The inability of BMT to correct the enzyme deficiency in the CNS could be attributed to the slow and incomplete engraftment of BM-derived cells in the adult brain. A general limitation of BMT is the lack of immunologically matched donors. Furthermore, response to BMT may be influenced by: (1) the type and number of engrafted donor cells; (2) the biochemical and physical properties of the secreted, correcting enzyme; (3) the efficiency of secretion and extracellular stability of the correcting enzyme (Dobrenis *et al.*, 1994); (4) the extent of uptake by target cells; and (5) the characteristics of the affected cells as well as the level of cell degeneration.

Somatic gene therapy of neurologic LSDs could become the preferred treatment if autologous stem cells could be engineered *in vitro* to constitutively express and secrete high levels of the correcting enzyme and become the source of the correcting enzyme when transplanted back in the patient. This procedure should overcome the main limitations of allogeneic BMT in humans: namely, the difficulty in finding HLA-matched donors, and the morbidity and mortality associated with irradiation, immunosuppression, and graft-versus-host disease. So far, this approach has been hampered by the low transduction efficiency of pluripotent stem cells and the inability to achieve long-term expression of the therapeutic protein *in vivo*. Some of these difficulties can now be circumvented by the use of improved viral vectors, virus purification conditions (Navarro *et al.*, 1996; Zolotukhin *et al.*, 1999), transduction procedures and viral packaging cell lines (Loiler *et al.*, 1997; Miller and Chen, 1996; Pan *et al.*, 1999; Sanyal and Schuening, 1999). Moreover, the expansion, transduction, and selection of BM stem cells *in vitro* have been facilitated by the use of specific cell culture conditions and selectable markers (Dardalhon *et al.*, 1999; Persons *et al.*, 1997).

Using combinations of these approaches, several laboratories have achieved improved, long-term expression of transferred genes in murine hematopoietic cells after transplantation of genetically-modified BM cells (Matzner *et al.*, 2000a; Matzner *et al.*, 2000b; Takenaka *et al.*, 2000; Takiyama *et al.*, 1999). These studies have helped to set the stage for trials of human stem-cell gene therapy in patients with Gaucher disease (Dunbar *et al.*, 1998; Schuening *et al.*, 1997). Nonetheless, the efficiency of this procedure in treating CNS disease needs careful evaluation. Alternative approaches of BMT and gene therapy are being developed specifically for the treatment of CNS manifestations. Neural stem cells have emerged as a suitable source of tissue for genetic modification and *ex vivo* gene transfer to the CNS (Conti and Cattaneo, 2002; Martinez-Serrano and Bjorklund, 1997; Snyder *et al.*, 1995; Snyder *et al.*, 1997). Cell-mediated and vector mediated transfer systems for correction of neurologic LSDs have also been reported (Daly *et al.*, 1999a; Daly *et al.*, 1999b; Elliger *et al.*, 1999; Taylor and Wolfe, 1997; Xiao *et al.*, 1997).

It is undoubted that much experimental work remains to be done and the availability of animal models of LSDs will facilitate the implementation of different therapeutic modalities and the assessment of their suitability.

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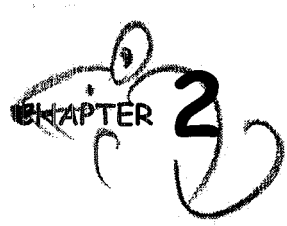

Introduction to the Experimental Work

INTRODUCTION TO THE EXPERIMENTAL WORK

We have generated mouse models of GS and GM1, initiated a comprehensive study of their phenotypes, and tested new therapeutic approaches to treat these diseases. These two models are complementary *in vivo* systems in which disease pathogenesis can be analyzed: the GM1 model is primarily a generalized nervous system disease, whereas the GS model is predominantly of a systemic nature with only specific brain regions been affected (Matsuda *et al.*, 1997; Zhou *et al.*, 1995) (Chapter 3).

G_{M1} gangliosidosis

In Chapter 3 we present the basic biochemical and pathological changes associated with a β-galactosidase deficiency in mice carrying a null mutation at the β-galactosidase locus. Overall, this mouse model represents a close phenocopy of the severe form of human G_{M1} gangliosidosis, with progressive and generalized central nervous system (CNS) involvement. However, unlike the infantile human patients, deficient mice do not develop an enlarged and vacuolated liver, and show no additional abnormalities in the systemic organs. Moreover, in spite of the pronounced accumulation of G_{M1} ganglioside and G_{A1} beginning early in life, knockout mice survive up to 10 months. The increased amount of G_{A1} in the brain is consistent with the existence of an active desialylation process in the GM1 model, as observed in β-hexosaminidase deficient mice (Elsea and Lucas, 2002; Suzuki and Proia, 1998). As the disease progresses, the mice develop severe ataxia, tremors, and abnormal gait resulting in hindlimb paralysis and premature death. In general, the extensive neuronal involvement in GM1 mice has provided a useful experimental tool to study the molecular bases of CNS pathology.

Mechanisms that influence pathogenesis, such as inflammation and cell death, have been reported in neurodegenerative disorders, including LSDs (Gervais *et al.*, 1999; Imaizumi *et al.*, 2001; Mattson, 2000; Myerowitz *et al.*, 2002; Sawa, 1999; Sherman and Goldberg, 2001). For example, a time-dependent massive apoptosis in the CNS is characteristic of Hex B deficient mice (Huang *et al.*, 1997), and progressive loss of a specific neuronal population, the Purkinje cells, is typical of the neuropathology of GS mice (Chapter 5-6).

Interestingly, in several neurodegenerative diseases, including our GM1 model (Chapter 4), cell loss or apoptosis occurs as a sporadic event affecting few cells at any given time during disease progression, although the primary enzyme deficiency is continuously present and involves many cells. Sporadic cell death is consistent with the activation of an ER stress response or UPR that is usually a lengthy event since damaged cells tend to survive a long time before they die. Conditions that disturb or alter ER homeostasis trigger a cascade of events that begins with the attenuation or inhibition of protein synthesis and upregulation of ER–resident chaperones, and end with the activation of specific caspases and eventually cell death. Although the entire process of ER stress-mediated apoptosis is not completely understood, an increase body of evidence implicates this mechanism in chronic neurodegenerative diseases such as Alzheimer dementia, Parkinson disease and Huntington disease (Paschen and Doutheil, 1999a; Paschen and Doutheil, 1999b; Paschen and Frandsen, 2001).

We have investigated whether a similar mechanism may underlie GM1 gangliosidosis because of the pattern of apoptotic cells seen in the CNS of the GM1 mice during progression of the disease. We first demonstrated that caspase 12 was upregulated both at the mRNA and protein levels in the spinal cord of GM1 mice starting at the age of ~3-4 months. Caspase-12 is a member of the ICE (interleukin-1beta converting enzyme)

subfamily of caspases, which is believed to be exclusively activated by an ER stress response, and to promote cell death (Bitko and Barik, 2001; Nakagawa and Yuan, 2000; Nakagawa *et al.*, 2000; Yoneda *et al.*, 2001). This finding encouraged us to test the levels of specific chaperones and other molecules that are known to be elevated under ER stress conditions. A time-dependent overexpression of the ER chaperone BiP/GRP78 in the spinal cord of the GM1 mice was indicative of the initiation of a cellular response to ER-stress.

Induction of BiP is known to be required to alleviate ER stress, suggesting a potential role of BiP in protecting affected cells from cell death. Recent reports postulate that the mechanism of protection consist in the formation of a complex between BiP, caspase-7 and caspase-12 upon ER stress activation that prevent the release of caspase-12, a death mediator, from the ER (Rao *et al.*, 2002). Moreover, overexpression of BiP attenuates the induction of the pro-apoptotic, stress-induced transcription factor CHOP (growth arrest and DNA damage C/EBP homologous protein) (Wang *et al.*, 1998). The activation of CHOP results in its heterodimerization with the C/EBP family of transcription factors, preventing transcription of C/EBP homodimer target genes and activating new downstream targets. Although CHOP specific targets have not been fully characterized, increased expression of CHOP has been linked to cell cycle arrest and cell death. We tested the expression levels of CHOP in spinal cord samples of GM1 mice and found that they were significantly higher than in age-matched wild type mice.

These results and the presence of apoptotic cells in spinal cord samples from GM1 mice, suggest that ER stress-mediated caspase-12 activation might contribute to the neuronal loss and overall neurodegeneration in this disease. In addition, the possibility that an ER stress response could occur as a result of lysosomal dysfunction implies that a feedback mechanism may exist between these two subcellular compartments.

Galactosialidosis

The mouse model of this disease recapitulates closely the biochemical and clinical features characteristic of GS in humans. PPCA null mice develop a generalized systemic disease affecting to some extent most of the visceral organs and the CNS. Although they are viable, GS mice display growth retardation, mate poorly and become edematous and ataxic as disease progresses, and die prematurely. The latter features are the consequence of the severe kidney involvement and sequential loss of cerebellar Purkinje cells, which are the most overt phenotypic abnormalities in this mouse model.

In Chapter 5 we have compared the expression of PPCA in normal mouse tissues with the occurrence of lysosomal storage in tissues of the GS mice. In most cases we found a close correlation between high expression of the PPCA mRNA and protein in normal cells and lysosomal storage in GS cells. Overt discrepancies were found primarily among neuronal cell populations, implying that individual cells may either metabolize a different spectrum of substrates, or have a different susceptibility to toxic by-products. Alternatively, PPCA may be expressed in selected cells primarily for its cathepsin A activity rather than its protective function towards β-galactosidase and neuraminidase. The detailed analysis of the GS phenotype has been instrumental for the accurate assessment of the efficacy of the therapeutic approaches that we have implemented in this mouse model (Chapter 6 and 7).

BMT studies suggest that BM-derived cells are capable of reaching different organs including the CNS and supplying normal enzyme to deficient cells, thereby restoring lysosomal function. However, response to BMT may be influenced by the amount of corrective enzyme that is secreted by normal cells, especially in the CNS, the extracellular stability of the corrective enzyme (Dobrenis *et al.*, 1994), the extent of uptake by target cells and their level of degeneration. We have tested some of these variables in proof-of-

principle studies by using transgenic BM cells overexpressing the therapeutic enzyme under the control of an erythroid- (LCR-β-globin) (Zhou *et al.*, 1995) or macrophage-specific (CSF-1R) promoter to correct the GS phenotype. This experimental approach was based on the hypothesis that higher levels of enzyme would provide effective treatment for the systemic and CNS manifestations in a shorter period of time. The results of these studies have validated this view, and unequivocally demonstrated that homogeneous populations of hematopoietic cells overexpressing the therapeutic enzyme can effectively cross-correct affected cells and improve/delay the CNS pathology in both BMT and crossbreeding experiments (Chapter 6).

The results obtained with BMT using transgenic BM cells supported the idea that *ex vivo* gene therapy could be a suitable approach for the treatment of GS. In Chapter 7 we tested whether retroviral gene delivery of PPCA resulted in the correction of the disease phenotype in GS mice. Deficient mice that received PPCA-/- hematopoietic progenitor cells retrovirally transduced to express PPCA, exhibited complete correction of visceral organs and functional amelioration of the cerebellar deficit. Most importantly, efficient uptake of the corrective enzyme was observed in visceral organs where the presence of PPCA in non-BM derived cells was indicative of cell-to-cell transfer. Although in the brain the expression of the PPCA was limited to the perivascular areas, it was sufficient to delay the onset of Purkinje cell degeneration and to slightly improve neurological symptoms.

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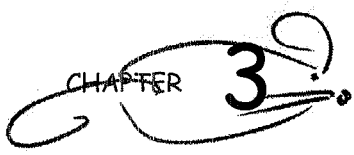

Generalized CNS disease and massive GM_1-gaglioside accumulation in mice defective in lysosomal acid β-galactosidase.

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Hum Mol Genet 1997 **6 (2)**:205-211

Human Molecular Genetics, 1997, Vol. 6, No. 2 205–211

ARTICLE

Generalized CNS disease and massive G_{M1}-ganglioside accumulation in mice defective in lysosomal acid β-galactosidase

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Received October 3, 1996; Revised and Accepted November 27, 1996

Human G_{M1}-gangliosidosis is caused by a genetic deficiency of lysosomal acid β-galactosidase (β-gal). The disease manifests itself either as an infantile, juvenile or adult form and is primarily a neurological disorder with progressive brain dysfunction. A mouse model lacking a functional β-gal gene has been generated by homologous recombination and embryonic stem cell technology. Tissues from affected mice are devoid of β-gal mRNA and totally deficient in G_{M1}-ganglioside-hydrolyzing capacity. Storage material was already conspicuous in the brain at 3 weeks. By 5 weeks, extensive storage of periodic acid Schiff -positive material was observed in neurons throughout the brain and spinal cord. Consistent with the neuropathology, abnormal accumulation of G_{M1}-ganglioside in the brain progressed from twice to almost five times the normal amount during the period from 3 weeks to 3.5 months. Despite the accumulation of brain G_{M1}-ganglioside at the level equal to or exceeding that seen in gravely ill human patients, these mice show no overt clinical phenotype up to 4–5 months. However, tremor, ataxia and abnormal gait become apparent in older mice. Thus, the β-gal-deficient mice appear to mimic closely the pathological, biochemical and clinical abnormalities of the human disease.

INTRODUCTION

Genetic deficiency of lysosomal acid β-galactosidase (β-gal) causes two phenotypically distinct disorders in humans, G_{M1}-gangliosidosis and Morquio B disease (1). G_{M1}-gangliosidosis is primarily a neurological disorder with progressive brain dysfunction. Clinically, infantile, juvenile and adult forms are recognized. The infantile disease is most rapidly progressive and involves not only the central nervous system (CNS), but also visceral organs with mucopolysaccharidosis-like features. Patients succumb to the disease usually within a few years. The visceral involvements are minor or absent in the older forms. The adult form is the most chronic, and radiologic bone abnormalities are often prominent. Morquio B disease occurs at varying ages and is characterized by severe bone deformities and an almost total lack of nervous system symptoms, except for those resulting from bone abnormalities, such as spinal cord compression. Patients with clinical manifestations that are intermediate between G_{M1}-gangliosidosis and Morquio B disease have also been reported.

At the neuropathological level, severe infantile G_{M1}-gangliosidosis patients exhibit distended neurons that contain typical lamellar inclusions referred to as membranous cytoplasmic bodies which are also found in other lipidoses (2,3). Although neurons are the primary target for storage, astrocytes may also appear abnormally vacuolated. Neuronal pathology in late onset forms is delayed and tends to be more severe in deeper structures of the brain than in the cortex (4). Inclusions in the liver are of fibrillar nature and are different from the lamellar bodies in neurons. Abnormal accumulation of G_{M1}-ganglioside and, to a much lesser extent, its asialo-derivative G_{A1}, in the brain is the most prominent biochemical feature (5,6). On the other hand, oligosaccharides derived from keratan sulfate and glycopeptides are stored primarily in visceral organs and are excreted abnormally into the urine (7). The human lysosomal acid β-galactosidase cDNA and gene have been cloned and

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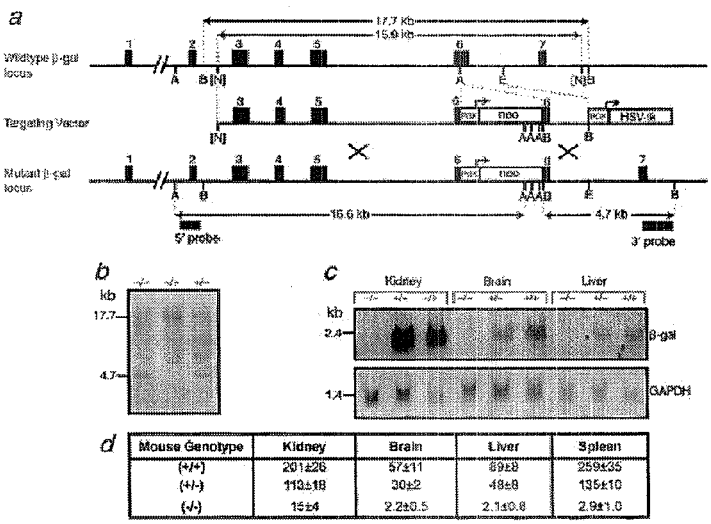

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Mouse Genotype	Kidney	Brain	Liver	Spleen
(+/+)	201±26	57±11	89±8	259±35
(+/-)	113±18	30±2	48±8	135±10
(-/-)	15±4	2.2±0.5	2.1±0.6	2.9±1.0

Figure 1. Targeted disruption of the murine β-gal locus by homologous recombination. (a) Structure of a portion of the β-gal gene, the targeting construct and the predicted structure of the disrupted β-gal locus. The targeting vector was constructed from genomic sequences found within a single λ clone containing 15.9 kb of the β-gal locus. Only relevant restriction sites are shown: (A) *Aat*II; (B) *Bam*HI; (E) *Eco*RI; [N] *Not*I—sites are not present in the gene, but are polylinker sites flanking the genomic clone. The numbered solid boxes are exons. The 5′ and 3′ probes used for determination of homologous recombination detect a 16.6 and 4.7 kb fragment in the mutant locus and a 14.6 and 17.7 kb fragment in the wild-type locus, respectively. (b) Southern blot analysis of *Bam*HI-digested genomic DNA from tail biopsies of F1 intercross progeny probed with the 3′ probe. A homologous recombinant mouse (–/–) showing the diagnostic 4.7 kb fragment is seen on the left, a wild-type (+/+) homozygous for a 17.7 kb band is in the middle and a heterozygous mouse is on the right. A non-specific band occurs in all lanes at a size of ~8 kb. (c) Northern blot analysis of 1-month-old mouse tissues. Total RNA (20 μg) from each tissue was run on a 1% agarose denaturing gel, transferred to ZetaProbe (Bio-Rad) and probed independently for β-gal using a 1.8 kb mouse β-gal cDNA fragment and for GAPDH using a 0.7 kb mouse GAPDH fragment. The data shown are a phosphor-image of 14 days for β-gal mRNA, and an autoradiograph for 24 h for GAPDH mRNA. (d) β-gal activity in tissues of 1–2-month-old mice. β-gal activity was measured in total tissue water homogenates using synthetic 4-methylumbelliferyl β-galactoside as a substrate. Activities are expressed as the mean±SD (nmol/h/mg) of four mice derived from chimeric clone 68.

characterized (8–11) and many disease-causing mutations have been identified in patients (12–21).

Naturally occurring canine, feline, bovine and ovine models for G_{M1}-gangliosidosis exist, but none is known among small laboratory animals (1). The canine and feline models appear to mimic the human phenotype to a large extent, but a faithful mouse model could facilitate study of the pathogenesis of the disease and its therapy. We have utilized gene targeting in embryonic stem (ES) cells to generate a mouse model of β-galactosidase deficiency. The model appears to be a close duplicate of human G_{M1}-gangliosidosis with respect to CNS involvement, since the mice present with pathological signs very early in life. This is reflected by the excessive accumulation of both G_{M1}-ganglioside and G_{A1}. In contrast to human infantile patients, the liver pathology is less apparent in the young mice. Although gross abnormalities are not visible during the first 4–5 months, the affected mice develop severe tremor, ataxia and an abnormal gait. A preliminary report from another laboratory has been presented in abstract form and describes the generation of a β-gal-deficient mouse line by disrupting the gene further towards the 3′ terminus. However, to the best of our knowledge, no details on phenotype pathology are available yet to compare with our model (22).

RESULTS

Generation of β-gal (–/–) mice

The β-gal gene consists of 16 exons and was inactivated by introduction of a neomycin resistance gene into the middle of exon 6 (177 bp) (Fig. 1a), which would ensure complete inactivation of the enzyme even if a truncated version was generated. A herpes simplex 1 virus thymidine kinase (*tk*) gene was inserted downstream of the gene to enable negative selection. Both selectable markers were placed in the same orientation as the β-gal gene. Following electroporation into E14 ES cells and selection with G418 and FIAU, genomic DNA from double resistant colonies was screened with a 3′ probe located outside the β-gal gene targeting sequences. Eight independent homologous recombinant ES cell clones were obtained from a total of 325 examined, giving a targeting frequency of 1:40. Two of these (clones 68 and 190) were injected into blastocysts and gave rise to two β-gal (–/–) mouse lines which show biochemical and pathological features that are essentially the same. At birth, the genotypic analysis (Fig. 1b) of 117 newborn animals (from 15 litters of heterozygous crossings) indicated a Mendelian

inheritance ratio among (–/–) (18%; $n = 21$), (+/–) (59%; $n = 69$) and (+/+) (23%; $n = 27$) mice. Thus, embryonic or fetal lethality did not occur. The β-gal (–/–) mice are fertile and produce normal size litters.

Biochemical features of the CNS in young β-gal (–/–) mice

Northern blot analysis of kidney, brain and liver total RNA detected no β-gal mRNA of the expected 2.4 kb size in (–/–) mice, while heterozygotes contained approximately half the level of wild-type mRNA when standardized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (Fig. 1c). No smaller truncated version equivalent to the first five and a half exons of β–gal was noted, suggesting that any generated mRNA was unstable.

Consistent with the absence of β-gal mRNA in β-gal (–/–) tissues, the residual activity in brain, kidney, liver and spleen using the fluorogenic artificial substrate was severely depressed. Activity levels ranged from 1% in spleen to 4% in brain compared with wild-type littermates (Fig. 1d). Kidney samples consistently displayed ~8% activity. This residual activity towards 4-methylumbelliferyl β-galactoside may be contributed by the other lysosomal β-gal, galactosylceramidase, which is genetically normal in these mice and has some activity toward this substrate (23,24). Heterozygotes contained ~50% activity compared with normal littermates.

Since β-gal is the first enzyme in the natural degradation pathway of G_{M1}-ganglioside, it was important to assess whether there was any β-gal activity towards the natural substrate in affected mice. The activity in the liver of a (–/–) mouse was undetectable compared with 85 nmol/h/mg measured in a wild-type mouse. This clearly indicates that there is no redundancy for the β-gal-catalyzed degradation of gangliosides.

G_{M1}-ganglioside and G_{A1} accumulate in the brain of β-gal (–/–) mice

Brain tissue from affected mice was analyzed for any alteration in lipid composition during progression of the disease. Thin layer chromatography (TLC) of total brain gangliosides from 3-week- and 3.5-month-old (–/–) mice clearly demonstrated a marked increase in G_{M1}-ganglioside, like that seen in an infantile patient (Fig. 2). Interestingly, a significantly higher level of G_{A1} occurred in affected mice compared with humans (5,6), suggesting that the murine neuraminidase is more active toward G_{M1}-ganglioside than the corresponding human enzyme. No other significant

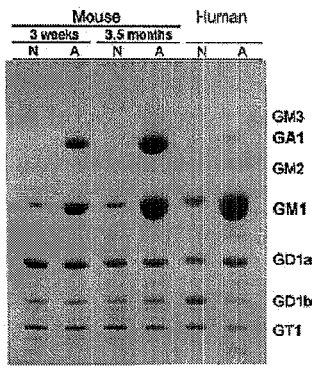


Figure 2. Thin-layer chromatogram of brain ganglioside fractions. The ganglioside fraction equivalent to 4 mg wet weight of brain was spotted for each sample and the components separated using chloroform:methanol:0.2% $CaCl_2$ (55:45:10, by vol). The plate was then sprayed with orcinol reagent in order to visualize gangliosides and G_{A1} with a similar sensitivity. Progressive accumulation of G_{M1}-ganglioside and G_{A1} in the affected mouse brain is evident. The accumulation of G_{M1}-ganglioside reaches that of the brain of a patient with infantile G_{M1}-gangliosidosis (age 8 months), while the degree of G_{A1} accumulation in the affected mouse brain is much more severe than in the human patient.

abnormalities regarding content or distribution of other lipids, including phospholipids, galactolipids (galactocerebrosides and sulfatides) and cholesterol, were seen in the brain of the affected mice (unpublished data).

In agreement with the qualitative analyses on TLC, the total amount of brain ganglioside sialic acid and G_{M1}-ganglioside increased rapidly and dramatically during progression of the disease from 3 weeks to 3.5 months of age (Table 1). Its level increased from 2.2 to 4.8 times that of normal controls over this period, which constituted 27–41% of total brain ganglioside sialic acid compared with 13–14% for normal mice. It is noteworthy that the G_{M1}-ganglioside values at 2.75 and 3.5 months were already nearly identical to those found in an 8-month-old human infant with G_{M1}-gangliosidosis. These results indicate that the lack of β-gal activity leads to the expected accumulation of G_{M1}-ganglioside, and has no effect on other lipid pathways.

Table 1. Brain G_{M1}-ganglioside content

	Control mice			Affected mice			Human G_{M1}-gangliosidosis[a]
	3 wks	2.75 mo	3.5 mo	3 wks	2.75 mo	3.5 mo	
Total sialic acid, μmol/g	3.21	3.58	3.25	4.01	6.30	6.88	6.05
(% normal)				(125%)	(176%)	(212%)	
G_{M1}-ganglioside, μmol/g	0.80	0.86	0.83	1.77	3.54	4.02	3.44
(% normal)				(221%)	(412%)	(483%)	
% Total sialic acid in G_{M1}-ganglioside	13	13	14	27	38	41	44

[a]Infantile form, 8-months-old.

β-gal (–/–) mice display neuropathology consistent with G_{M1}-gangliosidosis

The gross appearance of the brain was normal. On histological preparations, swollen neurons containing storage materials throughout the brain were strongly stained with periodic acid Schiff (PAS) as early as 3 weeks of age. By 5 weeks, neuronal storage had increased dramatically and was noted in almost all neurons in the cerebrum, cerebellum, brainstem, spinal cord and dorsal root ganglia (Fig. 3). Storage was particularly conspicuous in large neurons of both the central and peripheral nervous system. In the cerebellum, in addition to the perikarya, PAS-positive material was also noted in the dendrites of Purkinje cells (Fig. 3d). Electron microscopic examination of the cerebral cortical neurons at 3 weeks of age revealed pleomorphic inclusions consisting either of stacked or concentric lamellae, or of many small vesicles and complex lamellar structures (Fig. 3f). The histopathology and ultrastructural features of the neuronal inclusions in these β–gal (–/–) mice were closely similar to those of infantile G_{M1}-gangliosidosis in humans (1).

Unlike the human disease, affected mice showed no hepatosplenomegaly, and histologic examination demonstrated no conspicuous storage cells even in 3.5-month-old mice. Only minimal storage of oligosaccharides of the same size range as in humans (4–10 saccharides) occurred in the liver of affected mice, and there was essentially no progression in the accumulation from 3 weeks to 3.5 months. This finding is consistent with the minimal and nearly static liver pathology, if any, and also correlates well with the low level of abnormal urinary oligosaccharides compared with human patients (unpublished data).

