Gene therapy: prospects for glycolipid storage diseases

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Lysosomal storage diseases comprise a group of about 40 disorders, which in most cases are due to the deficiency of a lysosomal enzyme. Since lysosomal enzymes are involved in the degradation of various compounds, the diseases can be further subdivided according to which pathway is affected. Thus, enzyme deficiencies in the degradation pathway of glycosaminoglycans cause mucopolysaccharidosis, and deficiencies affecting glycopeptides cause glycoproteinosis. In glycolipid storage diseases enzymes are deficient that are involved in the degradation of sphingolipids. Mouse models are available for most of these diseases, and some of these mouse models have been used to study the applicability of *in vivo* gene therapy. We review the rationale for gene therapy in lysosomal disorders and present data, in particular, about trials in an animal model of metachromatic leukodystrophy. The data of these trials are compared with those obtained with animal models of other lysosomal diseases.

Keywords: gene therapy; glycolipid storage disease; sulphatide; metachromatic leukodystrophy

1. RATIONALE FOR GENE THERAPY IN GLYCOLIPID STORAGE DISEASES

In general, lysosomal storage diseases are considered good candidates for gene therapy and enzyme replacement. This is due to the particularities of the sorting pathway of lysosomal enzymes. Lysosomal enzymes are synthesized at the rough endoplasmic reticulum. Cotranslationally they acquire N-linked oligosaccharide side chains, which play an important part in their further sorting pathway. In the Golgi apparatus, lysosomal enzymes are specifically recognized by a phosphotransferase, which initiates a two-step process by which mannose residues of the N-linked oligosaccharide side chains become phosphorylated at position 6. Via these mannose-6-phosphate residues, lysosomal enzymes bind to mannose-6-phosphate receptors in the *cis*-Golgi apparatus. These receptors mediate the further vesicular transport of the enzymes to the lysosome. It is important to realize that mannose-6-phosphate receptors not only function in the transport from the Golgi to the lysosome, but are also present at the plasma membrane from which they can mediate the endocytosis and lysosomal delivery of extracellular lysosomal enzymes. The binding of lysosomal enzymes to their receptors in the Golgi apparatus is not complete. As much as 40% of newly syn-

thesized lysosomal enzymes are secreted, depending on the enzyme and cell type. The secreted enzymes can be endocytosed via the plasma-membrane-located receptors by neighbouring cells (for review on lysosomal enzyme sorting see Kornfeld (1992)). The possibility of enzyme transfer from one cell to the other has important implications for the therapy of lysosomal storage diseases, since a normal cell can provide the deficient enzyme for an affected cell causing a cross-correction of the metabolic defect (see figure 1). For many lysosomal storage diseases, this cross-correction was the rationale for bone marrow transplantations, which have been performed for many years. The bone marrow of a normal person transplanted into a patient provides the missing enzyme that, upon endocytosis by the patient's cells, corrects the metabolic defect.

Since it can be expected that the therapeutic effect will be greater the more enzyme the transplanted cell synthesizes and thus secretes, it seems reasonable to modify the cell genetically to produce large amounts of the missing enzyme in the patient. This can be achieved by viral transduction in the course of a gene therapy approach. Also, in this respect lysosomal enzymes are advantageous: there is, so far, no example in which even massive overexpression of a lysosomal enzyme exerts any toxic effect on the genetically modified cell. Therefore, in gene therapy trials there is no need to control the expression level of the enzyme.

Most of the glycolipid storage diseases (except for Fabry and subtypes of Gaucher disease) show massive central

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Figure 1. Intercellular transfer of lysosomal enzymes. Newly synthesized lysosomal enzymes are recognized in the Golgi apparatus by a phosphotransferase, which initiates the synthesis of mannose-6-phosphate (M6P) residues on the N-linked oligosaccharide side chains of the enzymes. Via these residues, the enzymes can bind to mannose-6-phosphate receptors, which mediate the further vesicular transport from the Golgi apparatus to the lysosome. Since binding to receptors is not complete, a considerable portion of the enzymes are secreted. Since the mannose-6-phosphate receptors are not only present in the Golgi, but also on the plasma membrane, extracellular enzymes can bind to receptors on neighbouring cells and become endocytosed and are delivered to the lysosomes. This mechanism allows for cross-correction of a metabolic defect between a donor and recipient cell. In gene therapy trials, the enzyme-donating cell is genetically modified, such that it overexpresses the enzyme the patient lacks. RER, rough endoplasmic reticulum.