DISCUSSION

We have established a mouse model of G_{M1}-gangliosidosis through targeted disruption of the β-gal gene. This disease has been reported in large domestic animals (reviewed in Suzuki *et al.*, ref. 1). The canine and feline models appear particularly faithful to the human disease, and manifest many of the pathological and biochemical abnormalities seen in our murine model. These animals have been carefully studied and utilized for experimental therapy. While, to some extent, they may more closely parallel the conditions in humans for transplantation, they are technically more difficult and costly to maintain and treat. Therefore, the availability of a small laboratory animal should greatly facilitate studies on disease pathology and therapy.

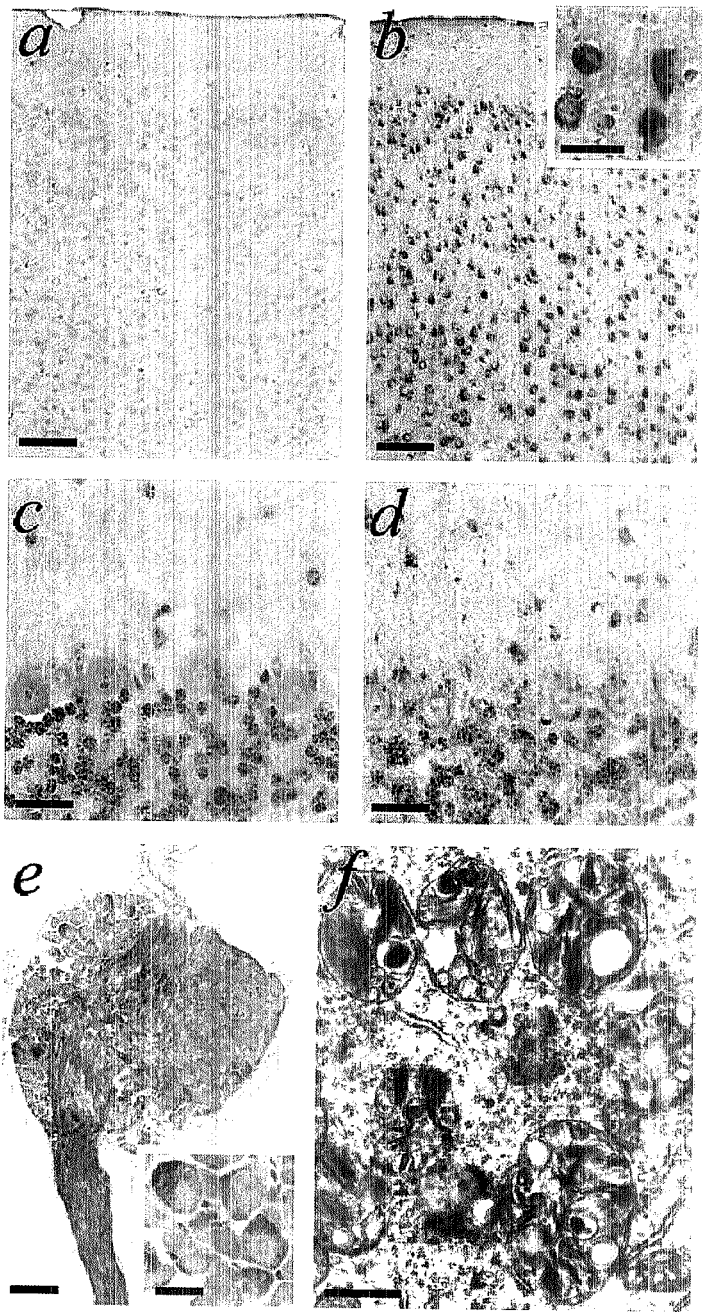
β-gal null mice show a complete absence of β-gal mRNA and low residual enzyme activity for the fluorogenic substrate in all tissues tested. G_{M1}-ganglioside is the primary substrate for the enzyme in the brain and no activity towards this compound is detected. As a consequence, there is rapid and progressive accumulation of G_{M1}-ganglioside in the CNS of deficient mice which is obvious already at 3 weeks of age. In addition, the asialo derivative of G_{M1}-ganglioside (G_{A1}) accumulates to a similar level during progression of the disease. In the murine models of Tay–Sachs and Sandhoff disease (deficiencies of β-hexosaminidase α and β subunits, respectively) (25,26), Proia and colleagues have recently obtained evidence that desialylation of G_{M2}-ganglioside to G_{A2} is a significant pathway in the mouse (26), unlike in humans (27). In the Tay–Sachs disease mouse, however, G_{A2} can be degraded further by the genetically normal β-hexosaminidase B (ββ) isoenzyme, while its degradation is blocked in the Sandoff disease mouse. The distribution of undegraded G_{M1}-ganglioside and G_{A1} in β-gal (–/–) mice thus parallels that of G_{M2}-ganglioside and G_{A2} in β-hexosaminidase B (–/–) mice. Active desialylation of both G_{M1}- and G_{M2}-gangliosides has been reported in mouse Neuro2a cells (28), but interpretation was ambiguous due to the transformed nature of the cells. Our results indicated that in the mouse, degradation of G_{M1}-ganglioside to G_{A1} is also a major pathway.

Contrary to G_{M1}-gangliosidosis patients, the liver pathology in deficient mice is much less pronounced. The liver in infantile patients is enlarged and contains numerous foamy cells with abnormal fibrillar inclusions which differ from the lamellar bodies of neurons (1). The major storage products are oligosaccharides derived from keratan sulfate and glycopeptides. In β-gal (–/–) mice, inclusions in the liver are not apparent even at the age of 3.5 months and storage of oligosaccharides is minimal. A possible explanation for this discrepancy is the presence of normal levels of the other β-gal enzyme (galactosylceramidase) which, in mice, may compensate, at least in part, for β-gal activity on some substrates. However, this seems unlikely since twitcher mice, deficient for galactosylceramidase, do not accumulate oligosaccharides (1), indicating that either this enzyme is not critical for catabolism of these hydrophilic substrates or that any activity towards these substrates can be fully compensated by β–gal. Therefore, it is possible that the metabolism of these hydrophilic substrates differs between mice and humans. While this may be the case in visceral organs, there seems to be no compensating activity towards G_{M1}-ganglioside, the primary β-gal substrate in the CNS.

The nervous system involvement is the most predominant feature in the mouse model and is consistent with that seen in early infantile patients. The significantly higher level of G_{M1}-ganglioside and G_{A1} in the brain of 3-week-old β-gal (–/–) mice suggests that the CNS disease may already be present at birth. Accumulation of storage products is rapid and their distribution is very widespread, with almost all neurons being affected to some extent. One would expect there to be major perturbations in mechanosensory and psychointellectual pathways as the disease progresses. As evidence of this, some of the mice are starting to display overt neurological problems including tremor, ataxia and abnormal gait beyond the age of 5 months. Recently, an 8-month-old mouse died, apparently from hemiparesis. It will be crucial to monitor subtle motor, sensory and intellectual functions in a significant number of animals to define accurately the time of onset and type of neurological aberrations that result, and their lifespan.

Overall, this mouse model for G_{M1}-gangliosidosis closely mimics the most fundamental aspects of the neuropathological

Figure 3. Histological analysis of brain from β-gal (–/–) and control (+/+) mice. Light microscopy of PAS-stained sections of the cerebral cortex (a and b), cerebellum (c and d) and a thoracic dorsal root ganglion (e) of 5-week-old wild-type (a and c) and β-gal (–/–) (b, d and e) mice, and an electron micrograph of inclusions in a cerebral cortical neuron from a 3-week-old β-gal (–/–) mouse (f). PAS-positive storage materials are conspicuous in the cerebral cortical neurons which appear swollen (b, insert), cerebellar Purkinje cells (d) and the large and medium neurons of dorsal root ganglia (e, insert) of the β-gal (–/–) mouse. Scale bar = 100 μm (a, b and e), 25 μm (c,d and inserts in b and e) or 0.5 μm (f).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and neurochemical abnormalities of the human disorder despite some distinct differences. In particular, because of the generalized CNS involvement, the β-gal (–/–) model will provide an excellent system in which to correlate neuronal storage with physiological effects, and in which to investigate CNS-targeted therapy.

MATERIALS AND METHODS

Targeting vector construct

A mouse acid β-gal cDNA (C57BL/6J) (29) was used as a probe to isolate the corresponding gene from a 129/SV mouse genomic λ library (Stratagene, La Jolla, CA). One clone of ~15.9 kb encompassed exons 3–7 of the mouse β-gal gene (Fig. 1a). The targeting construct essentially contained a neomycin resistance (*neo*) gene inserted into a unique *Aat*II site in exon 6 and a herpes simplex virus *tk* gene to enable positive/negative selection (30). To generate this, the 15.9 kb λ clone was cut with *Aat*II within exon 6 and the ends blunted. It was then digested with *Not*I (polylinker flanking site) and the 11.2 kb fragment containing exons 3 ,4, 5 and half of 6 was force-cloned into the pPNT (31) vector that had been cut with *Xho*I, blunted and then cut with *Not*I. The resultant construct was cut with *Bam*HI, between the *neo* and HSV *tk* genes, and into it was inserted a 2.2 kb *Aat*II–*Eco*RI fragment (downstream portion of exon 6 and part of intron 6) that was blunted and linked with *Bam*HI linkers. The final targeting construct was linearized with *Not*I.

Gene targeting in ES cells and generation of homozygous mice

Electroporation into E14 ES cells and Southern blot screening for homologous recombinant clones were performed as described (32). Chimeric mice were generated by injection of two independent clones into C57BL/6 blastocysts as described (33). Heterozygotes were mated to obtain homozygotes β-gal (–/–) mice.

β-gal activity assays

Mouse tissues were homogenized in 4 volumes of water and β-gal activity determined using an artificial 4-methylumbelliferyl β-galactoside substrate (34). Total protein concentration was determined using bicinchoninic acid (35) following the manufacturer's protocol (Pierce Chemical Co.). The assay for acid β-gal activity in total liver homogenates from the 3.5-month-old control and affected mice toward the natural substrate, G_{M1}-ganglioside, was performed as previously described (36).

Lipid analysis

Brain and liver samples were obtained from three affected mice and three control littermates aged 3 weeks, 2.75 and 3.5 months, and from a human infantile G_{M1}-gangliosidosis patient and one adult control subject. Lipid analyses were conducted on one cerebral hemisphere from each of the mice and on ~200 mg of human cerebral cortex. Lipid extraction, ganglioside isolation and lipid quantification were performed as described (37). Using chromatographic separation of total lipids on a 1 g silica gel 60 column, glycolipid G_{A1} eluted together with the ganglioside fraction. Thin-layer chromatograms were sprayed with resorcinol reagent and densitometric quantification of gangliosides was performed using a CAMAG II TLC scanner/CATS3 software. For visualization of gangliosides and G_{A1} on the same chromatograms, the orcinol spray which detects both lipids similarly was used instead of the sialic acid-specific resorcinol reagent.

Liver oligosaccharide analysis

The supernatant (50 000 *g* for 45 min) of 15% tissue homogenates were recentrifuged at 100 000 *g* for 2 h. Aliquots (5 µl) of the final supernatant were analyzed by TLC on high-performance silica gel 60 plates (Merck), developed for 3.5 h in *n*-butanol:acetic acid:water (3:3:2, by vol) and visually evaluated following spraying with the orcinol-sulfuric acid reagent (38).

Histological procedures

Mice were anesthetized with Avertin and perfused through the left cardiac ventricle with 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.4, and fixed overnight in the same fixative. The brain, spinal cord including dorsal root ganglia and liver were removed. Some were processed for routine paraffin sections and 4 µm thick sections were stained with hematoxylin/eosin. Others were placed in 30% sucrose in 0.1 M sodium phosphate, pH 7.4 for 2 days, and then embedded in TBS tissue freezing medium by rapidly freezing in liquid nitrogen. Cryosections (8 µm) were cut and stained with PAS reagent.

For electron microscopy, following perfusion as described above, the brain and spinal cord were processed, embedded, sectioned and examined, as previously described (37).

ACKNOWLEDGEMENTS

We are grateful to Dr Gerard C. Grosveld for his continuous support, to Drs Xiao Yan Zhou and Jan van Deursen for their guidance in the initial stages of ES cell gene targeting, to Christie Nagy and John Swift for blastocyst injections and chimeric breeding, to Peggy Burdick for help in typing the manuscript and Sjozef van Baal for his computer skills. This work was supported by grants from the Assisi Foundation of Memphis [328502-(C.N.H.)], the National Institutes of Health [NIH RO1-NS24289, P30-HD03110 (Ku.S.); NIH RO1–NS24453 (Ki.S.)], the ELA (M.T.V.) and by the American, Lebanese, Syrian Associated Charities (ALSAC) of St. Jude Children's Research Hospital (A.d'A., M.P.M.).

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ER-stress-mediated apoptosis in the CNS of GM_1 Gangliosidosis mouse model.

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Submitted for publication.

ER-STRESS-MEDIATED APOPTOSIS IN THE CNS OF GM_1 GANGLIOSIDOSIS MOUSE MODEL

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Lesions in the gene that encodes acid β-galactosidase are the underlying cause of the human lysosomal storage disorder G_{M1}-gangliosidosis. The characteristic features of G_{M1}-gangliosidosis include generalized central nervous system (CNS) involvement, rapidly progressive neurodegenerative symptoms, and premature death. Recent findings point to the involvement of the endoplasmic reticulum (ER) in the neuronal cell death observed in some acute and chronic neurodegenerative diseases. Conditions that alter the environment of the ER induce highly conserved cell-stress responses, which include transcriptional induction, translational attenuation, and degradation of unfolded proteins. In contrast, excessive and/or prolonged stress of the ER results in apoptosis. Here, we report an ER stress–induced apoptotic pathway in the mouse model of G_{M1}-gangliosidosis. The mRNA level of the stress-induced gene CHOP/GADD153 (C/EBP homologous protein, growth arrest and DNA damage-inducible gene 153) was elevated in spinal cord samples from 5- to 8-month-old mice with G_{M1} gangliosidosis. In addition, mRNA levels of the ER resident protein GRP78/BiP (78-kD glucose-regulated protein, immunoglobulin- –binding protein) were inversely regulated during the course of the disease compared to CHOP. We also demonstrated transcriptional induction of caspase-12, an ER-localized caspase that is activated in ER stress-mediated cell death. Immunoprecipitation and Western blot analyses confirmed the mRNA findings. The coordinate induction of CHOP and caspase-12 and the presence of apoptotic cells in spinal cord samples support the hypothesis that the activation of an ER stress–mediated apoptotic pathway contributes to the neuropathologic features of G_{M1}-gangliosidosis.

INTRODUCTION

G_{M1}-gangliosidosis is a severe neurodegenerative lysosomal storage disorder (LSD) that is characterized by generalized CNS involvement and progressive neurodegeneration. The primary biochemical and pathologic features of G_{M1}-gangliosidosis are the accumulation of abnormal amounts of the ganglioside G_{M1} in the brain and the formation of membranous cytoplasmic bodies in the neurons (Scriver *et al.*, 2001). Progressive mental and motor retardation is the first symptom of the disease, which worsens until the patient is in a state of decerebrate rigidity. Distinct clinical phenotypes of G_{M1}-gangliosidosis vary in the time of onset and severity of symptoms (Susuki Y., 2001). Our group and others have generated murine models of G_{M1}-gangliosidosis that closely recapitulate the human counterpart (Hahn *et al.*, 1997; Itoh *et al.*, 2001; Matsuda *et al.*, 1997). The CNS of β-galactosidase (β-Gal)–deficient mice shows an age-dependent accumulation of undegraded G_{M1} and GA1 metabolites that is accompanied by gradual deterioration of motor and mental function. However, the mechanism(s) that relate neuronal storage to neurodegeneration in G_{M1}-gangliosidosis has not yet been elucidated.

Factors that influence pathogenesis such as inflammation and cell death have been reported in LSDs and other neurodegenerative disorders (ref). Studies of a murine model of G_{M2}-gangliosidosis revealed a deregulated pattern of expression of genes involved in the inflammatory response that occurs during the late stage of the disease (Wada *et al.*, 2000). This inflammatory response coincided with the appearance of $TUNEL^+$ cells; however, the signaling pathway that triggers apoptosis in this system has not been elucidated. In addition, loss of specific neuronal populations is characteristic of the pathology of some LSDs; for instance, mice that have galactosialidosis experience a substantial loss of Purkinje cells (Hahn *et al.*, 1998; Zhou *et al.*, 1995).

Conditions that alter the homeostasis of the ER have been linked to neuronal injury (Paschen and Doutheil, 1999). The ER is sensitive to biological alterations such as glucose starvation, disturbance of intracellular storage of calcium, exposure to free radicals, and improper protein folding (Kaufman, 1999). Cells have evolved at least two specific mechanisms to maintain ER function

under conditions of environmental stress: translation attenuation and transcriptional activation of certain genes (Kaufman, 1999; Scheuner *et al.*, 2001; Sidrauski *et al.*, 1998). In response to biologic stress, cells activate the unfolded protein response (UPR); this process involves the activation of genes that encode ER protein chaperones, folding catalysts, and ER-associated protein degradation machinery (Gardner *et al.*, 2001; Hampton, 2000; Welihinda *et al.*, 1999). These responses decrease the demand for folding of newly synthesized proteins, increase the protein-folding activity, and thus prevent the aggregation of misfolded proteins in the ER compartment (Kaufman, 1999).

The UPR involves the coordinated transcriptional activation of sets of genes that encode either survival or cell-death responses. After the UPR is activated, the level of ER resident proteins such as BiP and GRP94, and the expression of genes associated with cell death (e.g., the pro-apoptotic transcription factor CHOP) increase (Kaufman, 1999; Ma and Hendershot, 2001). Under stress conditions that activate the UPR, a cell either adapts or dies. Irreversible damage caused by prolonged ER stress activates an apoptotic program, which requires the activation of caspase-12 (Nakagawa and Yuan, 2000; Nakagawa *et al.*, 2000). Caspase-12, a member of the interleukin-1β converting enzyme (ICE) subfamily of caspases, is ubiquitously expressed in mouse tissues; in normal cells, the subcellular localization of caspase-12 is restricted to the cytoplasmic side of the ER membrane, where it is cleaved upon activation (Bitko and Barik, 2001; Nakagawa *et al.*, 2000; Yoneda *et al.*, 2001). It has been postulated that activation of caspase 12 in response to ER stress contributes to cell death in ischemic brain and in chronic neurodegenerative diseases such as Huntington and Alzheimer disease (Ghribi *et al.*, 2001; Yuan and Yankner, 2000). Here we report that ER stress–mediated activation of the UPR is the cause of the neuronal cell death in the CNS of the murine model of G_{M1}-gangliosidosis (GM1 mice).

RESULTS

Motor deterioration of GM_1 mice

GM1 mice develop a severe motor impairment during disease progression, accompanied by difficulty to thrive and eventually paralysis of the hind limbs. Massive ganglioside and glycolipid accumulation is evident in virtually all neurons throughout the central and peripheral nervous system (Hahn *et al.*, 1997). Using a Roto-Rod performance test we monitor the gradual worsening of the motor abilities of the mutant mice compared to wild-type littermates (Fig.1). A measurement of balance and motor coordination was determined by the lapse of time that the animals stayed on a rod rotating at an increasing speed. At around 4 months of age, the motor abilities of GM1 mice started to deteriorate, as demonstrated by a decrease in the maximum performance time established for the test (3 min). Considerable motor and balance deficit was noticed in mutant mice that were older than 5 months. The Roto-Rod test could not be performed during the late stages of the disease, due to worsening of the symptoms.

Apoptosis in the spinal cord of GM1 mice

To relate this phenotype with the progressive storage of metabolites in the brain of the GM1 mice and neurodegeneration, we determined whether apoptotic cells were present in the CNS of *β-gal-/-* mice and whether the number of these cells varied during disease progression. Spinal cord sections were stained for the presence of fragmented DNA, a characteristic hallmark of apoptosis. Compared with age-matched samples from wild-type mice, the spinal cords of 3-, 5-, 7-, and 8-month-old GM1 mice showed a significantly higher number of apoptotic cells, which appeared to increase as the disease progressed (Fig.2). This phenomenon coincided with the appearance of gross phenotypic abnormalities in mice older than 3-months. However, no cluster of apoptotic cells were seen throughout the spinal cord even in old mice, but only sporadic apoptotic cells were detected. This pattern of cell death was indicative of an ER stress response.

Induction of CHOP and BiP in spinal cord of β-gal -/- mice

To test this hypothesis we assessed the induction of expression of the transcription factor CHOP and the ER-resident chaperone BiP in spinal cord samples from wild-type and GM1 mice. The levels of CHOP mRNA in 5-month-old mutant mice were comparable to that of wild-type mice (Fig.3). In contrast, in 7–month-old animals CHOP mRNA increased significantly and remained elevated during the late stages of the disease. BiP mRNA, on the other hand, was inversely regulated compared to CHOP mRNA: the level of BiP mRNA was elevated in GM1 mice of 4 to 6 months of age, and decreased to 90% that of wild-type mice as the disease progressed (Fig.4b). This profile of BiP activation is in tune with the function of this protein during the early phases of an ER stress response. In fact, an increased level of BiP following neuronal dysfunction has been linked to neuroprotection and resistance to cell death (Yu *et al.*, 1999)

To determine whether the variation in amounts of CHOP and BiP mRNAs was paralleled by a similar regulation of the proteins, Western blot analyses were performed on spinal cord lysates from knockout and wild type animals. A clear increase in the steady-state level of CHOP in 6- and 8-month-old *β-gal-/-* mice was detected that coincided with

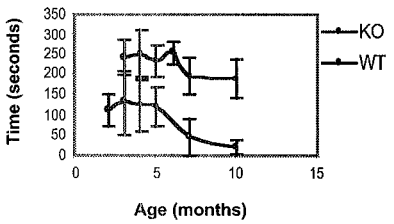

Figure 1. Coordination test of GM1 and wt mice on a rota-rod. Mice were placed on a rota-rod that accelerated from a 3 to 30 rpm, and the mice remained on the apparatus as recorded. The number of mice tested for each age ranged from 3 to 6.

the appearance of a lower Mol. Wt. band (Fig.3). The latter bands cross-reacted with antibodies specific for serine phosphate (data not shown) suggesting that CHOP activation is accompanied by phosphorylation of serine residues. This modification is known to result in feedback activation of CHOP transcription (Wang and Ron, 1996). Thus, both the transcription and translation of CHOP were upregulated. In contrast, the amount of BiP protein in spinal cord samples from the GM1 mice seemed to be comparable to that of wild types, but these results were likely due to the high steady-state levels of endogenous BiP (Fig.3a) (Kaufman, 1999).

The observed differences in the regulation of these two genes during the course of the disease suggest a balance between the pathways that regulate cell survival and cell death. Mice with G_{M1} - gangliosidosis get araund 7-10 months of age severe motor dysfunction, rapidly progressing to death (Hahn *et al.*, 1997). The increased expression of the pro-apoptotic gene CHOP and the decreased expression of the anti-apoptotic gene BiP coincide with the development of severe neuronal dysfunction, a finding that suggests that cell death contributes to the deterioration that is later observed.

Overexpression and Activation of Caspase-12

To determine whether ER stress induced cell death is mediated by activation of a caspase, we analyzed the pattern of expression of these proteases in spinal cord samples from GM1 mice older than 4 months. A clear induction of caspase-12 transcription was observed in 7-month-old knockout mice, and this increase of mRNA was associated with the development of severe neurological symptoms (Fig.5a,b). Caspase-12 is known to be essential for cell death induced by stress on the ER (Yuan and Yankner, 2000). Upon activation, a 60 kDa procaspase-12 is processed into various protein fragments of smaller size (42-and 35-kDa) that

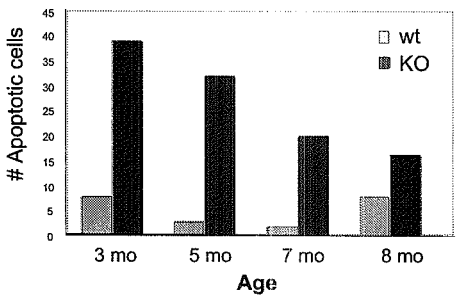

Figure 2. Apoptotic cell death in GM1 spinal cord. The number of TUNEL-positive cells per 6mm spinal cord length from GM1 and wild type littermates.

accumulate under conditions of ER stress (Rao *et al.*, 2002; Yuan and Yankner, 2000).

Immunoprecipitation analysis of spinal cord samples from wild type and GM1 mice of 6 and 8 months of age revealed an increase in the levels of procaspase-12 and of the active 42-kDa form only in the mutant mice (Fig.5c). These observations suggest that under conditions of prolonged ER stress, the sporadic but continuous cell death occurring in the spinal cord of GM1 mice is also preceded by activation of the caspase 12 pathway.

DISCUSSION

The mechanisms underlying neuronal dysfunction and neuronal degeneration in LSDs have not been elucidated. Neuronal cell death is associated with both acute and chronic neurodegenerative diseases, including Alzheimer, Huntington and Parkinson disease, and amyotrophic lateral sclerosis (Yuan and Yankner, 2000). These disorders are characterized by the deposition of protein aggregates or the formation of abnormal structures in specific neuronal populations (Imaizumi *et al.*,

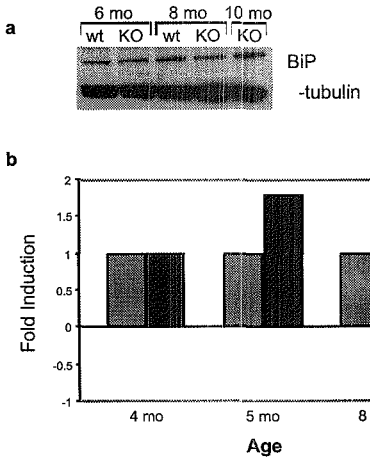

Figure 3. BiP expression in spinal cord samples of GM1 mice and littermate controls. **a**, Western blots of BiP protein in lysates (200 µg) from GM1 mice (KO) and wild type (wt) spinal cord samples. Note that protein levels are comparable when normalized with -tubulin. **b**, Differences in RNA expression levels are observed in GM1 spinal cord of 5-and 8- months old mice when analyzed by Northern blot and RPA and normalized with β-actin.

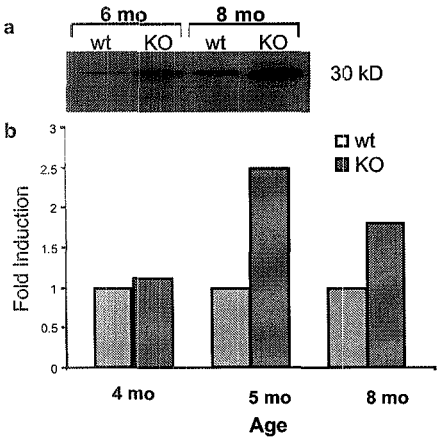

Figure 4. CHOP induction in spinal cord samples of GM1 mice. **a**, Protein levels of CHOP were analyzed by Western blotting 200 µg total lysates of spinal cord from 6- and 8-months wild type (wt) and deficient mice (KO). **b**, Northern blot and RPA analysis of CHOP and β actin of spinal cord samples at various ages of wild type and GM1 mice.

2001; Kaufman, 1999; Yuan and Yankner, 2000); both features are thought to be responsible for neuronal cell toxicity and the pathogenesis of these disorders. By analogy, abnormal accumulation of undegraded metabolites in lysosomes could be the origin of neuronal apoptosis in G_{M1}-gangliosidosis, although a direct correlation has not been demonstrated yet.

We have hypothesized that the neurodegeneration characteristic of GM1 mice could be the result of an ER-stress response. The observation that BiP, CHOP and caspase-12, are deregulated in these mice, confirmed this hypothesis. The fact that BiP is upregulated is no surprise considering that this molecular chaperone is one of the primary and most important molecules that control the early response of a cell to stress (Kaufman, 1999). BiP plays a pivotal role in preventing ER stress induced damage in order for the cell to survive. The increase in CHOP expression, on the other hand, coincides with the development of severe neuropathological symptoms and cell death. Several lines of evidence point to CHOP as a mediator of apoptosis upon ER stress: *Chop* -/- mouse embryonic fibroblasts exhibit significantly less apoptosis when challenged with ER stress-inducing agents (Zinszner *et al.*, 1998); CHOP-mediated apoptosis, following its transcriptional activation by ATF6, another ER stress induced transcription factor, has also been reported (Gotoh *et al.*, 2002). The precise signaling pathway following the activation of CHOP remains to be elucidated (Wang *et al.*, 1998) although a set of genes referred to as DOCs for downstream of CHOP have been identified that could be involved in this pathway. Our findings point to CHOP as the potential mediator of the neuronal apoptosis observed in GM1 mice. This hypothesis is further substantiated by the activation of caspase-12. Until recently, the only cellular compartments implicated in apoptotic processes were the mitochondrion and the plasma membrane. New studies have revealed that ER stress can induce cell death by a mechanism that is independent of the previously described apoptotic pathways (Rao *et al.*, 2002). The identification of a predominantly localized ER caspase family member, procaspase-12, whose activation is triggered specifically by disturbances in ER homeostasis, has implicated this subcellular compartment and in particular caspase-12 in the apoptotic execution (Nakagawa *et al.*, 2000).

The mechanisms by which β-galactosidase deficiency and, in turn, lysosomal accumulation of gangliosides could activate or render cells susceptible to an ER stress-mediated cell death are not fully understood. It is possible that neuronal accumulation of undegraded metabolites, in particular G_{M1} ganglioside, in the endosomal-

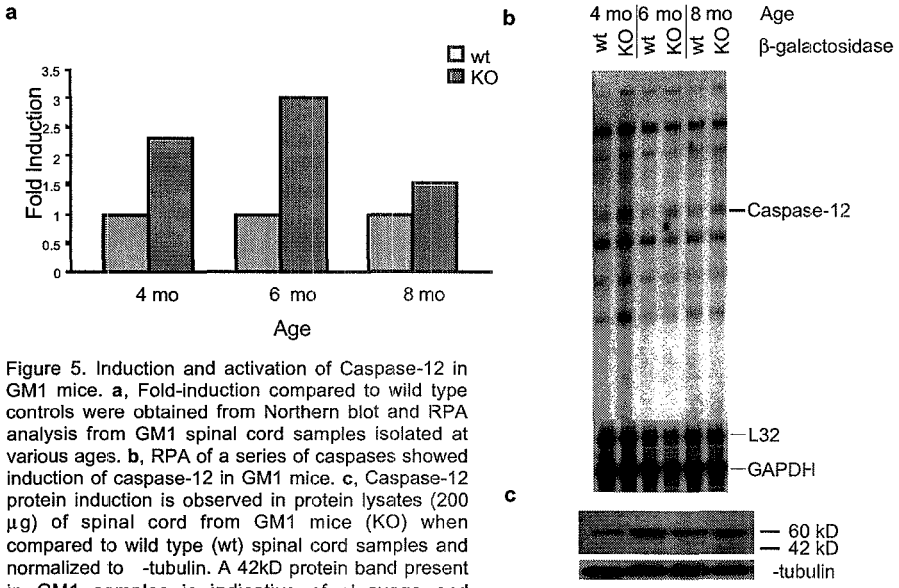

Figure 5. Induction and activation of Caspase-12 in GM1 mice. **a**, Fold-induction compared to wild type controls were obtained from Northern blot and RPA analysis from GM1 spinal cord samples isolated at various ages. **b**, RPA of a series of caspases showed induction of caspase-12 in GM1 mice. **c**, Caspase-12 protein induction is observed in protein lysates (200 µg) of spinal cord from GM1 mice (KO) when compared to wild type (wt) spinal cord samples and normalized to -tubulin. A 42kD protein band present in GM1 samples is indicative of cleavage and activation of procaspase-12.

lysosomal compartment hampers the overall degradative capacity of the organelle, results in a redistribution of the ganglioside or other by-products to different cellular sites, and disrupt ER homeostasis. Although the exact intracellular localization of the accumulated ganglioside during the course of the disease remains to be evaluated, we hypothesize that it may begin in the trans Golgi/endosomal network where the biosynthetic and degradative pathways of plasma membrane-derived glycosphingolipids converge (Kolter and Sandhoff, 1998; Wilkening *et al.*, 1998). In addition, G_{M1}-ganglioside has been implicated in intracellular efflux of Ca^{2+} (Ledeen *et al.*, 1998), and it is known that neuronal cell injury can be induced by alterations in the intracellular levels of Ca^{2+} (Paschen and Frandsen, 2001). Failure to overcome disturbances in calcium homeostasis may also contribute to neuronal susceptibility to ER stress-mediated cell death (Mengesdorf *et al.*, 2001). It is therefore possible that accumulation of G_{M1}-ganglioside affect Ca^{2+} levels and results in ER stress.

Overall our results have unraveled a novel pathway in G_{M1}-gangliosidosis that could explain the cause of neuronal death and could be applied to other neurodegenerative lysosomal disorders.

MATERIALS AND METHODS

Antibodies

Mouse monoclonal antibody (mAb) specific to GADD 153 (sc-7351) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rat monoclonal caspase-12 antibody was a gift of Dr. Junying Yuan, Harvard Medical School, Boston. Rabbit polyclonal BiP antibody (SPA-826) was obtained from StressGen. Mouse monoclonal -tubulin antibody (T 5168) was obtained from Sigma (St Louis, MO).

Mouse tissues collection

All animal procedures were carried out in accordance with the U.S. Public Health Service Policy on the Humane Care and Use of Laboratory Animals. Both wild type and β-gal KO mice were euthanized at the age of 3-8 months. For TUNEL assay, mice were perfused with 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.4. Spinal cords were processed for paraffin embedding and sequential coronal sections were cut. For RNA or protein extractions, the spinal cords were removed and snap-frozen in liquid nitrogen.

TUNEL assay

The TUNEL assay was performed on 7µm-thick coronal sections of 3- to 8-month-old animals. Apoptotic cells were detected on a total length of 6mm spinal cords by use of the ApopTag in situ apoptosis detection kit (Intergen, NY). Briefly, tissue sections were deparaffinized, rehydrated, and subject to Proteinase K treatment, 15 min at 25°C. Apoptotic cells were enzymatically labeled at the DNA level using terminal deoxynucleotidyl transferase (TdT) 1h at 37°C and revealed using anti-digoxigenin peroxidase. The counterstain was peformed in 0.5% (w:v) methyl green in 0.1M sodium acetate pH4.

Protein extraction and western blot

Approximately 50 mg of spinal cord tissue was homogenized, using a Teflon homogenizer (Thomas), in three volumes of low detergent buffer (0.05M Tris-HCl pH=7.5, 0.15M NaCl, 0.5% DOC, 0.5% NP-40, 0.02% sodium azide, 1 mM PMSF, 2 mg/ml aprotinin, 10 mg/ml leupeptin, 5 mg/ml pepstatin and 12.5 µg/ml of N-acetyl-Leu-Leu-Norleu-Al). The samples were briefly sonicated to reduce the viscosity. Protein concentrations were determined with the BCA protein assay reagent (Pierce Chemical Co). Protein extracts (200µg) were subjected to immunoprecipitation using specific antibody. Following an overnight incubation at 4°C, GammaBind-Sepharose (Amersham Pharmacia) was added to the samples and incubated at RT for an additional hour. Immunoprecipitated proteins were separated by SDS-PAGE (12.5% gel) under reducing conditions, followed by transfer to PVD membranes (Millipore). Membranes were incubated overnight in blocking buffer and probed for 3 hours at RT with either anti-CHOP mAb, anti-BiP anti-caspase-12, or anti-phosphoserin antibodies. -Tubulin was used as a gel-loading control. The blots were developed with the enhanced chemiluminescence kit (NEN-Perkin Elmer Life Sciences).

RNA extraction and Northern Blot Analysis

Total RNA and poly(A)+ RNA were isolated from spinal cords using TRIzol Reagent (Invitrogen) and Oligotex mRNA purification kit (Qiagen, Valencia, CA). mRNA (2µg) was separated on a 1% agarose gel (0.66M formaldehyde), blotted onto a Zeta-Probe-GT membrane (BioRad) and hybridized in ExpressHyb (Clontech) at 68°C with DNA or cDNA probes. The blots were washed according to the manufacturer's protocol (Clontech), followed by autoradiography. The quantification was performed by phosphorimager (Molecular Dynamics, Amersham Pharmacia).

RNase Protection Assay (RPA)

For the detection and quantification of the caspase-12 mRNA, the RPA was performed with the RiboQuant Multi-Probe RNase Protection Assay System (PharMingen), by using the mAPO-1 Multi-Probe Template Sets, according to the manufacturer's procedure. In brief, mAPO-1 probe ($3.6x10^5$ cpm/µl) and 15µg of each RNA sample were combined, ethanol precipitated and resuspended in hybridization buffer (PharMingen). For CHOP and BiP RPAs, the probes were obtained by PCR (CHOP nucleotides 26-522; BiP nucleotides 529 and 1019). In both cases the PCR primers included some non-homologous sequence. The CHOP and BiP riboprobes were synthesized from 1µg of purified PCR products by using the MaxiScript/T7 polymerase kit (Ambion, Austin, TX) and ^{32}P UTP (800 Ci/mmol, 10 mCi/ml, NEN Life Science, Boston MA), as recommended by the manufacturer. DNA templates were removed by Dnase I digestion. All ^{32}P-labelled riboprobes were separated by 5% polyacrylamide/8 M urea gel electrophoresis and gel purified. Specific radioactivities of the Chop and BiP probes were $1.6x10^4$, $1.8x10^4$cpm per 20 µg of total RNA, respectively. 250 bp of mouse β-actin was used as a gel-loading control. After hybridization, samples were ethanol precipitated, resuspended in gel loading buffer (PharMingen), denatured at 90°C for 3 min, and fractionated on a 7.5% polyacrylamide/8 M urea gel, followed by autoradiography. The quantification was performed on phosphorimager.

ACKNOWLEDGEMENTS

We thank Dr. Linda Hendershot, and Dr. Yanjun Ma for providing us with reagents and useful suggestions. We thank Linda Mann for her technical assistance. These studies were supported in part by National Institutes of Health grant RO1-GM60950, the Assisi foundation of Memphis, the Cancer Center Support Grant (CA 21765), and the American Lebanese Syrian Associated Charities (ALSAC) of St. Jude Children's Research Hospital.

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CHAPTER 5

Lack of expression only partially coincides with lysosomal storage in galactosialidosis mice: indirect evidence for spatial requirement of the catalytic rather than the protective function of PPCA.

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Hum Mol Genet 1998 **7 (11)**: 1787-94

Human Molecular Genetics, 1998, Vol. 7, No. 11 1787–1794

Lack of PPCA expression only partially coincides with lysosomal storage in galactosialidosis mice: indirect evidence for spatial requirement of the catalytic rather than the protective function of PPCA

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Received 29 May 1998; Revised and Accepted 19 July, 1998

Protective protein/cathepsin A (PPCA) is a pleiotropic lysosomal enzyme that complexes with β-galactosidase and neuraminidase, and possesses serine carboxypeptidase activity. Its deficiency in man results in the neurodegenerative lysosomal storage disorder galactosialidosis (GS). The mouse model of this disease resembles the human early onset phenotype and results in severe nephropathy and ataxia. To understand better the pathophysiology of the disease, we compared the occurrence of lysosomal PPCA mRNA and protein in normal adult mouse tissues with the incidence of lysosomal storage in PPCA(–/–) mice. PPCA expression was markedly variable among different tissues. Most sites that produced both mRNA and protein at high levels in normal mice showed extensive and overt storage in the knockout mice. However, this correlation was not consistent as some cells that normally expressed high levels of PPCA were unaffected in their storage capability in the PPCA(–/–) mice. In addition, some normally low expressing cells accumulated large amounts of undegraded products in the GS mouse. This apparent discrepancy may reflect a requirement for the catalytic rather than the protective function of PPCA and/or the presence of cell-specific substrates in certain cell types. A detailed map showing the cellular distribution of PPCA in nomal mouse tissues as well as the sites of lysosomal storage in deficient mice is critical for accurate assessment of the effects of therapeutic interventions.

INTRODUCTION

Lysosomal protective protein/cathepsin A (PPCA) is a serine carboxypeptidase that forms a high molecular weight multienzyme complex with acid β-D-galactosidase and *N*-acetyl-α-neuraminidase (1–6). PPCA's role in this complex is to facilitate the intracellular routing, lysosomal localization and activation of neuraminidase (7,8), and to stabilize β-galactosidase in the lysosomal environment (5,9). Genetic lesions in the PPCA gene that abolish its protective function cause severe loss of neuraminidase activity, and render β-galactosidase susceptible to rapid intralysosomal proteolysis (10,11). The resulting combined enzyme deficiency is the basis of the lysosomal storage disorder galactosialidosis (GS), which is characterized by the primary accumulation of sialylated oligosaccharides and glycopeptides in patient tissues and body fluids (12–15). Patients with GS present clinically with one of three forms of the disease (early infantile, late infantile or juvenile/adult), depending on their age at onset and the severity of their symptoms (16). The early onset patients, who completely lack PPCA activity, die within the first months of life from heart and kidney failure. The mouse model of GS closely resembles the phenotype of early infantile patients (17). Extensive vacuolation of certain cells in most organs is caused by the abnormal accumulation of undigested metabolites that result primarily from the severe secondary deficiency of lysosomal neuraminidase.

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As a member of the serine protease family, PPCA exerts cathepsin A activity at acidic pH and esterase/C-terminal deamidase activity at neutral pH. The catalytic activity of PPCA, which is fully separable from its protective function (18), is apparently important for the initial catalysis of selected bioactive peptides, including substance P, oxytocin and endothelin I (19–22). However, the physiological significance of this enzyme activity has not yet been proven. Recently, we identified the minimal promoter regions of the human and mouse PPCA genes (23). Transcription of the murine gene, unlike that of the human gene, gives rise to two differentially expressed transcripts that contain the same protein-encoding region. The more abundant 1.8 kb species is expressed in all tissues and originates from a promoter with features characteristic of housekeeping gene promoters (23). A minor murine-specific 2.0 kb mRNA is transcribed from a TATA box-containing promoter and is expressed at lower levels in specific tissues.

Here, we studied the *in vivo* expression of PPCA in adult murine tissues by using *in situ* hybridization and immunocytochemistry. Our aim was to correlate the distribution pattern of PPCA mRNA and protein in normal mice with the disease pathology in the knockout mouse. Our findings demonstrate that PPCA expression is not ubiquitous in normal adult tissues, but rather is confined preferentially to specific cells, especially those that are metabolically active. Most regions that express PPCA at high levels develop overt morphologic changes in the knockout mouse. Interestingly, however, there are also cell types where the correlation between gene expression and the occurrence of lysosomal storage is not maintained. We discuss how this result could suggest a role for the catalytic rather than the protective function of PPCA.

RESULTS

The distribution of PPCA mRNA and protein in the tissues of normal adult mice was analyzed using *in situ* hybridization and immunocytochemistry, respectively. The presence of lysosomal storage material in defined cell populations of GS mice was evidenced by periodic acid Schiff (PAS) staining and lysosomal vacuolation.

Brain

Despite a generalized expression of PPCA in the brain, regional and cellular differences were obvious. The frontal, parietal and occipital lobes of the cortex displayed a moderate signal throughout the inner five layers, whereas the external layer, which contains unmyelinated axons, was devoid of PPCA-specific signal (Fig. 1A). The inferior colliculus and cells within the meningeal layers also contained PPCA transcripts and protein. The expression in the cortex and the diencephalon was confined primarily to neuronal cell bodies rather than to nerve fibers. Sustained expression of PPCA mRNA was detected throughout the olfactory bulb, being most prominent at the level of the mitral cell layer (Fig. 1B). This was paralleled by strong punctated PPCA staining in the large mitral cells (Fig. 1C). Weak expression was present in the granular cells and the periglomerular neurons. The axons of the mitral cells are in contact with the olfactory cortex, which consists of five defined regions: the anterior olfactory nuclei, the tuberculum olfactorium, the pyriform cortical neurons, the amygdala and the entorhinal cortex. All of these areas, which belong to the limbic system, and in particular the tuberculum olfactorium and the pyriform nuclear cells, expressed PPCA mRNA and protein at high levels (not shown). The pyramidal neurons of the hippocampus and the granular cells of the dentate gyrus were also highly positive for both mRNA and protein (Fig. 1E and F). Only basal expression levels were detected in the caudate putamen, the thalamic and hypothalamic regions, and the substantia nigra (not shown). In the cerebellum, a strong positive signal was confined to the Purkinje cells, whereas the molecular and granular layers had significantly lower expression (Fig. 1H and I). The large neurons of the cerebellar nucleus also stained strongly. Like the cerebrum and the cerebellum, the brain stem expressed PPCA primarily in gray matter nuclei, containing the cell bodies of the different neurons. Both PPCA transcripts and protein were very prominent in the epithelial cells of the choroid plexus, whereas expression in the ependymal cells that line the cerebral ventricles was low (Fig. 1K and L). Strong expression was detected in the perivascular and leptomeningeal macrophages.

In general, PPCA expression in the brain correlated well with the distribution of vacuolated cells with lysosomal storage in the PPCA(–/–) mouse. PAS-positive cells appeared early in life. At 2 months of age, they were detected only in the anterior olfactory nucleus, the amygdala and the entorhinal cortex (not shown). Occasional Purkinje cells were also positive. As the disease progressed, the number of accumulating cells became more conspicuous in these regions; other neurons also appeared positive, including mitral cells (Fig. 1D), neurons of the 3rd–4th cerebral cortical layer, scattered cells of the thalamus and hypothalamus, and the large neurons of the cerebellar nucleus and brain stem (not shown). Interestingly, PAS-positive staining differed in different vacuolated cell types, probably reflecting variation in the composition of storage products. The choroid plexus, for example, showed clear morphological changes without evident PAS staining (Fig. 1M). Contrary to what might be expected from the high PPCA expression in the pyramidal neurons of the hippocampus and the granular cells of the dentate gyrus, very few PAS-positive or vacuolated cells were detected in these areas (Fig. 1G). This apparent inconsistency could be attributed to differences in the metabolic state of these cells or to the type of macromolecular substrates. Furthermore, there appeared to be a variable effect of the storage products on cell viability. Purkinje cells display few large PAS-positive granules prior to cell death (Fig. 1J), whereas cerebellar basket cells, and cells of the amygdala and anterior olfactory nucleus, become filled with PAS-positive material prior to obvious signs of cellular deterioration (not shown). It should also be noted that the undegraded products accumulate first in the Purkinje cells of the more anterior lobules which also die earliest. As the disease progresses, however, Purkinje cells in more posterior regions store and die. Loss of these cells contributes to the progressive ataxia seen in these mice (17). Interestingly, a similar feature was observed in other mouse models of lysosomal storage disorders such as Niemann–Pick A and C (24–27).

Visceral organs

PPCA mRNA expression was distributed throughout the liver (Fig. 2A). Immunocytochemistry clearly demonstrated that the protein expression was moderate in hepatocytes and high in Kupffer cells (Fig. 2B), reflecting the storage pattern detected in

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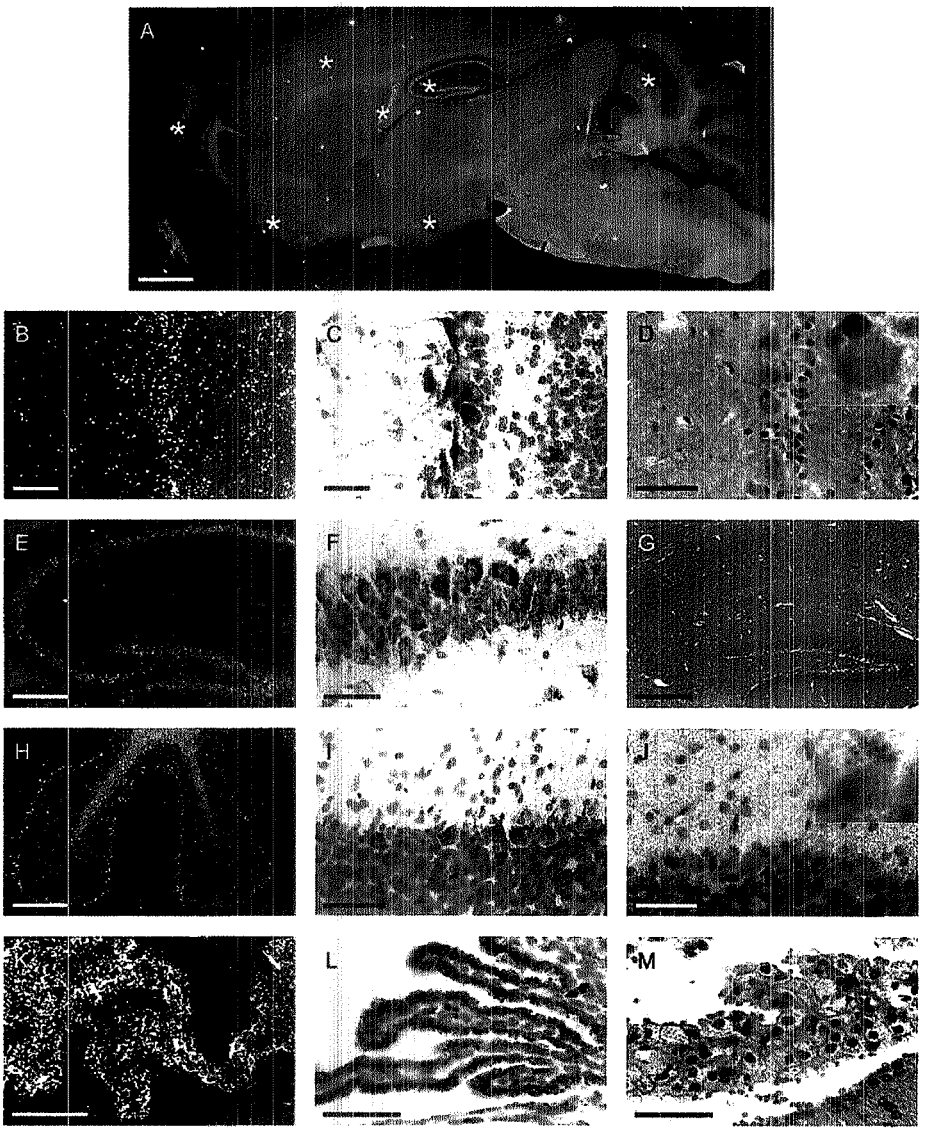

Figure 1. Comparison of PPCA mRNA and protein expression in wild-type mouse brain with lysosomal storage in PPCA(–/–) mouse brain. Sagittal brain sections demonstrate the localization of mouse PPCA mRNA expression by *in situ* RNA hybridization (**A**, **B**, **E**, H and K) and protein distribution by immunocytochemistry with PPCA antibodies (**C**, **F**, I and L) in relation to the accumulation of undegraded lysosomal products in the PPCA(–/–) mouse (D, G, J and M). (**A**) An overview of a dark-field image of a mouse brain; the asterisks mark the regions of high PPCA mRNA expression. Representative areas show PPCA expression and accumulated storage in the mitral cell layer of the olfactory bulb (**B–D**), the hippocampus and dentate gyrus (**E–G**), the Purkinje cell layer of the cerebellum (**H–J**) and the choroid plexus (**K–M**). Note the PAS-positive storage granules in the mitral (D) and Purkinje cells (J), the lack of storage in the hippocampus and dentate gyrus (G), and the PAS-negative storage vacuoles in the choroid plexus (M). Mice of age 2–6 months were used for *in situ* RNA hybridization and immunocytochemistry, and mice of 10–13 months were used for PAS staining although storage was visible in some cells earlier. All *in situ* RNA hybridization and PAS staining were performed on frozen sections, and immunocytochemistry was performed on paraffin sections. Scale bar = 1 mm (A), 250 μm (E, G and H), 50 μm (B–D, F and I–M).