nervous system involvement. Thus, the primary goal is to provide sufficient amounts of enzyme to the nervous system. Owing to the blood–brain barrier, it cannot be expected that enzyme delivered by cells derived from the transplanted bone marrow to the bloodstream will reach the brain. However, it has been shown that a fraction of brain microglia, the perivascular microglia, is bone marrow derived. In mice, six months after bone marrow transplantation *ca.* 23% of brain perivascular microglia were replaced by cells from the monocytic lineage derived from the donor bone marrow (Kennedy & Abkowitz 1997). Thus, bone marrow transplantation combined with viral transduction of bone marrow stem cells prior to transplantation seems to be promising in lysosomal storage diseases. Cells derived from the genetically modified stem cells will produce large amounts of enzyme secreted into the blood to supply the visceral organs. Some cells of the monocytic lineage will cross the blood–brain barrier and become resident perivascular microglia. These cells may be able to provide sufficient amounts of enzyme to the nervous system (Eto & Ohashi 2002).

Alternatively, gene therapy may also involve the direct transplantation of genetically modified cells into the nervous system, such as neural precursor cells or astrocytes. Yet another alternative is the direct injection of viral vectors into the brain with the aim of transducing a sufficient number of endogenous cells, which then serve as enzyme-producing platform cells (Consiglio *et al.* 2001; Shen *et al.* 2001). In glycolipid storage diseases, the effects of bone marrow transplantations with retrovirally modified stem cells, direct injections of viral vectors into the ventricular

space or brain and intravenous administration of viral vectors have already been examined.

2. GENE THERAPY IN A MOUSE MODEL OF METACHROMATIC LEUKODYSTROPHY

Metachromatic leukodystrophy is caused by the deficiency of ASA. This enzyme initiates the degradation of 3-*O*-sulphogalactocerebroside (sulphatide) through desulphation of a galactose moiety. Enzyme deficiency causes storage of sulphatide in various tissues, such as the distal tubules of the kidney, bile duct epithelia of the liver, neurons, and oligodendrocytes of the nervous system. Whereas the storage in liver and kidney has no major functional consequences, the storage in neuronal and glial cells causes massive demyelination with a wide variety of neurologic symptoms. The lipid storage pattern found in an ASA-deficient mouse is identical to that in humans. Except for storage in the nervous system, mice show a reduced axonal diameter, degeneration of neurons in the acoustic ganglion, and degeneration of fibres in peripheral nerves when the animals become older than 18 months of age. However, up to the age of 24 months the animals do not show the widespread demyelination in the central nervous system seen in humans. The phenotype of disease is thus much milder than in humans (Hess *et al.* 1996).

These animals were used for gene therapy experiments based on the transplantation of genetically modified haemopoietic stem cells (Matzner *et al.* 2000, 2002). Bone marrow was harvested from ASA-deficient mice and cultured in the presence of various growth factors. During

this period, cultured cells were transduced with a retrovirus conferring the overexpression of ASA on haemopoietic cells. In this retroviral vector, the ASA cDNA was under the control of the long terminal repeat and the vector did not include any resistance marker. Twenty-seven animals at five weeks of age were transplanted. The control group consisted of animals transplanted without retroviral transduction. ELISA-based quantification of ASA in blood taken at regular intervals from the tail vein allowed tracking of ASA expression from the integrated retroviral vector without the need to sacrifice the animals. One year after transplantation, approximately half the animals had lost expression of ASA, whereas in 50% of animals overexpression of ASA was maintained. Only the latter animals were analysed further. It is significant that none of these animals developed antibodies against arylsulphatase or tumours, which might be expected due to the random retroviral insertion observed. Animals were sacrificed *ca.* 1 year after transplantation, and ASA activity and cross-reacting material were determined in various organs. Tissue activities varied largely between animals but, on average, supranormal levels were obtained in liver, kidney, spleen and bone marrow. Surprisingly, even in brain 21% of normal enzyme activity, on average, was found. Upon treatment, sulphatide levels in liver were reduced by approximately one-third, whereas, on average, no sulphatide reduction was detected in kidney and brain. Closer examination, however, revealed a correlation between the tissue levels and reduction of storage. It became clear that *ca.* 200% of normal enzyme activity is needed to achieve a full reduction of storage in liver and kidney (Matzner *et al.* 2002).

In nervous tissue of patients suffering from metachromatic leukodystrophy the most sensitive indicator for the alteration of lipid metabolism is not the absolute amount of sulphatide rather than the ratio of galactocerebroside to sulphatide. When the ratio of these lipids was determined in the brain of treated animals, we found that indeed the ratio of these lipids had improved towards normal levels in treated mice. Thus, treatment also has an effect on the lipid metabolism of the central nervous system. However, it is insufficient to achieve a substantial clearance of sulphatide. In treated animals the axonal cross-sectional area, which is diminished in ASA-deficient mice, also returned to normal, which again indicates a positive therapeutic effect on the nervous system. This improvement, however, was only seen in the peripheral nerves and not in the optic nerve. Various behavioural tests performed with the treated mice showed no significant improvement, except that hyperactivity displayed by non-treated animals was clearly reduced in the treated group.