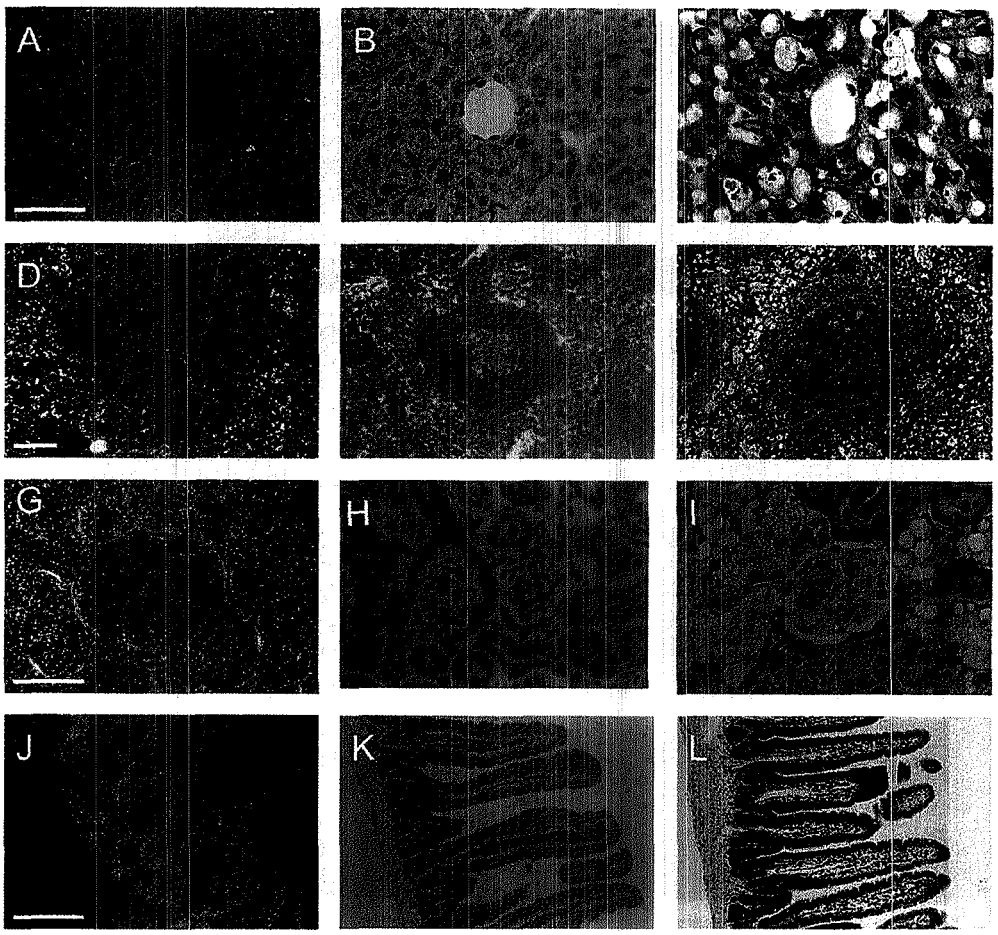

Figure 2. Comparison of PPCA mRNA and protein expression in wild-type mouse tissues with lysosomal storage in PPCA(–/–) mouse tissues. PPCA mRNA expression was analyzed in wild-type mice (2–4 months) by *in situ* RNA hybridization (A, D, G and J), the distribution of protein was detected by immunocytochemistry with PPCA antibodies (B, E, H and K) and lysosomal storage products were visualized in PPCA(–/–) mice (2–4 months) by PAS staining (C, F, I and L). Liver (**A–C**), spleen (**D–F**), kidney (**G–I**) and small intestine (**J–L**) are shown. Note that most visceral organ storage products are PAS-negative and appear as vacuoles. Scale bar = 50 μm (A–I), 150 μm (J–L).

the knockout mouse, where Kupffer cells are more affected than hepatocytes (Fig. 2C). The red pulp of the spleen showed higher protein and mRNA expression than the white pulp, probably because of the numerous macrophages that express high levels of PPCA (Fig. 2D and E). Consistent with this, the pathology of the spleen in the (–/–) mouse showed macrophages filled with vacuoles, whereas lymphocytes and megakaryocytes displayed little or no signs of storage. As a result of accumulating products in the liver and spleen, the affected mice develop hepatosplenomegaly, a clinical feature associated with the early and sometimes late infantile forms of the human disease (16).

In the kidney of wild-type mice, PPCA expression was high in the cuboidal epithelium of the proximal convoluted tubules and somewhat lower in the distal convoluted tubules of the nephron (Fig. 2G and H). The epithelial cells of Bowman's capsule were highly positive, whereas the glomerulus expressed very low levels that could be seen only weakly by immunocytochemistry (Fig. 2H). No expression was detected in the renal medulla. The epithelia of the proximal convoluted tubules and the Bowman's capsule were the first sites to store overtly in PPCA(–/–) mice (Fig. 2I). Interestingly, although the expression was very low in the glomerulus of the wild-type mouse, this region of the deficient

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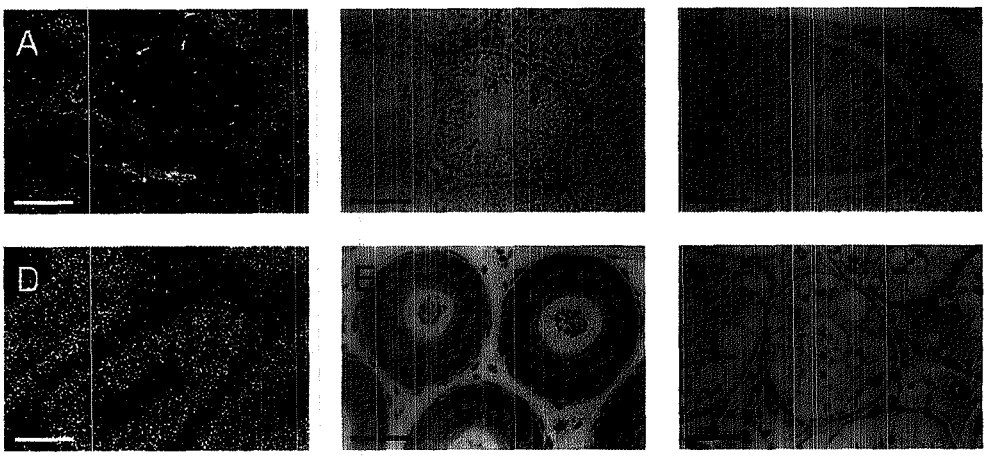

Figure 3. Comparison of PPCA mRNA and protein expression in wild-type mouse with lysosomal storage in PPCA(–/–) mouse male reproductive organs. PPCA mRNA expression was analyzed in wild-type mice by *in situ* RNA hybridization (A and D), the distribution of the protein was detected by immunocytochemistry with PPCA antibodies (B and E) and lysosomal storage products were visualized in the PPCA(–/–) mouse by PAS staining (C and F). Shown are the seminiferous tubules and interstitial tissue of the testis (**A–C**) and the caput of the epididymis (**D–F**). Mice ranged in age from 2 to 4 months. Scale bar = 90 μm (A–C), 35 μm (D–F).

mouse displayed high levels of storage (17). This severe renal pathology in knockout mice resulted in diffuse edema and closely paralleled the nephrotic syndrome seen in GS patients (16).

In the duodenum and jejunum of the small intestine, the crypts of Lieberkuhn expressed high levels of PPCA mRNA, but little protein, whereas the mucosal layer of the microvilli expressed moderately (Fig. 2J and K). In (–/–) mice, storage products or vacuolation were not detected in these cells (Fig. 2L). However, foamy macrophages were seen in the interstitial space of the mucosa, submucosa, muscular outer layer and the lymphoid Peyer's patches (not shown).

Heart and skeletal muscle appeared to have an overall, low PPCA expression. In muscle fibers, the knockout mouse displayed vacuolation only within endothelial cells and macrophages, but showed no signs of storage. The adrenal gland showed high expression in a specific rim of cells, probably the X zone, which is loosely defined between the cortical and the medullar cell layers (not shown). Cells in this layer secrete glucocorticoids and androgens, and are only present in sexually immature male and virgin female mice (28). PPCA(–/–) mice did not show detectable levels of storage products in the adrenal gland.

Reproductive system: testis and epididymis

Within the seminiferous tubules of the testis, PPCA expression was observed in the Sertoli cells, which provide nutrients to the developing spermatocytes. No expression was noted in the spermatogonia or spermatocytes (Fig. 3A and B). Moderate mRNA and protein levels were also detected in the interstitial cells of Leydig, which surround the seminiferous tubules and secrete testosterone under the influence of luteinizing hormone. Interestingly, the PPCA signal varied along the length of the epididymis. Most proximal to the testis, in the caput epididymis, PPCA levels were very high in the columnar epithelial cells of the ducts with little or no apparent expression in the supportive connective tissue (Fig. 3D and E). Further along through the corpus of the epididymis, the number of expressing cells decreased to the cauda, where only a few expressed high levels of the protein (data not shown).

In the (–/–) mice, extensive vacuolation occurred in all high expressing cells and, in addition, in the interstitial cells (Fig. 3C and F). Marked vacuolation of the epididymis and Leydig cells was already visible at 2 months of age, and these cells remained viable, but markedly affected, for the life of the animals.

Ovary and uterus

The zona pellucida and the granulosa cells, that surround the developing follicle and serve as support and/or feeder cells for the follicle, expressed high levels of PPCA transcripts (data not shown). The corpus luteum, which originates from the differentiated granulosa cells after follicle maturation and secretes estrogen and progesterone, displayed even higher levels of mRNA and protein, whereas expression was very low in the interstitial cells (not shown). High expression was also apparent in the columnar epithelial cells and branched tubular glands of the uterus and, during pregnancy, within the rapidly dividing endometrium/trophoblasts. In the affected mouse, foamy macrophages infiltrated the stroma of the ovary and uterus, but the secretory epithelial cells appeared normal (data not shown).

DISCUSSION

It is becoming increasingly clear that the notion of lysosomal genes as simple housekeeping genes (i.e. requiring little or no regulation) may be inaccurate. These enzymes are expressed at basal levels in virtually all cells, but are expressed differentially in specific cell types. For instance, murine prosaposin mRNA is seen in Leydig, Sertoli and peritubular cells but not in the

spermatogenic cells of the testis (29,30). In contrast, acid phosphatase is detected only in the spermatocytes of this organ (31). Acid lipase is expressed basally throughout the testis (32), while acid α-glucosidase is seen in the seminiferous tubules but not in the spermatogonia (33). In this study, we have identified another pattern of expression in the testis, that of PPCA being evident in Leydig and Sertoli cells but not in spermatogenic cells. Similar differential expression for PPCA mRNA and protein exists for virtually all other tissues of the body, being highest in metabolically active or phagocytic cells. This is particularly evident in specific subpopulations of neurons.

The distribution pattern of PPCA and other lysosomal enzymes necessitates some kind of regulation that cannot be anticipated by the housekeeping characteristics of their promoters. Regulation of each of these genes could occur at the transcriptional and/or post-transcriptional levels. It has been reported that human aspartylglucosaminidase is regulated predominantly at the translational level (34). Moreover, the activity of lysosomal enzymes that rely on other proteins or cofactors/activators for function may be modulated in a more complex fashion. For example, some lysosomal enzymes require other lysosomal proteins for intracellular transport and stability (neuraminidase/PPCA), activity (sphingolipid degradative enzymes/saposins, β-hexosaminidase/G_{M2}-activator) or substrate specificity (homo- and heterodimers of β-hexosaminidase) (8,29,30,35–37). Despite differences in the expression patterns of many lysosomal proteins, it is noteworthy that their interacting and cooperating proteins are usually co-expressed in specific cells. Little is known about the physiological and pathological stimuli or molecular mechanisms that affect the activity of lysosomal enzymes; however, it is clear that steroid hormones, such as androgens and estrogens, have a general inductive effect on the activity levels of many lysosomal enzymes (38–40).

Comparing PPCA levels in normal mouse tissues with the distribution of storage in PPCA knockout mice revealed that, in most cell types, a higher level of expression correlated with more extensive storage. This was particularly evident in the brain where specific neurons, such as the Purkinje and mitral cells, and the cells of the amygdala and entorhinal cortex displayed the most extensive storage. In some neuronal cells such as those of the hippocampus and dentate gyrus, however, this pattern was not maintained. Also, the glomerulus of the kidney expressed virtually no PPCA and yet that of the PPCA(–/–) mouse stored extensively. This apparent discrepancy between endogenous expression levels and the degree of storage in diseased mice could be due to a difference in the spectrum and amounts of substrates, derived during normal cellular processes. Alternatively, PPCA may be expressed in selected cells primarily for its enzymatic function on bioactive peptides, such as oxytocin, substance P or endothelin I (19–21), rather than for its ability to protect neuraminidase and β-galactosidase. In this latter situation, undegraded products would not be expected to accumulate in the knockout model. It is worth noting that the substance P receptor (NK-1 receptor) is well expressed in the hippocampus (41,42); however, it remains to be seen whether the primary requirement for PPCA in these cells is for its role in neuropeptide inactivation.

In PPCA(–/–) mice, we observed differences in the storage within different cell types. Purkinje cells accumulated only a few large PAS-positive storage granules prior to cell death, whereas cerebellar basket cells, and the cells of the amygdala and anterior olfactory nucleus, were almost completely full of fine storage granules before they deteriorated. Epithelial cells of the choroid plexus, renal proximal convoluted tubule and caput epididymis accumulated PAS-negative material and appeared to survive for the entire lifespan of the animal. From these results, it seems clear that some stored intermediates have more toxic effects on cell viability than do others, or that certain cells may be more vulnerable to toxicity. In this regard, both G_{M2}-ganglioside and one of its downstream degradation products, ceramide, have been implicated in the induction of apoptosis (43–47).

The lifespan of cells may also play a role in the correlation between PPCA expression and storage. In the small intestine, we found PPCA mRNA transcripts in the crypts of Lieberkuhn and expression of the protein only in the mucosal epithelium of the microvilli. This is consistent with the newly dividing mucosal cells, differentiating in the crypts to express PPCA transcripts and then translating these transcripts into protein as they move toward the tips of the microvilli. As might be expected, in PPCA(–/–) mice, storage products were never apparent in these cells, probably because their lifespan is too short to allow for accumulation at detectable levels.

The reproductive organs also differentially express PPCA, which is not surprising as lysosomal enzymes have been implicated in reproduction for many years (38,48–51). Both male and female mice express PPCA differentially at specific sites. In the knockout mice, the lack of PPCA considerably disturbs the reproductive capacity of the mice. Although fertile, they mate poorly, and the frequency of pregnancies is less than that for wild-type mice. Litter sizes and delivery, however, appear normal. We do not know at the moment whether this reduced number of pregnancies, which worsens with age, is a result of decreased fertility or whether it is secondary to pathological effects on other organs, including the brain, which may affect performance. It is noteworthy that one of the peptide hormones known to be inactivated by PPCA is oxytocin (19). The hormone is found in the corpus luteum, which also expresses PPCA at high levels, and stimulates growth during early blastocyst development (52). Oxytocin and its receptor are also important for parturition and lactation in mammals (53–60). It is unclear whether the absence of PPCA in the knockout mouse model hampers processing or clearance of oxytocin in the uterus, which could have detrimental effects on the continuing pregnancy. Curiously, it has been reported that mothers of human GS patients, who have 50% PPCA activity, often suffer from spontaneous abortions (61).

This detailed study highlights some important factors regarding the expression of lysosomal enzymes and the consequences of their functional loss. It is clear that there are marked differences in the expression levels of lysosomal enzymes in different cell types. Questions remain as to the specific cellular distribution of enzyme substrates and the differential toxicity of accumulated substrates in different cell types throughout the body. A detailed map of expression and storage will be extremely valuable in the accurate assessment of correction following therapeutic regimes.

MATERIALS AND METHODS

Materials

Chemicals were obtained from standard commercial suppliers. [α-^{35}S]UTP (>1000 Ci/mmol; Amersham), was used to prepare

riboprobes for *in vitro* transcription assays, which included either T7 or T3 RNA polymerase (Promega Biotec).

In situ RNA hybridization

FVB mice (8–12 weeks old) were anesthesized sublethally with avertin and subsequently perfused with freshly prepared 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Tissues were isolated and post-fixed for 2–4 h, before being processed for cryostat sectioning. Sections (10–14 µm) were placed on Superfrost/Plus (Fisher Scientific) glass slides and hybridized for 16–20 h at 50°C with a 272 bp murine PPCA ^{35}S-labeled riboprobe (position 904–1176 bp of the mouse PPCA cDNA), as previously described (23,62). This probe detects both the predominant ubiquitous 1.8 kb and the minor 2.0 kb PPCA mRNA. The washed slides were dipped in photographic emulsion (Kodak NTB-2) and developed after 5–8 days with Kodak D-19 developer and fixer. The slides were then counterstained with 0.1% toluidine blue and mounted.

Immunocytochemical and histological staining

Mice were perfused via the left cardiac ventricle at a rate of 3.5 ml/min for 2 min with phosophate-buffered saline (PBS) to remove circulating blood cells. They were perfused further for 15 min with 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.4. Tissues were removed and fixed for a further 4 h in the same fixative before being paraffin embedded. Tissue sections (10 µm for brain; 4 µm for all other tissues) were deparaffinized and rehydrated and the antigen was retrieved by microwave boiling in 0.1 M citrate, pH 6.0, and cooling for 30 min. The sections were then blocked in PBS (containing 0.05% Tween-20 and 0.1% bovine serum albumin) and 10% normal goat serum for 30 min before being incubated overnight at room temperature with anti-mouse PPCA antibodies. The latter were raised in rabbit against the native mouse PPCA precursor, overproduced in insect cells infected with a PPCA recombinant baculovirus construct. The polyclonal antibody preparation was shown previously to be monospecific for the mouse 54 kDa precursor and mature subunits (63). The Vector ABC–HRP system was used for detection. After a secondary antibody incubation of 2 h, endogenous peroxidase activity was removed by adding PBS containing 0.3% hydrogen peroxide and 0.02% sodium azide for 30 min. The ABC–HRP complex was then added for 1 h and visualized by adding a VIP substrate (Vector) for 3–8 min. Nuclei were counterstained with methyl green. For histological staining with hematoxylin/eosin, tissues were paraffin embedded (see above), and, for PAS staining, tissues were processed for frozen sectioning (17).

ACKNOWLEDGEMENTS

We are indebted to Dr Holly Soares for precious assistance with the *in situ* RNA hybridization. Thanks are also owed to Charlette Hill (Chill) for help in typing the manuscript. This work was supported by the Assisi Foundation of Memphis (328503) National Institutes of Health grants 1-RO1–DK32025-01 (to A.d.A.), 1-RO1-NS24533 and P30 HD 03110 (to K.S.). These studies were supported in part by the Cancer Center CORE grant CA-21765 and by the American Lebanese Syrian Associated Charities (ALSAC).

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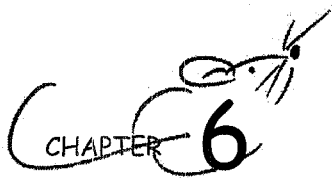

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Proc Nat Acad Sci 1998 **95**:14880-5.

Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 14880–14885, December 1998
Genetics

Correction of murine galactosialidosis by bone marrow-derived macrophages overexpressing human protective protein/cathepsin A under control of the colony-stimulating factor-1 receptor promoter

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ABSTRACT **Galactosialidosis (GS) is a human neurodegenerative disease caused by a deficiency of lysosomal protective protein/cathepsin A (PPCA). The GS mouse model resembles the severe human condition, resulting in nephropathy, ataxia, and premature death. To rescue the disease phenotype, GS mice were transplanted with bone marrow from transgenic mice overexpressing human PPCA specifically in monocytes/macrophages under the control of the colony stimulating factor-1 receptor promoter. Transgenic macrophages infiltrated and resided in all organs and expressed PPCA at high levels. Correction occurred in hematopoietic tissues and nonhematopoietic organs, including the central nervous system. PPCA-expressing perivascular and leptomeningeal macrophages were detected throughout the brain of recipient mice, although some neuronal cells, such as Purkinje cells, continued to show storage and died. GS mice crossed into the transgenic background reflected the outcome of bone marrow-transplanted mice, but the course of neuronal degeneration was delayed in this model. These studies present definite evidence that macrophages alone can provide a source of corrective enzyme for visceral organs and may be beneficial for neuronal correction if expression levels are sufficient.**

Lysosomal storage diseases are caused by a deficiency of hydrolases that are essential for the correct degradative function of lysosomes (1, 2). Patients with these diseases develop systemic organ pathology and neurodegeneration because of the progressive lysosomal accumulation of toxic metabolites in various tissues, including the brain. Therapeutic strategies have relied on the unique capacity of soluble enzyme precursors to be secreted by one cell type and internalized via receptor-mediated endocytosis, by other cells at distant sites. Methods such as enzyme replacement therapy, bone marrow transplantation (BMT), organoid implantation, and gene therapy have been attempted in patients and animal models (reviewed in refs. 3 and 4). Each approach presents inherent problems mainly related to the difficulty of correcting the central nervous system (CNS) pathology. BMT, which relies on available donors, has been attempted for treatment of patients with variable results (2, 5). In animal models, this procedure has proved efficacious in the amelioration of CNS pathology in some cases [e.g., canine mucopolysaccharidosis (MPS) I and feline α-mannosidosis] (6, 7), but offers little or no benefit in others (e.g., murine MPS VII, canine G_{M1}-gangliosidosis, and feline G_{M2}-gangliosidosis) (8–11). Other approaches, including ex *vivo* gene therapy, have suffered from poor transduction efficiencies, short-term or silenced gene expression *in vivo*, and the difficulty of delivering therapeutic protein to target cells (12, 13).

Our strategy, which overcomes many of these obstacles, is to generate transgenic mice that express the therapeutic protein at sustained levels in a specific BM cell lineage and to transplant their BM into deficient mice. The disease model used in these studies is galactosialidosis (GS) (reviewed in ref. 14), which is caused by a primary deficiency of protective protein/cathepsin A (PPCA). PPCA has carboxypeptidase/deamidase activity, forms a complex with lysosomal neuraminidase and β-galactosidase, and, when absent, leads to a secondary deficiency of both hydrolases. The GS mouse model closely mimics the human disease (15), developing extensive vacuolation of specific cells in most organs and oligosaccharidu ria. Transplantation of GS mice with BM from transgenic mice overexpressing human PPCA in the erythroid cell lineage resulted in complete correction of GS visceral pathology, but only minor amelioration of the brain disease. The latter was likely the result of expression/secretion of endogenous mouse PPCA by BM-derived macrophages that had infiltrated the brain (15).

Here, we have investigated whether BM-derived macrophages and microglia overexpressing the corrective protein might afford better correction of organs, including the CNS. The human colony-stimulating factor-1 receptor (CSF-1R) promoter (16) was used to drive expression of a human PPCA minigene specifically in macrophages of transgenic mice. We demonstrate that transgenic BM, transplanted into GS mice, is remarkably effective in ameliorating the disease process.

MATERIALS AND METHODS

Construction of the CSF-1R/Human PPCA Transgene. The human PPCA cDNA (17) was ligated to the rabbit β-globin splice site and polyadenylation signal and cloned into pIC20H (18). To enhance translation efficiency, the PPCA translation initiation sequence was replaced with that of the rabbit β-globin gene, thereby adding a *Hin*dIII site at the 5′ end of the cDNA. A 3.35-kb PPCA minigene was generated by replacing an internal 0.3-kb *Bam*HI cDNA fragment with the corresponding genomic fragment, including introns 4–6. A 5.3-kb

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Abbreviations: GS, galactosialidosis; PPCA, protective protein/cathepsin A; CSF-1R, colony-stimulating factor-1 receptor; BM, bone marrow; BMT, BM transplantation; CNS, central nervous system; H&E, hematoxylin/eosin.

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portion of the human CSF-1R promoter was obtained by cutting a genomic CSF-1R clone (kind gift of Thomas Look, St. Jude Children's Research Hospital, Memphis, TN) at the translation initiation site with *Nco*I and removing the ATG with S1 nuclease before digestion with *Spe*I. The PPCA minigene was inserted downstream of the CSF-1R promoter (Fig. 1*A*). The fragment (8.65-kb) used for DNA injections was excised with *Not*I/*Sal*I.

Generation of Transgenic Mice and Genotype Analysis. Transgenic mice were generated by using the FVB/NJ strain (19) and were identified by tail blots of *Hin*dIII-digested DNA probed with the 0.9-kb *Bam*HI fragment of the human PPCA gene. The copy number of the transgene in each founder was determined by comparison with defined amounts of the human PPCA minigene.

Enzyme Assays and Western Blot Analysis. Tissue lysates were assayed for cathepsin A, neuraminidase, and β-galactosidase activities (20). The same lysates also were run on 12.5% SDS-polyacrylamide gels (SDS/PAGE), under reducing conditions, and blotted onto Hybond-P poly(vinylidene difluoride) membranes (Amersham). Antibodies against the 32-kDa subunit of human PPCA (α32) were raised in rabbits (20) and affinity-purified against the human protein. Western blots were incubated overnight with purified α32 antibodies followed by a 2-hr incubation with horseradish peroxidase-conjugated, goat anti-rabbit secondary antibodies (Sigma). Binding was visualized by using a chemiluminescence substrate (NEN). The α32 antibodies have a high affinity for the human protein and crossreact poorly with mouse PPCA. Moreover, the human 32-kDa subunit on SDS/PAGE has a slightly lower molecular mass than its mouse counterpart.

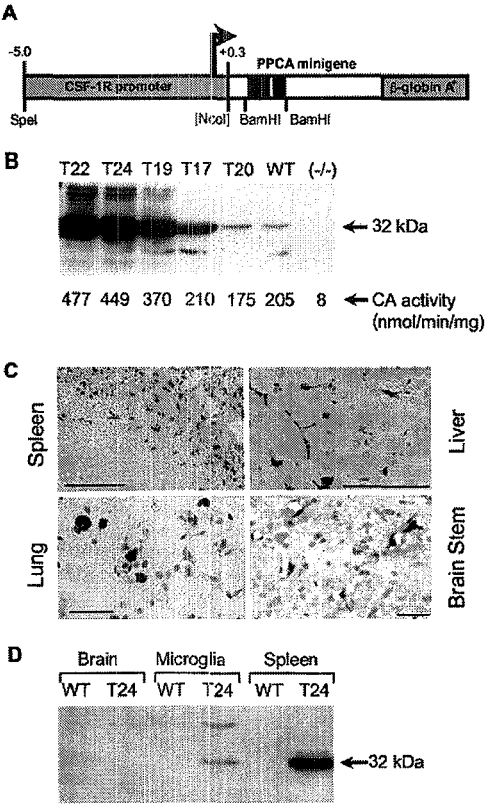

FIG. 1. Structure and expression of a CSF-1R-PPCA transgene in transgenic mice. (*A*) Schematic representation of the expression vector. (*B*) BM lysates were analyzed on a Western blot probed with α32 antibodies. (*B, Bottom*) Cathepsin A activity was measured in BM lysates from two mice of each transgenic line (age 2–6 months). These values are indicative of those obtained in three independent experiments. (*C*) Immunocytochemistry of transgenic mouse tissues. Transgenic mouse (T22) sections of spleen, liver, lung, and brain stem were probed with α32 antibodies. (*D*) Western blot comparing human PPCA expression in 20 μg of whole brain, a microglia-enriched cell population, and spleen from wild-type and transgenic (T24) mice. This blot is indicative of results obtained in three separate experiments.

Immunocytochemical and Histochemical Staining of Mouse Tissues. Mice were perfused with 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.4. Dissected organs were processed for paraffin embedding. Tissue sections were deparaffinized, rehydrated, and antigen-retrieved by microwave boiling in 0.1 M citrate, pH 6.0. The sections were blocked for 30 min and incubated overnight with either α32 or PEP-19 antibodies. Detection was performed with the ABC horseradish peroxidase system using a VIP (purple) or diaminobenzidine (brown) substrate (Vector). For Mac-1 antibody (rat anti-mouse CD11b-PharMingen) staining, fixed tissues were placed in a solution of 30% sucrose/0.1 M sodium phosphate, pH 7.4 for 24–48 hr at 4°C before embedding in tissue freezing medium (Triangle Biomedical Sciences, Durham, NC). Cryostat sections were probed with antibodies as above. Paraffin sections were stained with periodic acid/Schiff or hematoxylin/eosin (H&E) by using standard methods.