The same animal model was used in a study by Consiglio *et al.* (2001). This group used an alternative approach by injecting a lentiviral vector encoding ASA directly into the hippocampal region of ASA-deficient mice. Lentiviral vectors have the advantage that they also transduce non-dividing cells, whereas classical retroviral vectors depend on cell division for provirus integration. Consiglio *et al.* (2001) found that hippocampal neuronal degeneration was rescued in the treated animals and this results in an improved performance of treated animals in the Morris water maze. However, their findings are in conflict with those of other groups. The hippocampus of ASA-deficient

mice was examined by Hess *et al.* (1996) and independently by J. E. Mansson, Z. Pernber, M. Molander-Melin, V. Gieselmann and P. Fredman (unpublished data). These groups found no indication of hippocampal degeneration and thus the results of Consiglio *et al.* (2001) are somewhat enigmatic and require independent verification. Regarding the behavioural assays in this study, we consider it impossible to ascribe behavioural changes to circumscribed pathological improvements in particular brain regions, since the mice show considerable general neuromotoric coordination problems due to global brain affection by the widespread storage of sulphatide.

3. GENE THERAPY TRIALS IN MOUSE MODELS OF OTHER GLYCOLIPID STORAGE DISEASES

Excluding metachromatic leukodystrophy, *in vivo* gene therapy studies have also been reported for twitcher mice (Krabbe disease), β -hexosaminidase α -subunit-deficient mice (Tay-Sachs disease) and α -galactosidase A-deficient mice (Fabry disease).

Shen *et al.* (2001) injected recombinant adenovirus encoding galactocerebroside into the lateral ventricle of twitcher mice. This treatment was performed in animals of various ages. Treated animals showed slight improvements in motor functions, body weight and survival. However, these improvements were seen only when injection was performed at postnatal day 0 whereas no effects were observed after injection at postnatal day 15, which indicates the importance of correct timing of this gene therapy approach.

Tay-Sachs disease is due to β -hexosaminidase α -subunit deficiency, which causes storage of GM2 ganglioside. In a mouse model of Tay-Sachs disease adenoviral vectors were injected into the blood and transduced, preferentially, the liver. Since β -hexosaminidase A consists of two subunits encoded by different genes, two vectors—one for each subunit—were injected, because otherwise the subunit, which is not defective in the animals, was expected to be rate limiting in gene therapy. Adenovirus injection led to partial restoration of enzyme activity in all peripheral tissues, but not in the brain. Since this mouse model, due to peculiarities in murine GM2 ganglioside metabolism, stores only little lipid, it could not be determined whether these enzyme activities were sufficient to reduce storage (Guidotti *et al.* 1999). Lacorazza *et al.* (1996) genetically modified neural progenitor cells to overexpress hexosaminidase α -chain. They transplanted these cells into the brain of normal mice and demonstrated substantial *in situ* expression of the enzyme. Since these studies were performed with normal mice, however, it was not possible to demonstrate whether this approach may have any influence on the course of the disease.

The vast majority of trials of gene therapy have been performed in the mouse model of Fabry disease (for a review, see Siatskas & Medin 2001).

Fabry disease is due to a deficiency of α -galactosidase A, which results in the deposition of globotriaosylceramide. In contrast to most other glycolipid storage disorders, patients show no central nervous system involvement. Globotriaosylceramide accumulates predominantly in the endothelial cells of heart, kidney, liver and spleen. Patients suffer from renal, cardiac and cerebrovascular compli-

Table 1. Comparison of bone marrow transplantation-based gene therapy trials in mouse models of lysosomal storage disorders. (The table summarizes some data on enzyme activities and storage compound reduction taken from the respective references.)