Isolation of Microglia. Microglia were isolated from the brains of 7- to 10-day-old pups by fractionation on a discontinuous Percoll gradient (21). The enriched microglia fraction (90–95% pure) was washed in PBS and frozen as a pellet.

BMT. Recipient GS mice (C57BL/6×129/J×FVB/NJ) of ages 1, 2.5, and 7–9 months were lethally irradiated with 830 rad 24 hr before transplantation. One-3 homozygous CSF-1R–human PPCA transgenic mice served as BM donors while normal BM was obtained from wild-type FVB/NJ mice. BM cells were incubated on ice for 30 min with an anti-mouse CD3 antibody made in guinea pig (H57–597) (kind gift of Peter Doherty, St. Jude Children's Research Hospital). After being washed, the cells were subjected to complement lysis (five parts Low-Tox guinea pig complement/one part rabbit complement, Cedarlane Laboratories) at 37°C for 45 min to achieve T cell depletion. Five-hundred microliters of a cell suspension containing 4×10^7 cells/ml was injected via the tail vein. Treated mice were analyzed at various times (2–12 months) posttransplantation.

Analysis of Urinary Oligosaccharides. Urine samples were tested for the presence of undegraded oligosaccharides by using a FACE urinary carbohydrate analysis kit (Glyko, Novato, CA).

Coordination Testing on a Rota-Rod. Mice were trained twice on 2 consecutive days for a 5-min period at low speed (3 rpm) to become accustomed to the accelerating rota-rod treadmill (Stoelting). On the third and fourth days, they were placed on the rota-rod at accelerating speeds from 3 to 30 rpm (increments of 3 rpm/30 sec) and tested twice with a 5-min break between tests. The mice were kept on the apparatus for a maximum of 280 sec. For each mouse, a single measurement was calculated to represent the average performance of these four attempts. All testing was performed between 2 and 5 p.m.

RESULTS

CSF-1R Drives Human PPCA Expression Specifically in Macrophage-Derived Cells. To generate transgenic mice, we designed a construct with 5.3 kb of the human CSF-1R

promoter located upstream of a human PPCA minigene (Fig. 1*A*). Five independent transgenic founders were obtained with single integration sites for the transgene, ranging in copy number from 5 to 35 (i.e., T20, T17, T19, T24, and T22 had 5, 12, 15, 25, and 35 copies, respectively). They were bred to homozygosity before analysis. Human PPCA expression was assessed by measuring cathepsin A activity in the founder's BM. The three transgenic lines with the highest copy numbers gave activities 1.8- to 2.4-fold higher than controls (Fig. 1*B*, *Lower*). Similar values were measured in the spleen, whereas in other tissues, including liver, kidney, lung, and brain, there was little or no detectable increase in activity over endogenous levels (not shown). Consistent with these results, Western blots of transgenic BM (Fig. 1*B*) and spleen demonstrated high human PPCA expression that correlated with the transgene copy number. Lower, but significant, expression was seen in lung, liver, kidney, and brain, but not in testis (not shown). This variation of expression in different tissues could reflect differences in CSF-1R promoter regulation at sites where macrophage colonization/infiltration is required (22) and/or different demands for tissue macrophages in the animals at the time of sacrifice.

The distribution of the transgenic protein in the target cells was analyzed in tissue sections probed for human PPCA (Fig. 1*C*). Both in hematopoietic and nonhematopoietic tissues, human PPCA expression was confined solely to lysosomes (i.e., punctate staining pattern) of macrophages and macrophage-derived cells. When we used staining intensity as a measure of PPCA expression, alveolar macrophages (Fig. 1*C*, Lung) consistently expressed at higher levels than splenic macrophages (Fig. 1*C*, Spleen), which in turn expressed higher than Kupffer cells (Fig. 1*C*, Liver). Hence, the microenvironment of macrophages specifies the level of PPCA expression under the CSF-1R promoter.

In brain, moderately stained perivascular macrophages/microglia were found throughout the parenchyma, including the olfactory bulb, cerebrum, cerebellum, and brain stem (Fig. 1*C*, Brain Stem). Outside the blood-brain barrier, scattered positive macrophages were detected in the choroid plexus and leptomeningeal macrophages (not shown). Human PPCA, however, could not be visualized in ramified microglia, either because it was confined to the lysosomes of the very fine processes of these cells or because the level of expression was too low. To verify transgene expression, a purified preparation of microglia from wild-type and transgenic (T24) mice was analyzed by Western blotting (Fig. 1*D*). Human PPCA levels were clearly higher in the microglia-enriched transgenic sample compared with the total brain lysate, although expression was low compared with that in the spleen. Similar results were obtained with transgenic line T22 (not shown). Together these data demonstrate that the human CSF-1R promoter functions in the same tissue-specific manner in visceral tissues and brain as the endogenous gene from which it is derived (23–26).

Transplantation of GS Mice with Transgenic BM Corrects the Disease Phenotype. Affected mice were transplanted at 1 month of age with BM from either transgenic or wild-type mice and analyzed 2–12 months post-BMT. Treated mice appeared normal despite their smaller size, which is common for mice irradiated at an early age. Although GS mice developed ataxia at 5–6 months of age, no signs of ataxia were evident in the transplanted mice even 1 year after BMT. Oligosaccharidiuria was completely reverted by BMT (Fig. 2*B*) already after 1 month, and this correction persisted for the life of the animals. Mice with transgenic BM maintained levels of cathepsin A activity equal to or higher than wild-type mice in their BM, spleen, lung, and liver (Fig. 2*A*). No significant increase was seen in kidney and brain. However, considering that the distribution of endogenous PPCA in the latter tissues of wild-type mice is confined primarily to nonmacrophage-derived cells (27), restoration of enzyme activity in BMT mice might not be measurable even after efficient engraftment. Further, microglia turnover is slow and BM-derived transgenic macrophages may not yet have efficiently repopulated (28–30). Concomitant with restoration of cathepsin A activity was a proportionate increase in neuraminidase activity (not shown). We also noticed that, with the exception of cultured fibroblasts, tissues from PPCA(−/−) mice display a gradual increase in β-galactosidase activity during disease progression, and this activity is normalized upon engraftment after BMT (unpublished data). The mechanism underlying this phenomenon is still unclear.

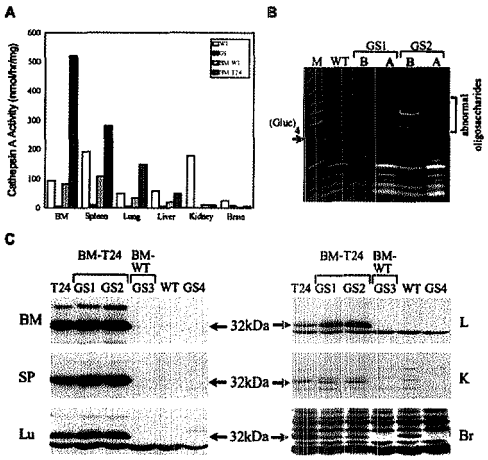

FIG. 2. Urinary oligosaccharides and human PPCA expression in GS mice after BMT. (*A*) Cathepsin A activities in tissues of BMT mice. GS mice, transplanted with either wild-type or transgenic (T24) BM at 2.5 months of age were sacrificed 2 months later, and tissue lysates were assayed for cathepsin A activity. (*B*) Urine samples were collected from 1-month-old wild-type and GS mice before BMT (lanes B) and 1 month after BMT (lanes A). Mouse GS1 and GS2 received wild-type and transgenic BM, respectively. A molecular weight ladder of glucose polymers (M), with the $(Glucose)_4$ for reference, is shown. Oligosaccharides larger than this tetrasaccharide are abnormal. (*C*) Western blot of tissues from BMT mice were probed with α32 antibodies: GS1 and GS2 mice received transgenic BM (BM-T24) and GS3 mouse received wild-type BM (BM-WT). The exposure time for the brain was 12 times longer than that for the other tissues.

Western blotting showed human PPCA only in those mice transplanted with transgenic BM (Fig. 2*C*). In spleen, lung, and liver, expression was consistently higher in BMT mice than in donor transgenic mice. This increase probably was caused by the extra macrophage recruitment of tissues to remove cellular debris from dead and dying cells. Interestingly, human PPCA was detected in the brain, but at a significantly lower level than for the transgenic animals, probably because of the slow turnover rate of microglia in BMT mice. These results clearly demonstrate that PPCA-expressing cells can infiltrate virtually any tissue.

To ascertain the improvement of tissue morphology after BMT, H&E staining was performed (Fig. 3, H&E). The extensive vacuolation observed in visceral organs of untreated GS mice (spleen, liver, kidney, and testis are shown as examples) was largely and similarly corrected by wild-type (BM-WT) and transgenic BM. However, in the kidney, transgenic BM (BM-T22) clearly afforded better correction than BM-WT, particularly in glomerular visceral epithelial cells that are known to require more therapeutic protein for correction (15), indicating that macrophage-derived human PPCA was secreted, effectively taken up, and processed into its active form. Interestingly, although the interstitial cells of the caput epi-

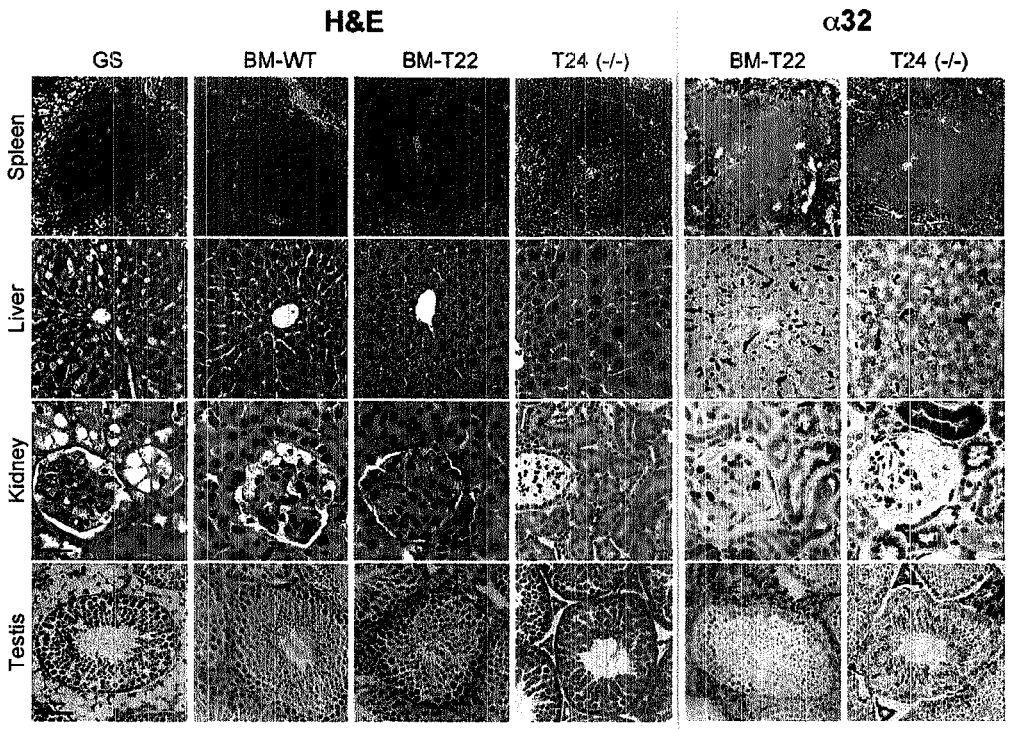

FIG. 3. Histology and immunocytochemistry of BMT and transgenic-knockout mice. GS mice (1 month old) were transplanted either with normal (BM-WT) or transgenic (BM-T22) BM and sacrificed 11.5 months later. Age-matched GS mice and transgenic-knockout [T24(−/−)] mice also were analyzed. Tissue sections were stained with H&E and labeled with α32 antibodies for immunocytochemistry. [Scale bar = 40 μm (kidney) or 80 μm (spleen, liver, testis).]

didymis were corrected, the tubular cells continued to store even in mice receiving transgenic BM (not shown). Immunocytochemistry confirmed the presence of human PPCA-expressing macrophages in every tissue tested (Fig. 3, α32 and BM-T22). This presence was most obvious in BM, spleen, lung, and liver. Notably, in the Bowman's capsule and proximal convoluted tubules of the kidney (Fig. 3, α32 and BM-T22) and in hepatocytes (Fig. 3, α32 and BM-T22), internalized protein accumulated in lysosomes to such an extent that it could be detected as punctate staining. In the testis, many PPCA-expressing macrophages were seen among the Leydig cells (Fig. 3, α32 and BM-T22), whereas relatively few infiltrated into the caput epididymis (not shown). This observation explains the complete correction of the testis compared with the epididymis. In general, visceral organ correction was impressive using this system, even if GS mice were transplanted late in life (7–9 months). These results could be relevant for future human applications. The transplanted mice displayed no obvious signs of an immune response to either introduce murine or human PPCA.

In the brain of transplanted mice, human PPCA expression was restricted to perivascular, leptomeningeal, and choroid plexus macrophages (Fig. 4 *A* and *B*). Although the choroid plexus was fully corrected, periodic acid/Schiff staining revealed that scattered neurons still accumulated undegraded products (not shown). Because of the regional distribution of accumulating cells in the CNS of GS mice (15), it is difficult to accurately estimate whether isolated neuronal cells have been cleared of storage. Therefore, we monitored the effect of BMT in the cerebellum that undergoes an obvious and dramatic loss of Purkinje cells in GS mice. The Purkinje cells in transplanted mice continued to store and die (2, 6, and 11.5 months post-BMT, not shown), indicating that the number of CNS macrophages/microglia and/or their level of secretion of corrective enzyme was insufficient to prevent their loss. More animals, sacrificed at different ages, will be required to determine whether a decreased rate of cell loss occurs after BMT considering that mice up to 11.5 months post-BMT performed at a level well above that of untreated GS mice when tested for motor coordination on a rota-rod (Fig. 5).

Breeding of CSF-1R Human PPCA Mice into the PPCA −/− Background. To assess the potential to correct this disease only by overexpressing macrophages, transgenic mice (T24) were cross-bred with GS mice to obtain transgenic knockout mice [T24(−/−)]. These mice developed no overt signs of disease and bred more prolifically than GS mice of a similar age. Analysis of their tissues by H&E staining [Fig. 3, H&E, T24(−/−)] and immunocytochemistry [Fig. 3, α32, T24(−/−)] revealed virtually identical results to those of mice receiving transgenic BM (BM-T22). The only difference was the better correction of glomerular cells in BMT mice, which may have resulted from these irradiated mice incurring damage, inducing an inflammatory response and hence acquiring a greater local concentration of PPCA-expressing cells. Alter-

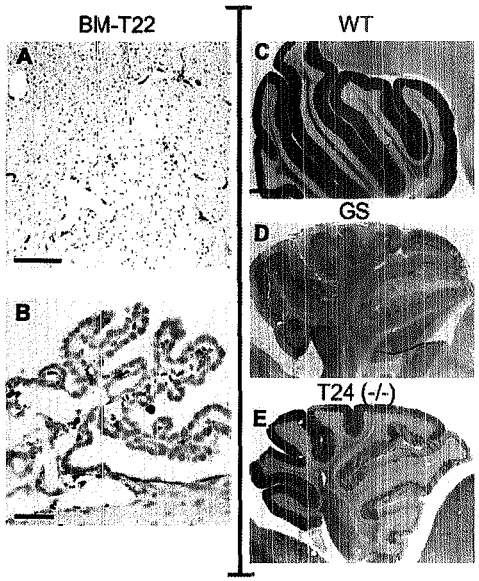

FIG. 4. Immunocytochemistry of the brain in BMT and transgenic-knockout mice. GS mice were transplanted with transgenic BM (BM-T22) and sacrificed 11.5 months later. Brain sections were stained with α32 antibodies and revealed expression in the perivascular macrophages of the brain stem (*A*) and macrophages of the choroid plexus (*B*). To visualize the Purkinje cells of the cerebellum, sections from 5-month-old (*C*), GS (*D*), and transgenic-knockout [T24(−/−)] (*E*) mice were stained with an anti-PEP-19 antibody. Note the marked loss of Purkinje cells in GS mice and the partial loss in T24(−/−) mice. [Scale bars = 60 μm (*B*), 120 μm (*A*), and 500 μm (*C–E*).]

natively, endogenous mouse PPCA in the transplanted transgenic BM may have contributed to the extra correction. In the cerebrum, cerebellum, and brain stem of T24(−/−) mice, PPCA-positive cells were present at perivascular and leptomeningeal sites (not shown). Again, we had difficulty detecting microglia by immunocytochemistry, and the results were virtually identical to those obtained for BMT mice. However, because these mice displayed no obvious disease symptoms and also performed better than GS mice on the rota-rod (not shown), we looked in detail at the morphology of their cerebella. Wild-type, GS, and T24(−/−) mice (Fig. 4 *C–E*) were analyzed by staining brain sections with an antibody

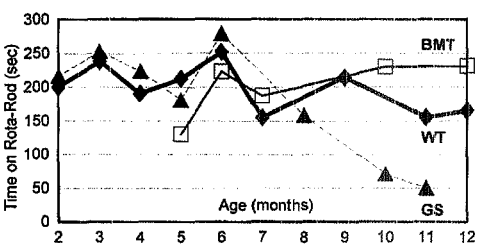

FIG. 5. Coordination tests of GS and BMT mice on a rota-rod. Mice were placed on a rota-rod that accelerated from 3 to 30 rpm, and the time they remained on the apparatus was recorded. The number of mice tested at each month of age ranged from 1 to 14. Note, few GS mice survive beyond the age of 11–12 months.

against PEP-19, a protein specific for Purkinje cells in the cerebellum (31). In GS mice, Purkinje cell loss proceeded in an anterior to posterior direction during disease progression, similar to that reported for the Niemann-Pick mouse model (32). Cell death occurred in patches, although the cells of the inferior lobule were largely spared even in very old mice (up to 13.5 months) and was preceded by the accumulation of intralysosomal periodic acid/Schiff-positive granules (not shown). In T24(−/−) mice, the rate of Purkinje cell death was slower than that in GS mice, which was evidenced by the dramatic difference of PEP-19-positive cells surviving in 5-month-old T24(−/−) mice compared with age-matched knockouts (Fig. 4 *D* and *E*). This apparent improvement of cerebellar pathology could be the result of small amounts of human PPCA expressed by microglia or perivascular macrophages throughout the brain of these mice after birth and/or during their development.

DISCUSSION

For some time, macrophages have been viewed as a vehicle to overexpress and deliver therapeutic proteins to virtually all regions of the body, including the brain. This feature is particularly important for lysosomal storage disorders whose pathology involves both visceral organs and the CNS. In this study, we investigated the overall expression of a lysosomal marker/therapeutic gene (PPCA) under the control of a macrophage-specific promoter, CSF-1R, in transgenic mice. For such a therapeutic regime to be effective, it is imperative that the promoter remains active in the macrophages as they enter different cellular microenvironments. CSF-1R is expressed solely in monocyte/macrophage-derived cells (33), including the microglia of adult mouse, rat, and human brain (23–26). Its tissue specificity is determined, for the most part, at the transcriptional level (16, 34, 35); however, little is known about the level of expression obtained with this promoter *in vivo* in tissues other than BM or peripheral blood.

We overexpressed the PPCA in cells derived from the BM macrophage lineage. Expression varied in different tissues or even in the same tissue, probably reflecting cell-specific CSF-1R promoter regulation upon differentiation or in response to signals from the surrounding microenvironment. In some tissues, PPCA levels correlated with copy number. It is unclear whether the CSF-1R promoter fragment used contains a locus control region-type element, to enhance and insulate the gene it activates from surrounding influences. After BMT in GS mice, both wild-type and transgenic BM corrected much of the visceral pathology, although there was a clear indication that overexpressing cells contributed to better correction. More compelling evidence came from the transgenic knockout mice, where virtually all visceral organs were corrected to some degree. We noticed that the level of human PPCA in transgenic mouse serum or that secreted from cultured transgenic BM macrophages was quite low (unpublished data), indicating that therapeutic amounts of the corrective protein may vary for different cell types. Many lysosomal enzymes are present in excess, and it is possible that some cells have a higher threshold requirement of enzyme for correction than others, for example, in these studies, the glomerular visceral and caput epididymis epithelial cells. Cells with a low threshold include the renal proximal convoluted tubule cells and Leydig cells. Interestingly, the wild-type glomerulus displays very low levels of endogenous mouse PPCA, whereas the proximal convoluted tubule epithelium expresses moderate levels and the epididymis high levels (27). Therefore, endogenous protein expression is not an indication of the overall requirement of this protein for normal metabolic processes. Further, the level of substrate in some cells may change as a result of the disease or there may be other barriers in certain cell types that prevent PPCA uptake.

One year after transplantation with transgenic BM, numerous PPCA-positive perivascular and leptomeningeal macrophages

were seen throughout the CNS. Similar results have been obtained by others (30, 36–38). However, affected neurons, such as Purkinje cells, continued to accumulate undegraded products and die. These cells are notoriously difficult to correct (39), although it was shown recently that BMT slowed their loss in Niemann-Pick disease mice (40). Transplanted GS mice, despite losing their Purkinje cells, performed better in motor coordination tests than their age-matched untreated siblings. This finding suggests that their ataxic phenotype and progressive lack of coordination is complex, involving not only neuronal but also significant visceral factors. In our transgenic knockout model, in which all perivascular macrophages and microglia expressed PPCA to some extent, Purkinje cell storage and death was delayed. This delay may be because of the presence of PPCA throughout development and implies that these neurons can take up the corrective protein. However, higher enzyme levels will be required to prevent cell death over an extended period. Alternatively, amelioration of this neuronal pathology may be secondary to the correction of peripheral organs, resulting in lower circulating levels of undegraded products.

GS mice, while fertile, mate poorly and consequently have few litters. Yet, transgenic knockout mice overexpressing the corrective protein specifically in erythroid (15) or macrophage-derived cells display normal pregnancy and delivery patterns (unpublished data). This apparent reduction in fertility is unlikely to be caused by neurologic disturbances in mating behavior, but rather visceral factors because virtually total amelioration of visceral pathology occurs. Further, correction of storage in Leydig cells, but not in epididymal cells, may contribute to this improvement.

We have shown that macrophages alone conclusively correct pathology of affected cells *in vivo*. It is noteworthy that wild-type macrophages, transplanted into mucopolysaccharidosis VII mice, seemed to partially ameliorate disease pathology, as reported in abstract form (41). Our results indicate that higher levels of expression afford a more timely and effective correction. It remains to be seen whether enough macrophages will ever repopulate the CNS to allow for neural correction even upon BMT at an early age. When considering the potential efficacy for cell therapy, issues to be taken into account include the level of expression/secretion of the therapeutic protein from donor cells, the number of donor cells either locally or in total, the capacity of target cells to take up the therapeutic protein, and the secondary correction of affected cells because of clearance of toxic products elsewhere. Furthermore, successful therapy for any given lysosomal disease may depend heavily on the properties of the individual enzyme, such as its stability in body fluids, whether it is membrane associated, and how efficiently it is secreted and reinternalized. Thus, the level of overexpression necessary to achieve a therapeutically beneficial response will be unique for each lysosomal storage disease being treated.

We are very grateful to Drs. Gerard Grosveld, William Walker, Carin Havenith, Peter Doherty, Tom Look, Jim Morgan, Richard Smeyne, and Susan Watson. We are indebted to Christy Nagy, John Swift, Charlette Hill, Erik Bonten, and Sjozef van Baal of St. Jude and Chris Starr of Glyko. This work was supported by the Assisi Foundation of Memphis, National Institutes of Health Grant DK-52025, the Cancer Center (CORE) Support Grant CA-21765, and the American Lebanese Syrian Associated Charities (ALSAC), St. Jude Children's Research Hospital.

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Functional amelioration of murine galactosialidosis by modified bone marrow hematopoietic progenitor cells.

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Blood 2002 **99 (9)**: 3169-78.

GENE THERAPY

Functional amelioration of murine galactosialidosis by genetically modified bone marrow hematopoietic progenitor cells

Thasia Leimig, Linda Mann, Maria del Pilar Martin, Erik Bonten, Derek Persons, James Knowles, James A. Allay, John Cunningham, Arthur W. Nienhuis, Richard Smeyne, and Alessandra d'Azzo

Protective protein/cathepsin A (PPCA), a lysosomal carboxypeptidase, is deficient in the neurodegenerative lysosomal disorder galactosialidosis (GS). *PPCA*$^{-/-}$ mice display a disease course similar to that of severe human GS, resulting in nephropathy, ataxia, and premature death. Bone marrow transplantation (BMT) in mutant animals using transgenic BM overexpressing the corrective enzyme in either erythroid cells or monocytes/macrophages has proven effective for the improvement of the phenotype, and encouraged the use of genetically modified BM cells for ex vivo gene therapy of GS. Here, we established stable donor hematopoiesis in *PPCA*$^{-/-}$ mice that received hematopoietic progenitors transduced with a murine stem cell virus (MSCV)–based, bicistronic retroviral vector overexpressing PPCA and the green fluorescent protein (GFP) marker. We observed complete correction of the disease phenotype in the systemic organs up to 10 months after transplantation. PPCA^{+} BM-derived cells were detected in all tissues, with the highest expression in liver, spleen, BM, thymus, and lung. In addition, a lysosomal immunostaining was seen in nonhematopoietic cells, indicating efficient uptake of the corrective protein by these cells and cross-correction. Expression in the brain occurred throughout the parenchyma but was mainly localized on perivascular areas. However, PPCA expression in the central nervous system was apparently sufficient to delay the onset of Purkinje cell degeneration and to correct the ataxia. The long-term expression and internalization of the PPCA by cells of systemic organs and the clear improvement of the neurologic phenotype support the use of this approach for the treatment of GS in humans. (Blood. 2002;99:3169-3178)

Introduction

Defective genes encoding specific lysosomal hydrolases are responsible for more than 40 disorders of the metabolism, known as lysosomal storage diseases (LSDs).[1] One of the glycoproteinoses, the autosomal recessive galactosialidosis (GS),[2,3] results from mutations in the *PPCA* gene,[4] causing a secondary deficiency of β-galactosidase and *N*-acetyl-α-neuraminidase.[3] Intracellular accumulation of sialyloligosaccharides and glycopeptides leads to the features characteristic of an LSD, including coarse facies, ocular cherry-red spots, vertebral changes, foamy bone marrow (BM) cells, and vacuolated peripheral blood lymphocytes.[2,5] In addition, most patients with GS experience severe neurologic damage characterized by ataxia, diffuse leukoencephalopathy, and mental retardation; death usually occurs within the first 2 years of life.[2]

Enzyme replacement that ameliorates or reverses systemic and neurologic defects is the goal of curative treatment for LSDs. This strategy is based on the observation that soluble enzyme precursors secreted by one cell type can be internalized via receptor-mediated endocytosis by deficient cells with consequent resolution of toxic catabolite accumulation, that is, correction "in trans." BM progenitor cells are an attractive source of corrective enzyme because of their potential to repopulate the recipient and to supply functional enzyme to cells in affected organs, including the central nervous system (CNS).[6] Allogeneic bone marrow transplantation (BMT) and syngeneic BMTs in affected patients and animal models effectively ameliorate visceral and bony lesions[7-13]; however, diseases with early, predominantly CNS involvement respond poorly.[8,14] Allogeneic BMT is still limited by difficulties in finding suitable HLA-compatible donors, high rates of nonengraftment, severe graft-versus-host disease, and other causes of transplantation-related morbidity and mortality.

The use of autologous hematopoietic progenitor cells (HPCs) that are genetically engineered to express a therapeutic gene could, in principle, circumvent some transplantation-associated obstacles. We have recently proven the feasibility of a BMT approach in our murine GS model. Early in life, *PPCA*$^{-/-}$ animals experience systemic and CNS abnormalities that closely mimic the severe form of GS in humans.[13,15] The animals develop severe nephropathy as well as the ataxia and neuronal degeneration characteristic of this disorder, and die prematurely.[13,15] Transplantation of BM cells that overexpress a *PPCA* transgene in either the erythroid or monocyte/macrophage lineage into lethally irradiated *PPCA*$^{-/-}$ animals resulted in complete correction of systemic pathology of GS.[13,16] Partial amelioration of the disease phenotype in the CNS was also observed, but a clear delay in the onset of the cerebellar phenotype was only achieved when transgenic mice were crossed into the *PPCA*$^{-/-}$ background.[16] Overall these results reinforced the feasibility of using ex vivo gene therapy for the treatment of GS.

From the St Jude Children's Research Hospital, Memphis, TN.

Submitted March 15, 2001; accepted December 4, 2001.

Supported by the Assisi Foundation of Memphis, National Institutes of Health grant RO1-DK 52025, the International Outreach Program at St Jude Children's Research Hospital (T.L.), the Cancer Center Support (CORE) grant CA 21765 from the National Cancer Institute, Phillip and Elizabeth Gross, and the American Lebanese Syrian Associated Charities (ALSAC) T.L., L.M., and M.d.P.M. contributed equally to this work.

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We have now tested whether genetically modified PPCA$^{-/-}$ BM cells could afford long-term expression of the therapeutic enzyme in transplanted *PPCA*$^{-/-}$ recipients and correct the disease phenotype. We report that *PPCA*$^{-/-}$ HPCs transduced with a retrovirus that expresses PPCA leads to complete correction of systemic organ damage, amelioration of CNS pathology, and functional correction of motor coordination defects in GS mice.

Materials and methods

Cell lines and vector construction

GP+E86, ecotropic packaging cell line,[17] 293T, and NIH3T3 cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), and antibiotics. We created the murine stem cell virus (MSCV)–PPCA retroviral plasmid by inserting human *PPCA* complementary DNA (cDNA) into a site that is 5′ of the internal ribosomal entry sequence[18,19] in the MSCV-GFP vector previously described[20,21] (Figure 1A).

Generation of an ecotropic virus producer line

Conditioned medium containing high-titer, amphotropic MSCV-PPCA particles was derived by cotransfection of 293T cells with the retroviral vector plasmid and helper plasmid containing *gag, pol,* and *env* genes driven by a Moloney leukemia virus long terminal repeat (LTR).[22] The conditioned medium was used to transduce the GP+E86 line, and viral producer cells were derived as previously described.[23] The viral titer was determined by serial dilutions of the conditioned medium in NIH3T3, followed by analysis of green fluorescent protein (GFP) expression. The titer was 5×10^6 particles/mL.

BMT

PPCA$^{-/-}$ donors were given intraperitoneal injections of 150 mg/kg 5-fluorouracil (Sigma Chemical, St Louis, MO) 48 hours before marrow harvest to mobilize the HPCs. BM cells were prestimulated with 20 ng/mL mouse interleukin (IL)–3, 50 ng/mL human IL-6, and 50 ng/mL mouse stem cell factor (R D Systems, Minneapolis, MN) in DMEM plus 15% heat-inactivated FBS. The hematopoietic cells were cocultured with irradiated (1200 cGy) viral producer cells in the same medium supplemented with 6 μg/mL polybrene for 48 hours. *PPCA*$^{-/-}$ recipients were lethally irradiated (850 cGy) 24 hours prior to tail-vein injection of the genetically modified BM cells. A ratio of 2 to 4 GS donors to 1 recipient was used. Samples of the GS BM cells, transduced with either MSCV-GFP or MSCV-PPCA, were analyzed by fluorescence-activated cell sorting (FACS; Becton Dickinson, Mountain View, CA) and the percentage of GFP$^+$ cells was determined (see "Results"). The initial GFP analysis of the second BM transduction showed an efficiency of only about 0.5%, similar to that of

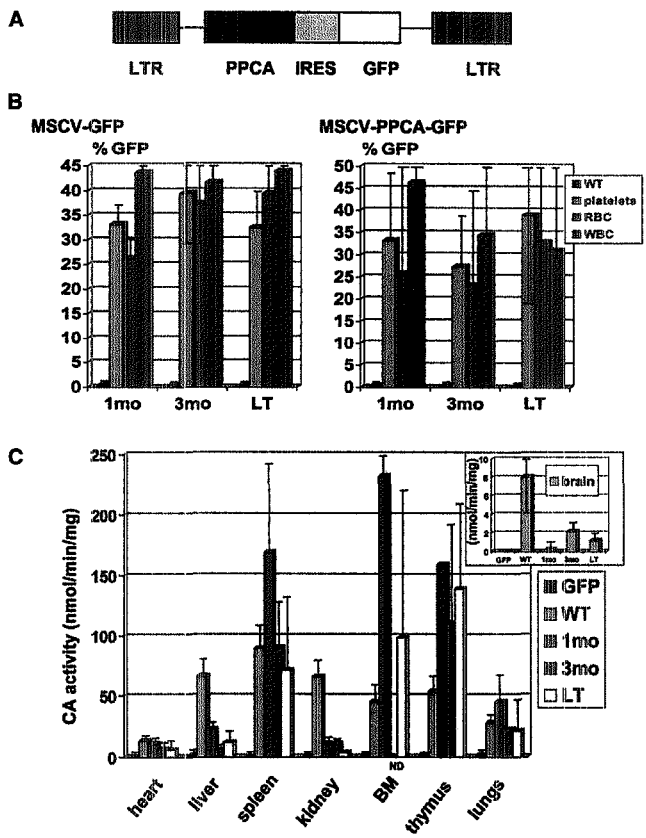

Figure 1. Retrovirally transduced PPCA$^{-/-}$ BM cells restore cathepsin A activity in transplanted *PPCA*$^{-/-}$ recipients. (A) Schematic diagram of the retroviral bicistronic construct encoding the human PPCA cDNA. In this vector, expression of both the human PPCA and the GFP marker is driven by the viral LTR. Translation of GFP is initiated at the internal ribosomal entry site (IRES). (B) Platelets, white blood cells (WBCs), and erythrocytes (RBCs), obtained from *PPCA*$^{-/-}$ mice transplanted with either MSCV-GFP– or MSCV-PPCA-GFP–transduced *PPCA*$^{-/-}$ BM, at different time points after treatment, were FACS sorted and analyzed for GFP expression; 1 month (1 month, 7 mice total); 3 months (3 months, 6 mice total); LT (long-term, ages 6, 8, 9, and 10 months, 5 mice total). (C) Cathepsin A (CA) activity was assayed in tissue-homogenates of MSCV-PPCA-GFP–treated mice, at different time points after treatment. Wild-type (WT, 4 mice total) and MSCV-GFP–treated *PPCA*$^{-/-}$ mice (GFP, 4 mice total), ranging in age between 3 and 8 months, were used as positive and negative controls, respectively. The level of CA activity was independent from the age of the wild-type or MSCV-GFP–treated mice. The inset shows the CA activity in brain homogenates of *PPCA*$^{-/-}$ treated and untreated mice, as well as controls. For the CA activity in the thymus of 1-month–treated mice, only one tissue sample was collected and measured; 1 month (1 month, 4 mice total); 3 months (3 months, 3 mice total); LT (long-term, ages 6, 8, 9, and 10 months, 5 mice total). We observed considerable variation in the measured CA activity between mice of the same age group, likely due to differences in engraftment of the transplanted BM cells. The presented data are average values with a typical high and low limit of ± 25% to 50%, which is significantly above the CA activity of the GFP controls. The bars represent SDs.

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untransduced cells. This value did not correlate with the GFP expression levels detected subsequently in peripheral blood cells (PBCs) of the mice receiving transplants with this transduced marrow and with their cathepsin A activity in tissues (Figure 1B,C), which was in the same range of the activity in the other 4 groups of mice receiving transplants. Therefore, we assumed that the initial FACS analysis was erroneous.

Determination of GFP expression in PBCs

Recipients were bled by orbital sinus puncture at 1, 3, 6, and 8 to 10 months after BMT. Blood (20 μL) was collected in 1 mL cold phosphate-buffered saline (PBS) for FACS analysis of erythrocytes and platelets. For analysis of lymphocytes, erythrocytes were lysed in Gay solution and propidium iodide was added.[23]

Enzyme activity assay

Tissues were homogenized in water. Cathepsin A activity was measured with the synthetic dipeptide substrate Z-Phe-Ala as described earlier.[24] Total protein concentration was determined with the bicinchoninic acid reagent (Pierce Chemical, Rockford, IL).

Histochemical analysis

Antibodies against the 32-kd subunit of human PPCA (α-32) were raised in rabbit and affinity purified against the human protein.[25] This antibody was shown to selectively recognize the human PPCA protein and does not cross-react with the endogenous murine PPCA.[16] Paraffin-embedded tissue sections were deparaffinized and hydrated; antigen retrieval was accomplished by boiling the sections in 0.1 M citrate, pH 6.0. After a 20-minute blocking process, the sections were incubated overnight with either α-32 or anti-GFP (α-GFP, Clontech Laboratories, Palo Alto, CA) antibodies followed by washing and incubation with goat-antirabbit IgG secondary antibodies (Pharmingen, San Diego, CA) for 2 hours at room temperature. Antigen-antibody complexes were detected with the ABC horseradish peroxidase system, which uses a VIP (purple) or diaminobenzidine (brown) substrate (Vector, Burlingame, CA). For PEP19 staining, serially sectioned, cerebella were processed and incubated with anti-PEP19 antibodies[26] (a kind gift of Dr James Morgan, Developmental Neurobiology, St Jude Children's Research Hospital) as above.

Purkinje cell count

Counting of Purkinje cells was performed following the method described in Smeyne and Goldowitz.[27]

Results

Expression of retrovirally encoded PPCA in GS BM cells

To investigate whether genetically modified HPCs can correct the murine GS phenotype, we constructed a MSCV-based bicistronic vector containing *PPCA* cDNA that was linked by an internal ribosomal entry site to the gene encoding the GFP marker (MSCV-PPCA, Figure 1A). An identical vector carrying only the *GFP* gene was used as a control (MSCV-GFP). We first determined that the total number of BM cells harvested from *PPCA*$^{-/-}$ donors, aged 2 to 6 months, was similar to that of cells from wild-type, age-matched mice and that the different lineages were correctly represented. Total *PPCA*$^{-/-}$ BM was then transduced with either MSCV-PPCA or MSCV-GFP ex vivo, to assess the transducibility of deficient cells versus normal BM. In 2 pilot experiments performed before BMT, the transduction efficiency of *PPCA*$^{-/-}$ BM cells, calculated on the basis of GFP expression, was 15% and 20% with the MSCV-PPCA vector, and 19% and 39% with the MSCV-GFP vector. In parallel, a 15-fold increase in cathepsin A activity was measured in transduced PPCA$^{-/-}$ BM cells compared to untreated cells or cells transduced with the MSCV-GFP vector. We also assessed the level of cathepsin A activity in lysates of clonogenic progenitor colonies that were positive for GFP as visualized by fluorescence microscopy. Cathepsin A activity was more than 100-fold higher in MSCV-PPCA^{+} colonies than in MSCV-GFP–transduced colonies.

Correction of the murine GS phenotype by genetically modified *PPCA*$^{-/-}$ BM cells

To evaluate the effects of enforced PPCA expression in vivo, we transplanted *PPCA*$^{-/-}$ BM cells transduced with either MSCV-PPCA or MSCV-GFP into lethally irradiated, 3- to 6-week-old GS mice. In 5 independent transplantation experiments, the transduction efficiency of either the MSCV-PPCA or the MSCV-GFP retrovirus was calculated on the basis of GFP expression in FACS-sorted cells, immediately before transplantation. With the exception of the second BM transduction experiment (see "Materials and methods"), the transduction efficiency of the MSCV-PPCA virus was 28% (experiment [exp] 1); 23% (exp 3); 19% (exp 4); and 17% (exp 5). The transduction efficiency of the MSCV-GFP virus ranged from 19% to 44%. GFP-expressing cells of the erythroid, myeloid, or lymphoid lineage were detected by FACS analysis of peripheral blood samples, collected at different time points after transplantation. Regardless of the vector used, the percentage of gated cells expressing GFP varied between 18% and 40% in erythrocytes, 20% and 61% in platelets, and 20% and 56% in lymphocytes (Figure 1B). To estimate the levels of the therapeutic enzyme in different transplanted mice, cathepsin A activity was assayed in tissue homogenates from organs of recipients at various time points after BMT. For as long as 10 months after treatment, increased cathepsin A activity was detected in most tissues; the highest levels were measured in spleen, BM, and thymus, but also liver, kidney, and heart had persistent and increased activity compared to the knockout or BMT-GFP–treated mice (Figure 1C). Cathepsin A activity in total brain lysates, which is usually low also in wild-type samples, was only marginally increased and varied among animals receiving transplants, probably because of the uneven distribution of engrafted cells that expressed the corrective enzyme. Although transgene expression differed among recipients, the level of enzyme was apparently sufficient to correct or ameliorate the histologic changes consistent with PPCA deficiency (Figures 2-6).

Correction of systemic pathology in PPCA$^{-/-}$ mice after transplantation of MSCV-PPCA–transduced BM cells

In contrast to the *PPCA*$^{-/-}$ untreated mice, mice transplanted with the MSCV-PPCA–marked BM had no systemic manifestations of disease; they had a normal gross appearance, a shiny fur, lack of diffuse edema, and inflammation of the eyelids, no tremor, or ataxic movements up to 10 months after BMT. These features become evident in *PPCA*$^{-/-}$ mice starting at the age of 3 to 4 months.[13,15,16] To assess the effect of PPCA-expressing BM cells on organ morphology, we performed histologic and immunohistochemical analyses of tissue sections. The combined use of an anti-PPCA antibody, specific for the human PPCA, and an anti-GFP antibody enabled us to discriminate between cells expressing the transgene and cells that have internalized the corrective enzyme (Figure 3). Transplanted mice were analyzed at 1 to 10 months after treatment. Systemic correction was observed in all MSCV-PPCA–transplanted animals, although the number of PPCA^{+} cells varied slightly in different mice, according to the transduction efficiency

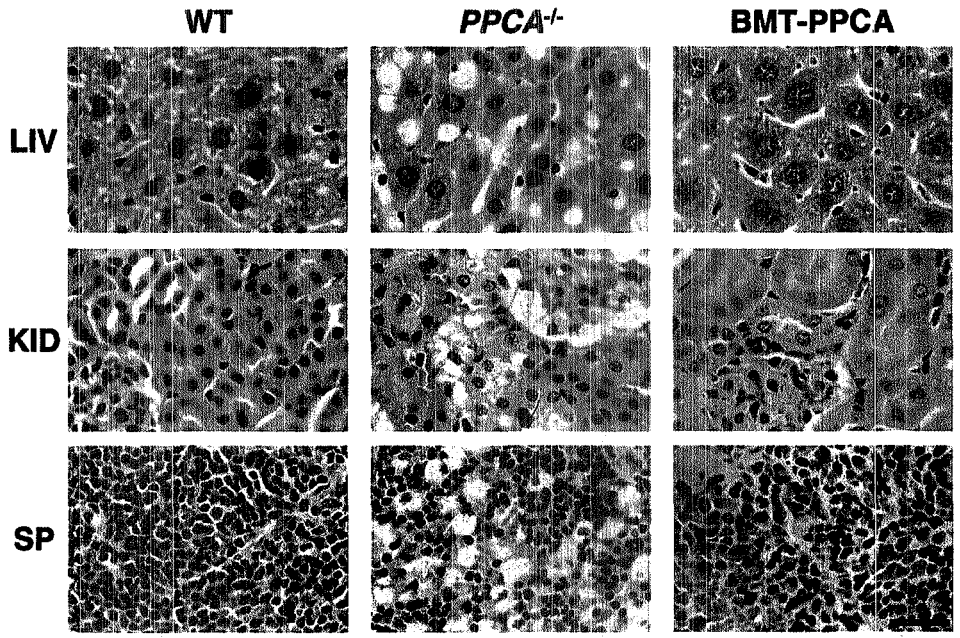

Figure 2. Histology of systemic organs from BM-transplanted GS mice. Organs from $PPCA^{-/-}$ mice transplanted with total $-/-$BM transduced with the MSCV-PPCA (BMT-PPCA) retrovirus were isolated at different time points after treatment. Hematoxylin and eosin-stained tissue sections of the liver (LIV), kidney (KID), and spleen (SP) from a BMT-PPCA–treated $PPCA^{-/-}$ mouse killed at 9 months after treatment, and from age-matched wild-type and $PPCA^{-/-}$ mice revealed the complete restoration of normal tissue morphology with BM expressing PPCA, compared to the extensive vacuolation present in the $PPCA^{-/-}$ control mouse. Size bar corresponds to 30 μm.

and the repopulating capacity of retrovirally marked BM cells. PPCA expression persisted long term, indicating that sufficient numbers of HPCs were transduced. As predicted by the levels of cathepsin A activity in various organs, we detected high expression of PPCA in tissues of hematopoietic origin; in the spleen the distribution of PPCA-expressing cells was similar to that observed in previous studies[13,16] (Figure 3, α-32 panel). This resulted in full reversal of the morphologic changes that remained apparent in untreated GS mice (Figure 2, BMT-PPCA and $PPCA^{-/-}$). Clearance of storage material occurred in the liver, both in Kupffer cells and in the hepatic parenchyma (Figure 2, BMT-PPCA). Staining of adjacent sections with the macrophage-specific anti–Mac-1 antibody (not shown) confirmed that the BM-derived Kupffer cells were highly positive for PPCA (Figure 3, α-32 panel). In addition, hepatocytes displayed a PPCA-specific punctate staining characteristic of lysosomes; this finding indicated that PPCA was actively internalized (Figure 3, α-32 panel). Foamy histiocytes and vacuolated endothelial cells and hepatocytes persisted in the untreated mice of similar age (Figure 2, $PPCA^{-/-}$). In the kidney, one of the most severely affected organs in GS, PPCA-specific immunostaining was observed throughout the renal parenchyma (Figure 3, α-32 panel). This feature was associated with complete resolution of lysosomal storage in the proximal tubular and glomerular epithelia that instead was still evident in $PPCA^{-/-}$ mice (Figure 2, BMT-PPCA). Strong immunostaining was also seen in the pulmonary alveolar macrophages, the heart, the thymus, and the salivary glands (data not shown). In all examined organs, the number of PPCA-expressing cells exceeded that of GFP^+ cells (Figure 3, α-GFP panels) that represented the population of transduced BM-derived cells that repopulated the organs. These observations implied that efficient cell-to-cell transfer of PPCA had occurred, resulting in the clearance of lysosomal storage and correction of the systemic phenotype.

Amelioration of the pathologic changes in the CNS of recipient mice

Regional distribution of CNS abnormalities in murine GS[13,15] makes it difficult to accurately estimate whether isolated neuronal cells have been cleared of storage material. To ascertain the effects of transplanted, genetically corrected cells on the CNS phenotype, we performed histologic, immunochemical, and enzymatic analyses of the CNS at various time points after transplantation. Comparison of brains from mice that received MSCV-PPCA–transduced BM cells with those from $PPCA^{-/-}$ mice revealed a significant amelioration of the pathologic phenotype (Figure 4, BMT-PPCA and $PPCA^{-/-}$). In the regions most affected by PPCA deficiency, including the cerebellar nuclei, the lateral geniculate nuclei, and the amygdala, the amount of storage material appeared reduced in recipients of MSCV-PPCA–transduced BM cells (Figure 4, BMT-PPCA). The overall brain architecture was overtly improved in these mice, likely because the endothelial cells and perivascular macrophages were largely corrected. In accordance with this finding, immunoreactive PPCA in the brain was primarily restricted to leptomeningial, perivascular macrophages, and the vascular structure of the choroid plexus (Figure 5, α-32). This

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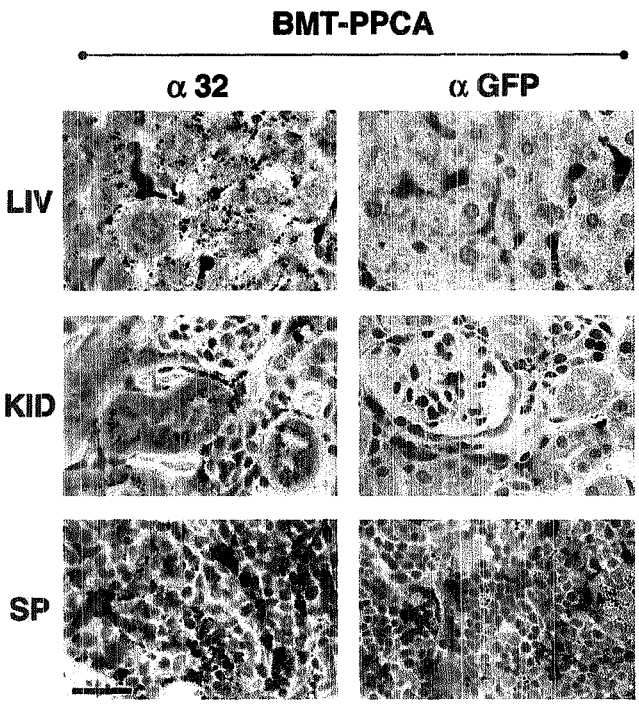

Figure 3. Immunostaining of tissue sections from BM-transplanted GS mice with α-32 and α-GFP antibodies. Numerous human PPCA-expressing cells were detected by immunostaining with α-32 antibody, monospecific for the human protein (left panels). In the liver of a BMT-treated mouse killed at 9 months after treatment, strong immunostaining was detected in Kupffer cells, as confirmed by staining of adjacent sections with the macrophage-specific anti-Mac-1 antibody. The clear punctate staining of the hepatocytes demonstrated internalization of the corrective enzyme by these cells. In the kidney the presence of the human PPCA was detected in the proximal convoluted tubules and Bowman capsule. Numerous macrophages and splenocytes in the spleen of transplanted mice were positive for the human protein. Staining of the same tissues with α-GFP antibody (right panels) was restricted to cells in locations consistent with their being of hematopoietic origin. Size bar corresponds to 30 μm.

expression pattern coincided with that observed with α-GFP antibody (Figure 5, α-GFP), although PPCA expression was more widely distributed than GFP expression, and occasional neurons displaying a clear punctate staining were observed only with the α-32 antibody (Figure 5).

The relatively small number of PPCA-expressing cells detected in neural tissues was in agreement with the low levels of cathepsin A activity measured in total brain lysates. However, given the overall improvement of brain morphology, it is apparent that only small amounts of enzyme are required for amelioration of brain pathology.