organ	Fabry disease α -galactosidase A (Takenaka <i>et al.</i> 2000)		metachromatic leukodystrophy ASA (Matzner <i>et al.</i> 2002)	
	activity (% of normal)	effect on storage material	activity (% of normal)	effect on storage material
liver	30%	reduction to normal	ca. 100–150%	reduction to 70%
kidney	< 10%	reduction to 27%	ca. 100–150%	no reduction
spleen	80%	reduction to 25%	—	—
brain	0%	no reduction	20%	no reduction

organ	galactosialidosis cathepsin A (Leimig <i>et al.</i> 2002)		Niemann–Pick type A sphingomyelinase (Miranda <i>et al.</i> 2000)	
	activity (% of normal)	effect on storage material	activity (% of normal)	effect on storage material
liver	20%	reduction to normal	65%	reduction to 5%
kidney	< 10%	reduction to normal	0%	no reduction
spleen	—	—	100%	reduction to 10%
brain	11%	partial reduction	0%	no reduction

cations. Various gene therapy protocols have been examined in a mouse model of this disease. Since the central nervous system is not the therapeutic target in Fabry disease, gene therapy protocols aiming at peripheral organs are also feasible. For that reason Jung *et al.* (2001) injected adeno-associated viral vectors encoding α -galactosidase A directly into the portal vein of enzyme-deficient mice. These vectors transduced the liver of injected animals *in vivo*, leading to ca. 20–35% of normal enzyme activity in the liver. Considerable amounts of α -galactosidase A were also found in other peripheral organs. Globotriaosylceramide storage in spleen, heart and lung was reduced to between 66% and 81%, whereas little reduction was seen in kidney. This study demonstrates that genetic modification of tissues other than bone marrow may also be a suitable approach.

A similar approach was taken by Ziegler *et al.* (1999) who intravenously injected an adenoviral vector encoding α -galactosidase A without targeting specific organs. This led to an increase of enzyme activity in all tissues and significant reduction of lipid storage. Finally, the same vector was used for instillation into the lung (Li *et al.* 2002). This led to viral modification of lung tissue only, but enzyme was found in various other tissues, which caused an amelioration of globotriaosylceramide storage. These trials demonstrate that in glycolipid storage diseases without central nervous system involvement various virally modified peripheral organs can serve equally successfully as enzyme sources.

Transplantation of bone marrow cells transduced with a retrovirus encoding α -galactosidase A led to long-term enzyme corrections (Takenaka *et al.* 2000). Animals were followed for up to 26 weeks post-transplant. In spleen 80%, and in liver and heart 30%, of normal enzyme activity was achieved in treated mice. By contrast, in kidney the activity did not even reach 10% of normal activity. No activity was found in brain. Thus, there is considerable variation in the distribution of the enzyme among visceral organs. In liver and heart, globotriaosylceramide storage was reduced close to control levels, whereas in all other peripheral organs only partial reduction was observed. In

correlation with the low amounts of enzyme found in the kidneys, only slight amelioration of lipid accumulation was found in this organ.

Table 1 summarizes some data from bone marrow stem-cell-based gene therapy trials in four lysosomal storage disorders, two glycolipid storage diseases (Takenaka *et al.* 2000; Matzner *et al.* 2000, 2002), one lipid storage disease (Miranda *et al.* 2000) and one disorder of glycoprotein degradation (Leimig *et al.* 2002). In all these trials bone marrow cells were transduced with a retrovirus *ex vivo*, were subsequently transplanted and analysed after long-term reconstitution ranging from 5 to 11 months. Comparison of these data reveals some interesting differences. First, it becomes apparent that depending on the enzyme and storage compound, the amounts of enzyme necessary for correction of the metabolic defect vary between the particular enzyme/substrate combinations. Thus, as little as 10–30% of normal enzyme activity can result in at least partial clearance of the storage compound in models of Fabry disease and galactosialidosis. By contrast, supranormal levels of enzyme activity are needed in the case of ASA-deficient mice. Second, there are striking differences in the distribution of enzymes in the organs of the various animal models. Since all these enzymes are sorted by the mannose-6-phosphate receptor pathway, these results clearly demonstrate that factors other than mannose-6-phosphate influence the distribution of enzymes substantially. Whereas in the case of α -galactosidase A and sphingomyelinase no enzyme could be found in the brain, cathepsin A and ASA could be delivered to the brain. In the case of cathepsin A, this also led to a decrease of storage compounds in brain and a significant improvement of neuropathology.

These data reveal that the feasibility of gene therapy has to be examined for every enzyme deficiency separately and that results of various animal models simply cannot be transferred to each other.

The data of all these trials show that in general the storage in peripheral organs can be positively influenced by bone marrow stem cell gene therapy, whereas there is currently no example among the lipid storage diseases that

central nervous system accumulation can be decreased. Thus, in most of the glycolipid storage diseases the unresolved question is the effective widespread and long-lasting delivery of the missing enzyme into the brain. The dissatisfying outcome of bone marrow transplantation-based therapeutic trials suggests that other strategies must be employed. These involve direct injection of viral vectors into the brain and transplantation of enzyme-overexpressing cells into the brain.

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GLOSSARY

ASA: arylsulphatase A