Correction of the cerebellar defect in BM recipient mice

A dramatic and progressive death of Purkinje cells occurs in the cerebellum of the GS mice, starting at the age of 3 to 4 months, and is one of the most overt consequences of this disease in the mice. Purkinje cells are lost in an anteroposterior and mediolateral fashion, the anterior lobes being the ones that are affected most and sooner. We have used this feature as a marker to monitor reduction in the neurologic damage after BMT. Serial sections of cerebella from mice that received MSCV-PPCA–transduced BM cells were compared with sections from wild-type and $PPCA^{-/-}$ mice. The appearance of Purkinje cells in PPCA-corrected mice was determined at 9 months after BMT by staining serial sections of the cerebella with an antibody against PEP19.[25] Purkinje cells were clearly more numerous in treated mice than in age-matched PPCA mutant animals (Figure 6). To quantify our observations, Purkinje cells were counted in these transplanted mice as well as in one of the 3-month–treated group, and compared to 3- and 9-month-old $PPCA^{-/-}$ mice and age-matched controls. Purkinje cells were counted at 2 levels: (1) in the paravermis at the point where the lateral cerebellar nuclei first become obvious (medial) and (2) in the hemisphere at the level of the dorsal cochlear nucleus (hemisphere). In the medial region wild-type mice averaged 392 ± 19 Purkinje cells/section, whereas in the hemisphere the number was 362 ± 16. As expected, in the 3-month-old $PPCA^{-/-}$ animal only a small number of Purkinje cells were lost: 24% in the medial and 21% in the hemisphere sections (Figure 7). The total numbers were practically identical in the 3-month-old–treated mouse, because the variations in the different cerebellar regions were too small to be detected. At this time point, there was also little variation in Purkinje cell number between the anterior and posterior lobes of the cerebellum. In contrast at 9 months, we observed a dramatic loss of Purkinje cells in the $PPCA^{-/-}$ mouse. In the midline the total loss was 79%, but it was clearly more dramatic in the anterior lobes of the cerebellum than in the posterior lobes, with a loss of 93% and 61% of the cells, respectively. After BMT, the rescue of Purkinje cells in the 9-month-old mice varied in different cerebellar lobes, but the total number of cells was substantially greater than that of age-matched $PPCA^{-/-}$ mice (Figure 7). In the medial cerebellum, the overall loss of Purkinje cells in BMT recipient mice was 44% of controls. In the anterior lobes of the BMT-treated medial cerebellum, 55% of the cells were missing, whereas in the posterior lobes only 30% were. In the cerebellar hemisphere, the overall loss of Purkinje cells in the BMT-treated mice compared to the wild-type mice was 60%, with the anterior lobes showing a

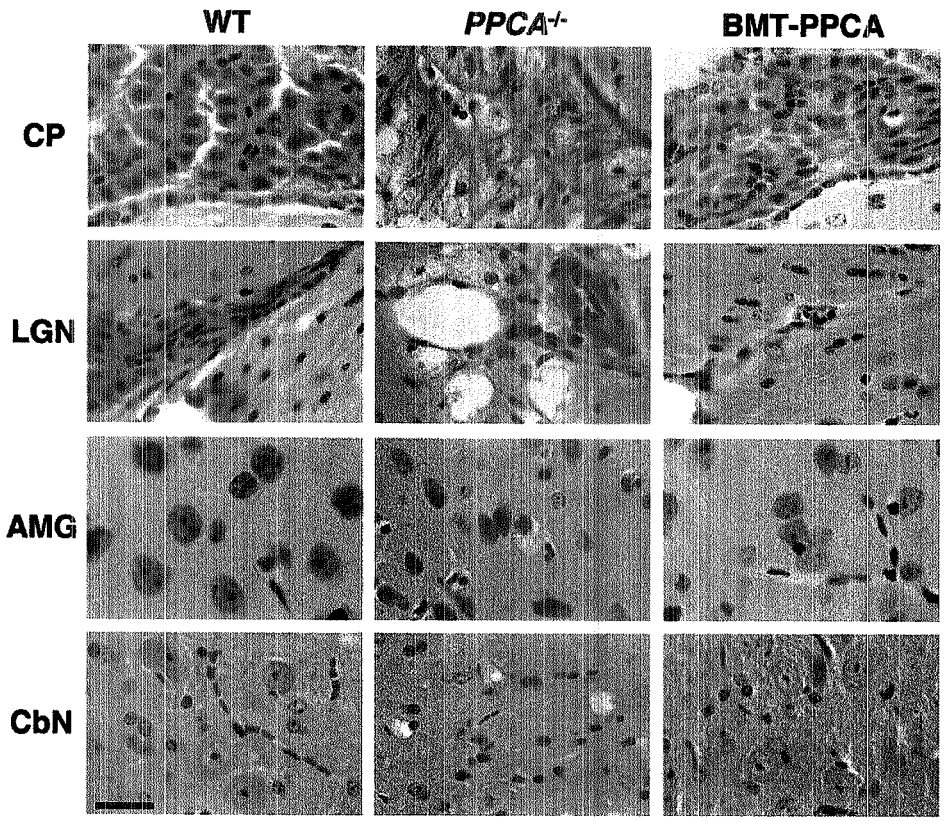

Figure 4. Histology of 4 regions of the brain from BM-transplanted GS mice. Hematoxylin and eosin staining of the choroid plexus (CP), the lateral geniculate nucleus (LGN), the amygdala (AMG), and the deep cerebellar nucleus (CbN) isolated from BMT-PPCA–treated GS mouse at 9 months after transplantation showed clear reduction of storage in neural cells compared to an age-matched GS animal. The overall brain architecture was clearly improved due to the clearance of storage material in endothelial cells and perivascular and leptomeningeal macrophages. Storage in some of the neurons in the amygdala and cerebellar nucleus was also cleared. Size bar corresponds to 30 μm.

66% loss and the posterior lobes 51% loss of Purkinje cells. These results support the notion that BMT of genetically modified cells in GS mice delays the progressive loss of Purkinje cells characteristic of the GS mice.

Discussion

Transplantation of normal HPCs has been exploited for the treatment of LSDs because BM progenitor cells can differentiate and repopulate target organs, including the CNS, providing a permanent source of normal enzyme. The overall outcome of allogeneic and syngeneic BMT in patients and animal models has indicated that this procedure is relatively effective in alleviating the systemic manifestations of the disease and in stabilizing bone lesions, especially if BMT is performed early in life.[10,28-32] Correction, however, is often incomplete, suggesting that higher local levels of gene expression may be required in some organs. Moreover, diseases that have an early onset and involve predominantly the CNS respond poorly to BMT, albeit that some variation in outcome has been observed among disease subtypes.[10] The difficulty to correct the CNS is attributed to the slow and incomplete engraftment of BM-derived cells into the adult brain[33]; it may also depend on the amount of enzyme secreted by normal cells, the extracellular stability of the enzyme,[10] and the extent of uptake by target cells. This conclusion is supported by our previous finding that complete systemic correction and partial amelioration of the brain pathology occur in GS mice that received transgenic BM in which cells of the monocyte/macrophage lineage were modified to overexpress a PPCA transgene.[16] In these studies, neurologic abnormalities, including the loss of cerebellar function, were dramatically delayed when the transgenic mice were crossed into the *PPCA*$^{-/-}$ background.[16]

Building on these observations, we have now tested the hypothesis that a similar or better outcome could be obtained in a gene therapy setting, if sustained and long-term expression of the transgene could be achieved. These studies allowed us to examine the feasibility of such an approach for treatment of GS patients.

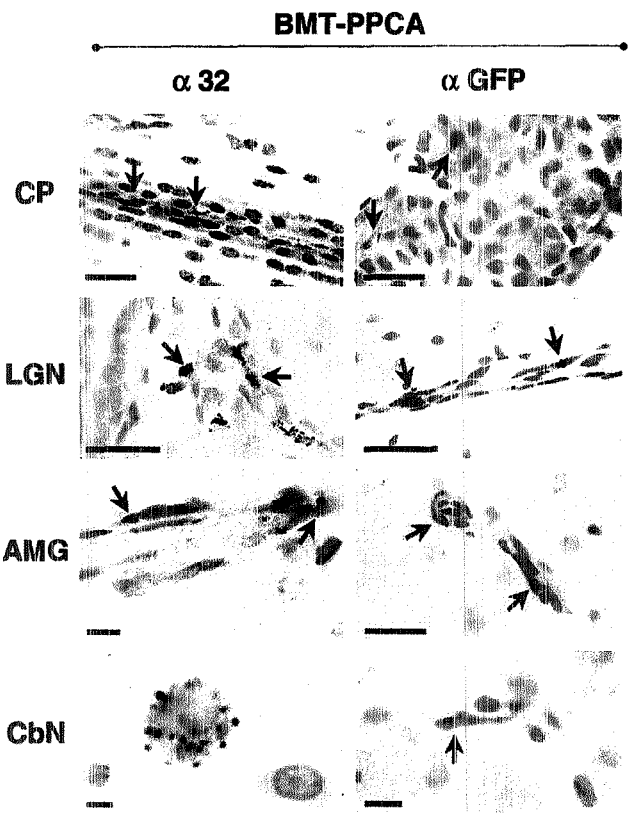

Figure 5. Immunostaining of 4 brain regions from BM-transplanted GS mice with α-32 and α-GFP antibodies. α-32 immunostaining of brain sections derived from BMT-PPCA–transplanted mice at 9 months after treatment revealed numerous PPCA+ endothelial cells, perivascular macrophages, sparse neurons as well as the cuboidal epithelium of the choroid plexus and its macrophages (indicated by arrowheads). Similar but more restricted immunostaining was detected in sections of the same regions stained with α-GFP antibody. Size bars correspond to 40 μm.

Somatic gene therapy of neurologic LSDs could be, in fact, the preferred treatment if autologous HPCs could be engineered in vitro to constitutively express and secrete high levels of the correcting enzyme. Early studies in animal models have been disappointing with persistence of the lysosomal defect and only negligible amelioration of the disease phenotype.[34] These results have been attributed to ineffective transduction of HPCs, insufficient level or silencing of transgene expression, immune depletion of the enzyme, or a combination of these factors.[35-37] However, some of these difficulties can now be circumvented by the use of improved viral vectors like the one used in the treatment of α-galA-deficient mice.[38] In addition, MSCV-based retroviral vectors have been shown to selectively and efficiently infect HPCs.[21-23,39] This vector system has been recently applied successfully for the treatment of arylsulfatase A–null mice.[40] Interesting and encouraging studies have recently shown delayed onset of clinical signs and amelioration of the functional and physical defects in the mucopolysaccharidosis (MPS) VII mouse model, using in utero transplantation of fetal liver cells and nonablative neonatal BMT, respectively.[41,42]

We have established stable hematopoiesis in GS mice using *PPCA*$^{-/-}$ BM transduced with an MSCV-based bicistronic retrovirus expressing both PPCA and the GFP marker. One of the main findings is the capacity of MSCV to mediate long-term high transgene expression, which is indicated by the analysis of PPCA and GFP levels in tissues and PBMCs of the reconstituted animals. It is noteworthy that the level of cathepsin A activity varied considerably between recipients. These differences can be attributed to varying ratios of human PPCA expressing HPCs, which dictate the relative number of differentiated hematopoietic cells secreting the enzyme. Heterogeneity of retroviral LTR-mediated expression can be a function of the site of integration into the genome within heterochromatin or euchromatin,[43] position effect variegation,[44] and progressive silencing of retroviral-mediated expression.[43] Our MSCV vector, although modified to enhance expression,[20] may still be subjected to silencing.[45] Further modifications in the LTR regulatory elements[45] or inclusion of additional regulatory elements into the vector genome[46] or both may be necessary to ensure persistent, high-level, retroviral-mediated gene expression in large animal models and in patients with LSDs.

Despite the variability in levels of enzyme among recipients we can conclude that sustained and long-term expression of PPCA, generated by the MSCV retroviral cassette, undoubtedly contributed to the prevention/correction of storage in the GS organs, including the CNS. Most importantly, the extent of correction of

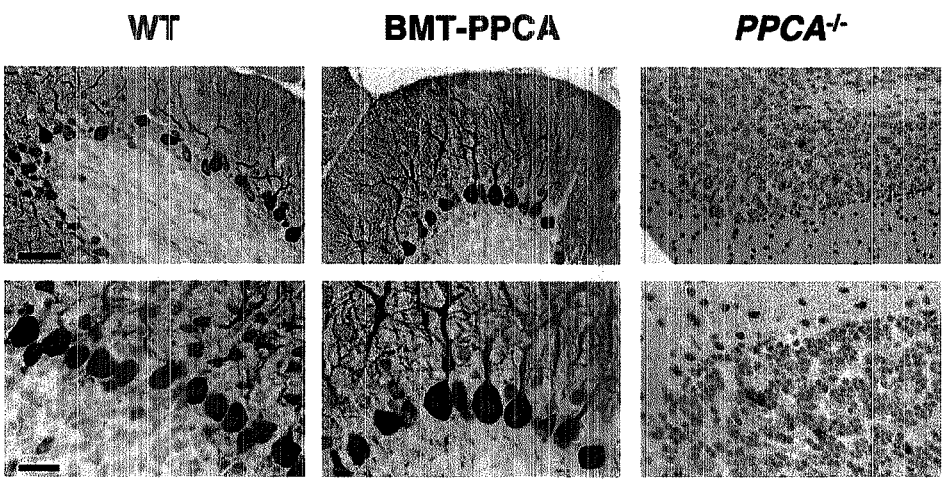

Figure 6. PEP19 staining of cerebellar sections from BM-transplanted GS mice. Serial sections of the cerebellum from a 9-month-old GS mouse transplanted with MSCV-PPCA–marked BM cells were stained with anti-PEP19 antibody. Note the dramatic loss of Purkinje cells in an age-matched GS mouse and the significant number of these cells that are retained in the treated animal. Size bars correspond to 60 μm for the upper panels and 30 μm for the lower panels.

the phenotype, including the cerebellar defect, observed in BMT-treated GS mice is comparable to that observed in crosses between *PPCA*$^{-/-}$ and transgenic mice overexpressing the corrective enzyme in the monocyte/macrophage lineage.[16] In a mouse model of Niemann-Pick disease, although neurologic dysfunction was corrected in part after transplantation, no histologic resolution of storage was observed and the mice died of the disease.[47] The quantitative analysis of Purkinje cell present in BMT-treated GS mice correlates well with their motor coordination skills. *PPCA*$^{-/-}$ animals demonstrate a progressive deterioration in motor coordination skills as measured by a standardized rota-rod treadmill assay, whereas PPCA-corrected mice, at all tested time points after transplantation, perform better on the rota-rod treadmill, albeit that the statistical analysis failed to detect a significant effect in these results due to a small sample population. The functional amelioration of the cerebellar deficit in GS recipients of retrovirus-transduced BM cells was associated with significant amelioration of the histopathology observed in *PPCA*-deficient animals, although the persistence of neuronal cells with storage throughout the CNS in these mice suggests that further steps are needed to achieve complete correction.

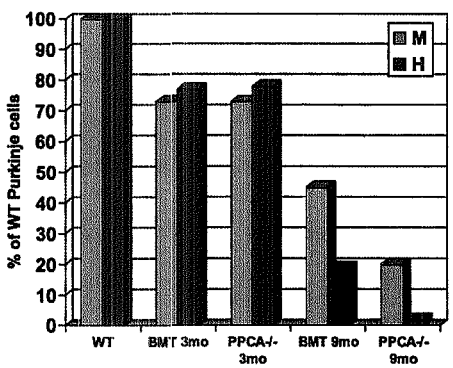

Figure 7. Purkinje cell counts in BM-transplanted GS mice. Purkinje cells were counted in recipient mice at 3 and 9 months after treatment and compared to 3- and 9-month-old *PPCA*$^{-/-}$ mice and age-matched controls. Purkinje cells were counted at 2 levels: (1) in the paravermis at the point where the lateral cerebellar nuclei first become obvious (M, medial), and (2) in the hemisphere at the level of the dorsal cochlear nucleus (H, hemisphere). The values are expressed as percentage of Purkinje cells counted in the control group.

Like the BMT approach, gene transfer into animal models of MPS I, MPS VII, Niemann-Pick disease, and metachromatic leukodystrophy has resulted in only partial correction of the enzyme deficiency in the brain although improvement in neurologic function could not be documented.[48] Several features have been implicated in the poor response, including differences in disease type, the age of the animals at the time of transplantation, and the use of irradiation. To simulate a potential clinical intervention, we used total body irradiation (TBI), a conditioning modality important for engraftment in patients undergoing allogeneic HPC transplantation. TBI negatively affects neuronal development in infants. However, an unfavorable outcome due to TBI must be balanced with the ability of this procedure to disrupt transiently the integrity of the blood-brain barrier, and allow the entry of corrected cells into the CNS. Thus, it is possible that the high initial levels of enzyme that we achieved in this population and the early age at the time of treatment permitted the correction of a significant proportion of affected cells. Further studies in our model will be required to determine if sublethal doses of radiation allow a similar outcome.

The combination of the high dose of cells, levels of HPC transduction, and appropriate cellular expression of the corrective enzyme might have played a crucial role in the histologic and functional correction of the CNS pathology in treated mice. Given the devastation of the cerebellar cortex in untreated *PPCA*$^{-/-}$ animals,[13] and the vulnerability of Purkinje cells to storage-mediated damage, it was surprising to observe the preservation of cerebellar architecture in our treated mice. Although estimates of

gross cathepsin A activity are not significantly different from those observed in the transgenic BMT model, our results suggest that a crucially higher threshold of protein expression was reached by using the genetic modification approach than by using the transgenic BMT method. Additional studies are required to determine the exact mechanism of enzyme delivery by genetically modified HPCs. In addition, substrate mobility, accessibility, and accumulation rate must be determined before a more comprehensive picture evolves.

Acknowledgments

We are grateful to Gerard Grosveld for his continuous support, Tommaso Nastasi for his expert assistance in the preparation of the histology figures, Hongjun Wang for assistance in the preparation of the revised manuscript, and Charlette Hill for help in typing and editing the manuscript.

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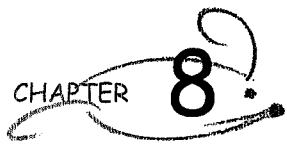

Discussion

Discussion

G_{M1}-gangliosidosis

The GM1 mouse model, which we have generated, represents a faithful copy of the severe infantile form of the disease, as 100% of the mice have gross neurologic impairment. Tremors, ataxia and abnormal gait, which rapidly progress to rigidity and paralysis of the hind limbs, become apparent beyond the age of 5 months; death occurs at about 7-10 months of age. Accumulation of G_{M1}-ganglioside in the CNS of deficient mice is apparent already in newborns, implying that the process had started during embryonic development. Ganglioside levels in 3.5-month old deficient mice are comparable to those measured in 8-month-old human patients at a terminal stage of the disease. In spite of this the deficient mice present with severe neurological symptoms later in life and survive longer. In addition, the asialo derivative of G_{M1}-ganglioside (GA_1) accumulates to a similar extent during the course of the disease. This feature of the GM1 model is also found in the G_{M2} gangliosidosis mice (Elsea and Lucas, 2002). Unlike humans, desialylation of the gangliosides by a sialidase appears to be an alternate catabolic pathway in the mouse. We have no indication at the moment as whether the abnormal storage of GA1 also contributes to the neuropathology of the disease.

The mechanisms underlying neuronal dysfunction and neuronal degeneration in LSDs have not been fully elucidated. Neuronal cell death is the central feature of both acute and chronic neurodegenerative diseases, including Alzheimer, Huntington and Parkinson disease, and amyotrophic lateral sclerosis (Yuan 2000). The deposition of protein aggregates or the formation of abnormal structures in specific neuronal populations (Imaizumi *et al.*, 2001; Kaufman, 1999; Yuan and Yankner, 2000) characterizes these disorders; both features are thought to be responsible for toxicity to neurons and the pathogenesis of these disorders. By analogy, abnormal accumulation of undegraded metabolites in lysosomes could be the origin of neuronal apoptosis in many neurodegenerative LSDs, including G_{M1}-gangliosidosis, although a direct correlation has not been demonstrated yet.

Recently, a series of articles have brought about the notion that ER dysfunction may be involved in the pathogenesis of neuronal cell injury in acute and chronic diseases of the brain (Paschen and Frandsen, 2001). Conditions that alter the ER environment have the potential to induce cellular damage. In order to overcome ER dysfunction, cells induce highly conserved stress responses, which include increased levels of molecular chaperones, transcriptional induction, translational attenuation, and degradation of unfolded proteins (Ma and Hendershot, 2001). In contrast, excessive and/or prolonged stress results in apoptosis (Rao *et al.*, 2002).

We have demonstrated that the neurodegeneration characteristic of GM1 mice could be the result of an ER-stress response. The observation that some of the molecular players involved in this process were deregulated in our mice, namely BiP, CHOP and caspase 12, brought us to our current experimental model (Fig. 3) that could be in principle applied to other LSDs and is supported by recently published data. The fact that BiP is upregulated in GM1 mice is no surprise considering that this molecular chaperone is one of primary and most important molecules that control the early response of the cell to stress (Kaufman, 1999).

The increase in CHOP expression in the GM1 model coincides with the development of severe neuropathological symptoms. Several lines of evidence point to the role of CHOP as a mediator of apoptosis upon ER stress: *chop* -/- mouse embryonic fibroblasts exhibit significantly less apoptosis when challenged with ER stress-inducing agents (Zinszner *et*

al., 1998); CHOP-mediated apoptosis following its transcriptional activation by ATF6, another ER stress induced transcription factor, has also been reported (Gotoh *et al.*, 2002). Although a set of genes referred to as DOCs for downstream of CHOP have been identified, the precise signaling pathway following the activation of CHOP remains to be elucidated (Wang *et al.*, 1998). However, our findings involve CHOP as potential mediator of the neuronal apoptosis observed in our mice. This hypothesis is further substantiated by activation of caspase-12. Until recently, the only cellular compartments implicated in apoptotic death processes were the mitochondrion and the plasma membrane. New studies have revealed that ER stress can induce cell death by a mechanism that is independent of the previously described apoptotic pathways (Rao *et al.*, 2002). The identification of a predominantly localized ER caspase family member, procaspase-12, whose activation is triggered specifically by disturbances in ER homeostasis, has implicated this subcellular compartment and in particular caspase-12 in the apoptotic execution (Nakagawa *et al.*, 2000).

The mechanisms by which β-galactosidase deficiency and in turn lysosomal accumulation of gangliosides could activate or render cells susceptible to an ER stress-mediated cell death are not understood. It is possible that neuronal accumulation of undegraded metabolites, in particular G_{M1}-ganglioside, in the endosomal-lysosomal compartment hampers the overall degradative capacity of the organelle, results in a redistribution of the ganglioside or other by-products to different cellular sites, and disrupts ER integrity. Although the exact intracellular localization of the accumulated ganglioside during the course of the disease remains to be evaluated, we hypothesize that may begin in trans Golgi/endosomal network where the biosynthetic and degradative pathways of plasma membrane-derived glycosphingolipids converge (Kolter and Sandhoff, 1998) (Figure 3). In addition, neuronal cell injury induced under conditions associated with both increase and decrease in cytoplasmic calcium has been previously reported (Paschen and Frandsen, 2001). Failure to overcome disturbances in calcium homeostasis may also contribute to neuronal susceptibility to ER stress-mediated cell (McCullough *et al.*, 2001; Nakagawa and Yuan, 2000; Nakagawa *et al.*, 2000). It is noteworthy that we observed differential expression of a number of genes that are known to be involved in calcium regulation (data unpublished) by representational difference analysis of brain samples from GM1 mice compared to age-matched wild types.

Overall our results have unraveled a novel pathway in G_{M1}-gangliosidosis that could explain the cause of neuronal death, and could be applied to other neurodegenerative lysosomal disorders. Further analysis of the contribution of the ER stress apoptotic pathway and the exact signaling pathway(s) involved in the neurodegeneration of G_{M1}-gangliosidosis are needed in order to gain a complete understanding of the pathogenesis of this disease. It is the hope that these studies will eventually contribute to the development of a suitable therapy.

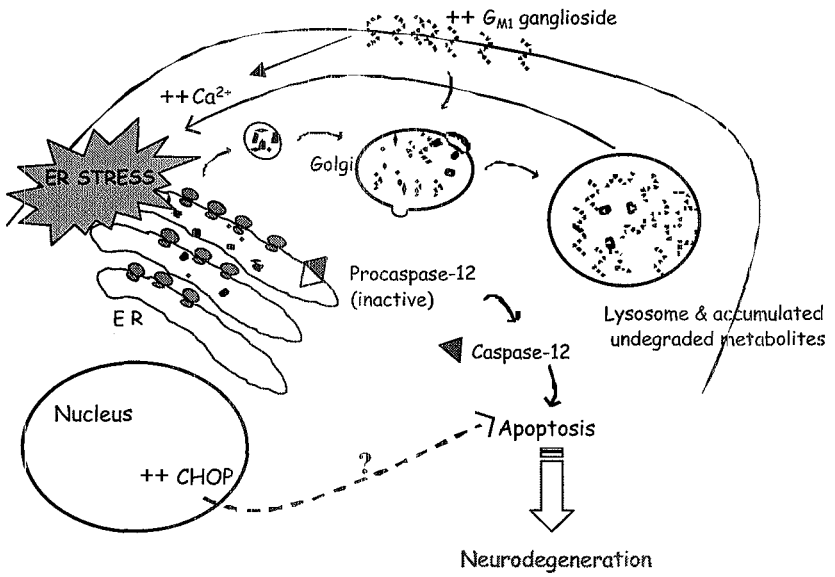

Figure 3. A proposed model for the pathophysiology of G_{M1} gangliosidosis.

Galactosialidosis

The use of bone marrow transplantation in LSDs as a source of the corrective enzyme in LSDs has been partially successful primarily due to the failure to correct the CNS pathology. The incomplete amelioration is attributed to brain incomplete engraftment, stability of the enzyme, and possible to amount of enzyme secreted by the donor cells. This conclusion is sustained by the improved overall pathology observed in GS mice transplanted with BM of transgenic mice overexpressing PPCA in the erythroid lineage (Zhou *et al.*, 1995). Minor amelioration of the brain disease was observed in treated mice, that most probably resulted from the expression of PPCA or secretion by BM-derived macrophages that had infiltrated the brain.

In line of this observation and to improve the correction of the CNS, we studied whether BM-derived macrophages and microglia overexpressing PPCA might afford better correction of disease organs in GS mice, including the CNS. Macrophages are suitable vehicles for the delivery of the corrective protein as they are mobilized in response to environmental stimuli and repopulate virtually all organs, including the brain (Hickey and Kimura, 1988). In bone marrow-transplanted GS mice expression exclusively in the monocyte/ macrophage lineage results in complete correction of visceral organs and reduction of oligosaccharide secretion in the urine. Therefore, expression in macrophages was sufficient to clear and prevent storage accumulation in the majority of the organs. In contrast, throughout the CNS, neurons remain affected, and Purkinje cells continued to accumulate undegraded products and die. Transplanted GS mice, despite their loss in Purkinje cells, improved motor coordination compared with untreated GS mice. The relative overall improvement is suggestive of the contribution by low levels of PPCA, particularly from perivascular and leptomeningeal macrophages. Although expression of PPCA was observed in transgenic mice, transplanted animals do not show high levels of the protein in

the brain. The reason for the low expression of PPCA in the treated mice could be explained by a low turnover of microglia or, as reported recently, due to the potential renewal from a different source of stem cells, the neural stem cell (Asahara *et al.*, 2000). Interestingly, in our transgenic knockout model, motor deterioration was improved and Purkinje cell death was delayed. This delay in pathophysiologic symptoms may result from the presence of PPCA expression during development and implies that if low levels of corrective enzyme that reach the brain or are expressed there, could result in the amelioration of the neuropathology in GS mice and most likely in the human disease. Similar results have been reported in BMT studies of the mouse model of Sandhoff disease, in which treated mice showed improvement of neurological symptoms, delayed neuronal cell death, and prolonged life spans (Wada *et al.*, 2000).

Encouraged by the observed neuropathology amelioration in GS mice after BMT, we hypothesized that a better outcome could be achieved by a sustained expression of the corrective protein in a gene therapy setting. Gene modification of hematopoietic progenitor cells (HPCs) has the advantages of being transplanted autologously without immunological consequences, and accessibility of donor HPCs (Asahara *et al.*, 2000). These features overcome some of the major hurdles in the clinical application of stem cell therapy. Galactosialidosis as a single gene deficiency disease could benefit from the transfer *ex vivo* of the corrective gene via the use of viral vectors. Most reported attempts to transduced HPCs for gene therapy protocols have used retroviral vectors (Marshall, 2001). Retroviral vectors exhibit the following advantageous properties; the ability of efficiently transduced HPCs, contain a moderately DNA-carrying capacity and low immunogenicity. The retroviral vector murine stem cell virus (MSCV) has been recently used successfully as an expression vector and has been shown through the expression of green fluorescent protein (GFP) to efficiently infect HPCs (Marx *et al.*, 1999; Persons *et al.*, 1997; Persons *et al.*, 1998). We constructed a murine stem cell virus (MSCV)-based, bicistronic retroviral cassette overexpressing PPCA and the green fluorescent protein (GFP) marker.

The use of this construct result in sustained high level expression of PPCA in most tissues of transplanted GS mice. The overexpression of the corrective protein particularly in the hematopoietic tissues was sufficient for the complete reversal of lysosomal accumulation. Overall, PPCA expression was more evident than expression of the GFP marker, indicative of corrective protein secretion and successful internalization by adjacent cells. Although PPCA expression was detected in endothelial cells and perivascular and leptomeningeal macrophages, resulting in improvement of the overall brain architecture, numerous neuronal cells with lysosomal storage were found throughout the CNS. Regardless of the absence in increase of PPCA enzymatic activity in transplanted brains, Purkinje cells are retained and prevention in degeneration of motor coordination was observed in treated animals. Although high levels of protein expression comparable to those achieved using the transgenic BMT method were observed using genetically modified HPCs, only relatively small numbers of PPCA-expressing cells were found in neural tissues. The low protein expression in CNS was in agreement with the persistence of affected neurons. These results suggest that only partial amelioration of the CNS can be achieved with this methodology and point to the requirement of alternative strategies for the complete correction of the neuropathology.

The attempts in the treatment of lysosomal storage disorders such as enzyme replacement therapy, adeno-associated vectors, substrate deprivation therapy, and bone marrow transplantation have not achieved complete correction of the neuropatholic symptoms (Jeyakumar *et al.*, 2001; Marshall, 2001; Ready, 2002). The shift to the use of lentiviral vectors, in particular their ability to infect non-dividing cells, provide new alternatives for the correction of CNS storage (Woods *et al.*, 2002). The use of recombinant feline immunodeficiency virus in glucoranidase-deficient mice resulted in reversion of

cellular inclusions of the brain, which was also accompanied by the reversal of mouse behavioral phenotype (Brooks *et al.*, 2002). Although, an in depth evaluation of safety issues remain to be assessed.

The recent discovery of neural stems cells; their capacity of reintegration into the host cytoarchitecture, migration, and relatively easy transducibility and manipulation has revolutionized the concept of gene transfer (Wartiovaara, 2000). Neural stem cells, as gene delivery vehicles may provide new strategies against neurodegenerative disease. Considering the complexity of LSDs, it is likely that the development of a curative strategy for these disorders will include a combination of strategies. In which case, the choice of which combination of strategies to use may be dependent on the biological properties of the protein and on the biological bases of the pathology.

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Summary

SUMMARY

Although the hallmark of LSDs in tissues is the presence of vacuolated cells, the cellular and molecular consequences of the intralysosomal accumulation of various metabolites are largely unknown. Common pathological features include severe psychomotor delay, visceromegaly, growth retardation and early death. Variations in disease penetrance for both systemic and nervous systems have been documented, and likely reflect differences in the metabolic needs of individual cell types that, in turn, may depend on the selective nature of the primary defect. G_{M1}-gangliosidosis and galactosialidosis represent perfect examples of this concept with a severe and generalized CNS involvement in G_{M1}-gangliosidosis and multisistemic manifestations and regionalized neuronal involvement in galactosialidosis. The large number of genetically engineered mouse models of LSDs, currently available combined with spontaneously occurring animal models, is used as an advantageous experimental tool to elucidate disease development. These studies are focused in the use of G_{M1}-gangliosidosis and galactosialidosis animal models to achieve a better understanding of the molecular bases of LSD's and to use genetic approaches as curative therapy.

G_{M1}-gangliosidosis is a glycolipidosis caused by lysosomal β-gal deficiency that affects mainly infants. The severe form of GM1 is characterized primarily by growth retardation, progressive neurologic deterioration due to extensive brain atrophy, visceromegaly, and skeletal dysplasia. Abnormal amounts of G_{M1}-ganglioside and, to a lesser extent, its asialo-derivative (G_{A1}) accumulate in the brain, and oligosaccharides derived from glycoproteins and keratan sulfate are excreted in the urine. In Chapter 3 we report the generation of a mouse model of G_{M1}-gangliosidosis. This model represents a faithful copy of the severe infantile form of the disease, as 100% of the mice have gross neurologic impairment. Tremors, ataxia and abnormal gait, which rapidly progress to rigidity and paralysis of the hind limbs, become apparent beyond the age of 5 months; death occurs at about 7-10 months of age. Accumulation of G_{M1}-ganglioside in the CNS of deficient mice is present already in newborns, implying initiation of lysosomal accumulation during embryonic development and strongly suggesting that *in utero* therapeutic intervention would be necessary in treating this disease.

In Chapter 4 we proposed ER stress–induced apoptotis as a contributing mechanism underlying neuronal dysfunction and neuronal degeneration in G_{M1}-gangliosidosis. Neuronal cell death has been reported as the central feature of both acute and chronic neurodegenerative diseases. Recently, a series of articles have brought about the notion that ER dysfunction may be involved in the pathogenesis of neuronal cell injury. Furthermore, excessive and/or prolonged stress could result in apoptosis by activation of a predominantly localized ER caspase. Procaspase-12 is a member of the caspase family whose activation is triggered specifically by disturbances in ER homeostasis. We have demonstrated that the neurodegeneration characteristic of GM1 mice could result from caspase-12 induced apoptosis upon activation of the ER-stress pathway. The observation that some of the molecular players involved in this process were deregulated in our mice, namely BiP, CHOP and caspase-12, brought us to the hypothesis of an ER stress response initiated by an impaired lysosomal degradative machinery. Overall our results have unraveled a novel pathway in G_{M1}-gangliosidosis that could explain the cause of neuronal death, and could be applied to other neurodegenerative lysosomal disorders.

In Chapter 5 we present correlation study between the expression of PPCA in normal mouse tissues and the occurrence of lysosomal storage in tissues of the GS mice. We found that a close correlation between high expression of the PPCA mRNA and protein in normal cells and lysosomal storage in deficient cells predominate in the majority of the tissues examined. Overt discrepancies were found primarily among neuronal cell

populations, implying that individual cells may either metabolize different spectrum of substrates, or have a different susceptibility to toxic by-products. On the other hand, expression of PPCA in selected cells could be representative of cell demands for the proteolytic activity rather than the protective function towards β-galactosidase and neuraminidase. The detailed analysis of the GS phenotype has provided the essential groundwork to evaluate the efficacy of the therapeutic approaches that we have implemented in this mouse model.

Chapters 6 and 7 evaluate the use of genetically modified BM as therapy. We studied the use of BMT as a permanent source of normal enzyme, based on the unique feature of lysosomal enzyme precursors to be secreted in small quantities and to be reinternalized via cell surface receptors. In Chapter 6 we reported the use of donor BM modified to exclusively express PPCA in the monocyte/ macrophage lineage in view of their ability to respond to environmental stimuli and repopulate virtually all organs, including the brain. In bone marrow-transplanted GS mice expression of the corrective enzyme exclusively in the monocyte/ macrophage lineage resulted in complete correction of visceral organs and reduction of oligosaccharide secretion in the urine. Therefore, expression in macrophages was sufficient to clear and prevent storage accumulation in the majority of the organs. In contrast, we observed that throughout the CNS of transplanted GS mice, neurons remain affected. Despite their loss in Purkinje cells, BMT mice exhibited improved motor coordination compared with untreated GS mice. Moreover, in GS mice with PPCA transgenic background, motor deterioration was improved and Purkinje cell death was delayed. These results implied that expression of the protein during development could result in considerable amelioration of the neuropathology.

Finally, in Chapter 7 we reported the use of autologous stem cells engineered *in vitro* to constitutively express and secrete high levels of the correcting enzyme when transplanted back in GS mice. We constructed a murine stem cell virus (MSCV)-based, bicistronic retroviral cassette overexpressing PPCA and the green fluorescent protein (GFP) marker. The use of (MSCV)-based vector resulted in sustained high level expression of PPCA after treatment in most the tissues of transplanted GS mice. The overexpression of the corrective protein particularly in the hematopoietic tissues was sufficient for the complete reversal of lysosomal accumulation. Most importantly, PPCA expression was observed in cells in which GFP marker expression was absent, indicative of corrective protein secretion and successful internalization by adjacent cells. In the CNS, although PPCA expression was limited to perivascular and leptomeningeal macrophages and neuronal lysosomal storage was present, functional amelioration of the cerebellar deficit was observed in treated animals. Although, an increase in PPCA enzymatic activity was not apparent, it was sufficient to delay the onset of Purkinje cell loss and prevent motor coordination degeneration. Regardless of the absence in increase of PPCA enzymatic activity in transplanted brains, Purkinje cells were retained and prevention in degeneration of motor coordination was observed in treated animals.

Acknowledgments

Certainly, I feel very grateful for the professional and personal experiences that brought me to this point and that could only have gotten accomplished with the support of some key people.

Sandra, you always provided me with every resource you had at hands. Thank you for your support and generosity. I did not only accomplish many things, I was fortunate to have fun while obtaining them.

Prof. Galjaard I appreciate you acting as my promoter and your interest in my education even when you really should be enjoying the efforts of your long and strong professional carrier.

I have met so many wonderful people and in addition I had the privilege of working with them. Erik, you were the first person I met, always so calm and cool. Thanks for everything! Robert and his discotheque after 6:00 PM was really an experience. Aarnoud and his distinctive laughter are permanently recorded in my memory. Chris, Jean, Thasia and Linda, we were really a good team. Stephanie and Jake, both young and tender souls that while performing science became insane and both got married. Jimmy, Jimmy, Jimmy! It was almost impossible to ignore you. Natalie, always so determined. Anto your height is inversely proportional to your positive energy. TOMMASO, do you know?!! Do you! (Also to you Cintia.) Huimin, it was fun having you as my neighbor. Ivan, I still do not comprehend if you only "seemed" quiet or was it Jimmy the bad influence? Alessandra, my partner, I am sorry we could not spend more time together. Thank you Angela for your hospitality and more importantly Tia's recipe. To Guri and Duran, best of luck to you.

Gerard, thanks for sharing a really great department.

Andy, for the corrections and for the best babysitting ever (second only to Rhonda's)!

To the friends I made along the way! **All of you!** You made everything so much easier!

Mi familia, por tener la suerte de haber nacido Martin Matos.

Eddie, la verdad es que no me equivoque contigo!

GRACIAS!

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Martin M del P*, Tessitore A*, and d'Azzo A. 2002. ER-stress-mediated apoptosis in the CNS of GM_1 Gangliosidosis mouse model. (Submitted for publication)
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ABSTRACTS

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Appendix

Table 1. Knockout mouse models of lysosomal disease±

Human disease	Deficiency	Human phenotype	Mouse phenotype
Aspartylglucosaminuria	Aspartylglucosaminidase	▪ Excretion of glycoasparganines in the urine and storage material in liver, brain, kidney and epithelial cells. ▪ Slowly progressive with severe mental retardation, decrease stature and coarse features. Mild motor clumsiness. ▪ Shorter life spans (35-45 yr.).	▪ Excretion of glycoasparganines in urine and storage in lysosomes of the kidney, liver, skin and brain. ▪ Slightly affected earning and memory abilities and mild skeletal abnormalities. ▪ Normal motor coordination ▪ Normal life spans. ▪ Ref: Jalanko A,, *et al.*, 1998
Cholesteryl ester storage disease (CESD) and **Wolman's disease** (WD)	Lysosomal acid lipase	▪ Lysosomal storage of cholesteryl ester and triglycerides in liver, adrenal glands, small intestine and other organs. ▪ WD: severe infantile form, hepatosplenomegaly, malbsorption, steatorrhea, abdominal distention, adrenal calcification, and failure to thrive. Death before 1yr. of age. ▪ CESD: later-onset with milder clinical symptoms. Hepatomegaly and premature arteriosclerosis. Deposition of only cholesteryl ester in various tissues. Survival beyond middle age.	▪ Accumulation of cholesteryl ester and triglycerides as in WD. ▪ Lower body weight. ▪ Survive until reproductive age. ▪ Biochemical phenotype of the more severe form, WD and survival of CESD ▪ Ref: Du H *et al.*, 1998
Fabry disease	β-Galactosidase A	▪ Deposition of neutral glycosphingolipds in the liver, heart, spleen, kidneys and vascular endothelial cells. ▪ Paresthesis in the extremities, corneal dystrophy, angiokeratoma, vascular disease of the heart, kidney and brain. ▪ Premature mortality.	▪ Extreme lipid accumulation in liver and kidney. ▪ No overt clinical phenotype. ▪ Appear normal through 10 weeks of age. ▪ Ref: Oshima T *et al*, 1997
Galactosialidosis	Protective Protein Cathepsin A (PPCA) Secondary deficiency: -galactosidase and acid neuraminidase	▪ Excretion of sialyloligosaccharides and vacuolation in visceral organs and in cells of central and peripheral nervous system. ▪ Early infantile: fetal hydrops, edema, visceromegaly and skeletal dysplasia. Death within first yr. ▪ Late infantile: hepatosplenomegaly, growth retardation and cardiac involvement. Absence of neurological signs. ▪ Juvenile/adult: progressive neurological defects, myoclonus, ataxia, macular cherry red spots, angiokeratoma and skeletal dysmorphia.	▪ Presence of urinary oligosaccharides and vacuolated cells in various organs. ▪ Neuraminidase activity was deficient as in the human disease, while enzymatic activity of b-galactosidase was variable. ▪ Coarse facies, edema, ataxia and tremors. ▪ Life span of 12 mo. of age. ▪ Pathological symptoms of severe early onset with prolonged survival. ▪ Ref: Zhou XY *et al*, 1995

Gaucher disease	Glucocerebrosidase	▪ **Accumulated glucocerebroside of characteristic twisted tubular structure.** ▪ Type I: characterized by the lack of central nervous system involvement. ▪ Type II, has an early onset along with CNS involvement and culminates in death in the first years of life. ▪ Late onset, Type III, has a slower progression of the neurological symptoms with common clinical manifestations consisting of hepatosplenomegaly, bone lesions and in some cases patients present lung or other organ involvement.	▪ **Mice died 24 h after birth and manifest clinical symptoms that are consistent with nervous system dysfunction.** ▪ Symptoms are similar to the progression seen in patients of Gaucher disease Type II. ▪ Fulminant phenotype. ▪ Ref: Tybuleewicz VLJ *et al*, 1992
Glycogen storage disease type II/Pompe disease	β-Glucosidase	▪ Infantile onset: cardiomegaly, hypotonia and hepatomegaly. ▪ Fatal cardiorespiratory failure by 2 yr. age. ▪ Adult onset: slowly progressive, and myopathy. Shorter life spans (20-60 yr. of age). ▪ Childhood/ juvenile: skeletal muscle involvement, lack of cardiac involvement. Slowly progressive course with short life spans. ▪ The amount of residual activity correlates inversely with severity of symptoms.	▪ Biochemical identical. ▪ Progressive lysosomal glycogen accumulation in heart, skeletal muscle and other tissues. ▪ Exon 6-deleted mutant/ Cre-Lox P: developed locomotor abnormalities around 7 mo. of age. ▪ Exon 6-inserted mutant: impaired mobility around first mo. of life. Survived beyond 1 yr. ▪ Exon 13-inserted mutant: phenotype normal up to 9 mo. when developed gait abnormalities. Survived beyond 1 yr. ▪ Mimic early onset by genetic, biochemical and pathological criteria, but adult onset in clinical course, onset and life span. ▪ Ref: Bijvoet AG *et al*, 1998
GM1-gangliosidosis	-Galactosidase	▪ Accumulation of GM_1 ganglioside in neurons. ▪ Neurological disorder of progressive brain dysfunction. ▪ Infantile type: rapid progression, visceral organ involvement with MPS-like symptoms (abnormal facies, dyostosis multiplex, heart, vision, hearing malfunction and mental retardation. Death within first years. ▪ Adult form: bone abnormalities and lack visceral organ involvement.	▪ Diffuse neuronal storage. ▪ GM1- and GA1- ganglioside accumulation in neurons. Patients observed far less G_{A1} ganglioside storage. ▪ Spastic dysplegia, tremors, ataxia and abnormal gait. Minimal involvement of visceral organs. ▪ Life spans of 7-10 mo. of age. ▪ Ref: Hahn CJ *et al*, 1997
GM2-Gangliosidosis, Tay-Sachs disease	-Hexoxaminidase β-subunit (Hex A)	▪ Accumulation of GM2-ganglioside and MCB in neurons. ▪ Infantile form (total absence of enzymatic activity) Rapid progressive neurodegenerative symptoms (mental and motor deterioration). ▪ Lack of visceral organ involvement. ▪ Death in early childhood (4 yr. of age).	▪ Regional storage to GM2-ganglioside. ▪ Biochemical and pathological features as the human disease. ▪ Phenotypical normal and fertile ▪ Lack of visceral organ involvement. ▪ Normal life span. ▪ Ref: Yamanaka S, 1994, Coeh-Tannoudji M, 1995, Taniike M *et al*, 1995

GM2-Gangliosidosis, Sandhoff disease	-Hexoxaminidase -subunit (Hex A and B)	▪ Accumulation of GM2 ganglioside and presence of MCB in the CNS. ▪ Infantile form, total absence of enzymatic activity: Rapid progressive neurodegenerative symptoms (mental and motor deterioration). Visceral organ involvement. ▪ Death in early childhood (4 yr. of age).	▪ Widespread vacuolation in neurons. ▪ Accumulate GM2- and GA2-gangliosides. ▪ Severe impaired motor function and gait abnormalities. ▪ Visceral organ involvement. ▪ Life span around 4 mo. of age ▪ Ref: Sango K *et al*, 1995
GM2-Gangliosidosis, GM2- activator deficiency	GM2-activator protein	▪ Accumulation of GM2- ganglioside and MCB in neurons. ▪ Infantile form (total absence of enzymatic activity): death in early childhood (4 yr. of age). ▪ Rapid progressive mental and motor deterioration.	▪ Regionalized accumulation of GM2-ganglioside and slight storage of GA2 ganglioside, including the cerebellum. ▪ Defects in balance and coordination and subtle neuronal dysfunction. ▪ Normal life span. ▪ Ref: Liu Y. *et al* 1997
Infantile Neuronal Ceroid Lipofuscinosis or Batten's disease	Palmitoyl-protein thioesterase 1	▪ Accumulation of autofluorescent material in the brain and other tissues of characteristic granular osmiophilic deposits (GROD). ▪ Progressive psychomotor retardation, visual failure and seizures. ▪ Death within first decade of life.	▪ Widespread autofluorescent deposits in the brain and characteristic GROD structures. ▪ Neuronal cell loss and apoptosis. ▪ Viable and fertile. ▪ Spasticity, motor abnormalities and myoclonic seizures. ▪ Death by 10 mo. of age. ▪ Ref: Gupta P *et al*, 2001
β-Mannosidosis	β-Mannosidase	▪ Elevation in the serum and urine of oligosacharides. ▪ Enlargement of lysosomes from accumulated metabolites. ▪ Progressive mental retardation, impaired hearing, dyostosis multiplex, immune defects, lens opacities, muscular hypotonia, macroglosia, and prognathism. ▪ Symptoms manifest within the first year. ▪ Death within the first decade of life. ▪ No correlation found between genotype and phenotype.	▪ Elevated urinary secretion of mannose-containing oligosaccharides. ▪ Accumulation in the liver, kidney, spleen, testis and brain. ▪ Restricted neuronal storage. ▪ Mice did not develop the disease until12 mo. of age. ▪ Attenuated phenotype. ▪ Ref: Stinchi S *et al*, 1999
Metachromatic leukodystrophy (MLD)	Arylsulfatse A	▪ Accumulation of sulfatide in various organs including the brain. ▪ Mayor pathological feature: demyelination. ▪ Infantile type: blindness, loss of speech, quadriparesis, peripheral neuropathy and seizures. Patients die within first years. ▪ Adult onset: behavioral disturbances and dementia. ▪ Severity of the disease correlates with residual activity.	▪ Accumulation of sulfatide in various organs including the brain. ▪ Lack of demyelination. ▪ Milder phenotype with only subtle abnormalities after 1 year. ▪ Normal life spans. ▪ Biochemical abnormalities as the human disease. ▪ Ref: Hess B *et al*, 1996

MPS I*	β-L-iduronidase	▪ Glycosaminoglycan excretion. ▪ Hurler syndrome: severe mental retardation, hepatosplenomegaly, dyostosis multiplex, corneal clouding, and cardiac involvement. Death in early childhood. ▪ Scheie syndrome: milder symptoms, corneal clouding, hearing loss, and mild visceral involvement. Normal life spans.	▪ Glycosaminoglycan excretion and widespread lysosomal storage. ▪ Facial abnormalities and dyostosis multiplex. ▪ Remain normal trough 5 mo. of age. ▪ Symptoms resemble severe MPS in humans with an attenuated phenotype. ▪ Ref: Clarke L *et al*, 1997
MPS VI or Maroteaux-Lamy syndrome	Arylsulfatase B	▪ Granular inclusion bodies in leukocytes and urinary secretion of dermatan sulfate. ▪ Dysmorphic skeletal abnormalities, dyostosis multiplex, macrocephaly, hepatosplenomegaly, corneal clouding, hernias, thickening of the skin, and cardiac involvement. ▪ Short life span (20-30 years).	▪ Granular inclusion bodies in leukocytes and urinary secretion of dermatan sulfate. ▪ Symptoms worsen around 9-12 mo. of age. ▪ Do not manifest hepatosplenomegaly. ▪ Milder phenotype. ▪ Mortality at about 15 mo. of age. ▪ Ref: Evers M *et al*, 1996
Niemann-Pick disease	Acid sphingomyelinase	▪ Sphyngomyelin and cholesterol accumulate in reticuloendothelial system. ▪ Hepatosplenomegaly ▪ Type A: severe neurovisceral storage Death by 3 yr. of age. ▪ Type B: little or lack of neurological manifestation Survive until adulthood.	▪ Mouse models of Otterbach, et. al and Horinuchi et.al. have biochemical identical features. ▪ Atrophy of the brain, cerebellar dysfunction, and lack of hepatosplenomegaly. Storage in visceral organs and neurons. ▪ Disease manifestations at around 8-12 weeks ▪ Life span of about 6 mo. of age. ▪ Similar features of Type A of the human disease. ▪ Ref: Otterbach B *et al*, 1995, Horinouchi K *et al* 1995
Pycnodyostosis	Cathepsin K	▪ Vacuolation containing collagen fibrils. ▪ Diffuse bone sclerosis, short stature, dysmorphic appearance, dental abnormalities and predisposition to bone fractures. ▪ Life span is normal.	▪ Accumulation of partially degraded products in osteoclast. ▪ Osteopetrotic phenotype with ultrastructural, histological and radiological impaired reabsorption of the bone matrix. No suppressed growth observed. ▪ Ref: Saftig P *et al*, 1998

Sialidosis	Acid neuraminidase	▪ Urinary excretion of syalilated oligosaccharides and proteinuria. ▪ Infantile form: mucopolisaccharidosis and glomerulopathy. Severe nephropathy, progressive edema, splenomegaly and kyphosis. Exhibit early death and stillbirth. ▪ Type I: late onset, cherry red spots, myoclonus, and visual failure. Death around 30 yr. of age. ▪ Type II: skeletal dysphasia, hepatosplenomegaly, moderate to severe mental retardation. ▪ Juvenile/infantile subtype of Type II: progressive visceromegaly, dyostosis multiplex and mental retardation. Death around 20 yr. of age.	▪ Oligosaccharidurla and prominent vacuolation. Lack of proteinuria. ▪ Minor bone abnormalities, deformities of the spine, and extramedullary hematopoiesis. ▪ 27% of knockouts died around 21 days of age. ▪ Life spans between 8-12 mo. of age. ▪ Molecular and biochemical features of severe sialidosis with decrease mortality. ▪ Ref: de Geest N *et al*, 2002
Total Sphingolipid Activator Deficiency	Prosaposin (pro SAPs)	▪ Accumulation of sulfatide and other sphingolipids. ▪ Clinical aspects similar to MLD and severe type of Gaucher disease. ▪ Decrease activity of glucosylceramidase and galactosylceramidase.	▪ Extensive neuronal storage trough the cerebrum, cerebellum, brainstem, spinal cord and retinal ganglion. ▪ Cellular inclusions similar to those of SAP deficiency. ▪ Around 10% of knockout die in utero. ▪ Tremors and ataxia. ▪ Animals mimic the biochemical changes with milder phenotype.

± Adapted from Scriver et. al. (ref) with additional data from references (Kolter 1998, Elsea, 2002, Susuki, 1998).
* MPS indicates mucopolysaccharidosis.

Propositions belonging to this thesis:

GM1 Gangliosidosis and Galactosialidosis: pathogenesis and therapy

1. Successful gene therapy will require the application of many different techniques, tailored to the disease that is being approached.
   D. Balicki and E. Beutler. Reviews in Molecular Medicine. 2002.

2. Persistent UPR activation leading to caspase-12 activation in GM1-gangliosidosis might explain the cause of neuronal cell death.
   This thesis.

3. The dogmatic view of an ever-immutable neural tissue in mammals is now been replaced by the notion than indeed cell turnover occurs in the mature CNS, thanks to the persistence of precursor cells that possess the functional characteristics of bona-fide neural stem cells within restricted brain areas.
   F. H. Gage. Science. 2000.

4. Inflammation may play an important role in the pathogenesis of the gangliosidoses as suggested by the observation that progressive CNS inflammation coincided with the onset of clinical signs in mouse models.
   Jeyakumar M., *et al.* Brain. 2003.

5. General blockade of caspases sensitizes rather than protects against TNF-induced lethality, indicating that caspase-dependent protective pathways are essential to counteract TNF-induced lethal signaling pathways *in vivo*.
   Cauwels A., *et al.* Nature Immunology. 2003.

6. Recovery of function rather than protection from disease is a key goal for any effective therapy for human lysosomal storage disease, because most patients are diagnosed well after onset of CNS disease.
   W. S. Sly and C. Vogler. PNAS. 2002.

7. The demonstration of the Protective protein/cathepsin A proteolytic activity in the degradation of lysosome-associated membrane protein type 2a (lamp2) suggests that Cathepsin A has an important regulatory function in chaperone mediated autophagy.
   Cuervo A.M., *et al.* EMBO 2003.

8. The research that will develop from the humane genome project will force us as a society to thoroughly evaluate the consequences that this knowledge will bring about.

9. Half of the battle is won when one decides to fight it.

10. Talent is a gift from nature and not to use it to benefit society is simply egoistic.

11. Draw, produce, create. Don't criticize yourself. Don't cringe when someone looks right through your drawings-and don't fly off on wings of ecstasy when someone else loves them. You are working for yourself here. And your Mum, of course.
    Q. Blake and J. Cassidy. Drawing for the Artistically Undiscovered.

Maria del Pilar Martin, June 11, 2003.