Gene therapy of lysosomal storage diseases

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Introduction

Lysosomal storage diseases, representing to about forty distinct genetic entities, mostly autosomal recessive, are due mainly to abnormalities of the catabolism of macromolecules: glycolipids, glycoproteins, glycosaminoglycans, etc. The defect of degradation of these molecules results in their accumulation and a cascade of metabolic abnormalities affecting the cell. They are generally associated with severe psychomotor retardation, hepatosplenomegaly, skeletal deformities, corneal opacities or neurological disorders, and often lead to an early fatal outcome [1]. However, cases with late clinical manifestations also exist: the adult forms.

Each one of these diseases, considered individually, is rare, but their overall frequency is 1/5,000 births. Some have been delineated as clinical syndromes for more than a century, but their aetiopathogenic mechanisms have only been elucidated over the last few decades. In 1881, Warren Tay described the clinical characteristics of infantile amaurotic idiocy and, in 1896, Bernard Sachs described its familial nature, giving their names to Tay-Sachs disease. It was also at the end of last century, in 1882, that Philippe Gaucher presented a doctoral thesis reporting the first case of hepatosplenomegaly with reticulo-endothelial overload and gave his name to this syndrome. The description of lysosomes by Christian de Duve in 1955 is now forty years old and the discovery of "correction factors" by Elisabeth Neufeld in 1971 almost 25. Over the following years, these correction factors have been identified to correspond to the deficient lysosomal enzymes in each type of disease.

Subsequent research by several teams, including those directed by William Sly and Stuart Kornfeld, led to the discovery, in 1977, of mannose-6-phosphate as a recognition marker of lysosomal enzymes and elucidated their transport from the Golgi apparatus to lysosomes via specific receptors: mannose-6-phosphate (Man-6-P) receptors [2]. These receptors, also expressed on the cell surface, mediate the endocytosis of lysosomal enzymes secreted into extracellular spaces (Fig. 1). In fact, part of synthesised lysosomal enzymes (about 10%) is excreted and the possibility of "uptake" by neighbouring cells is important to be considered for gene therapy. This explains the possibility of distant or cross-correction of enzyme deficiencies. The discovery of the Man-6-P receptor also helped to elucidate certain pathogenic mechanisms involved in lysosomal transport disorders, such as mucolipidosis II [3].

The subsequent identification of the enzyme deficiencies responsible for these diseases allowed their precise diagnosis (Table I). Progress in foetal sampling techniques now allows early antenatal diagnosis of the majority of these diseases [4]. The chromosomal locus of most of the genes responsible has now been identified. Most of the genes have been cloned. Their precise genotyping, now possible, is most useful either for epidemiology [5], prognosis, genotype-phenotype correlation as exemplified by Gaucher disease [6], or diagnosis, particularly in atypical cases, such as pseudo-deficiencies [7]. Molecular genotyping is also useful for the detection of heterozygotes [8].

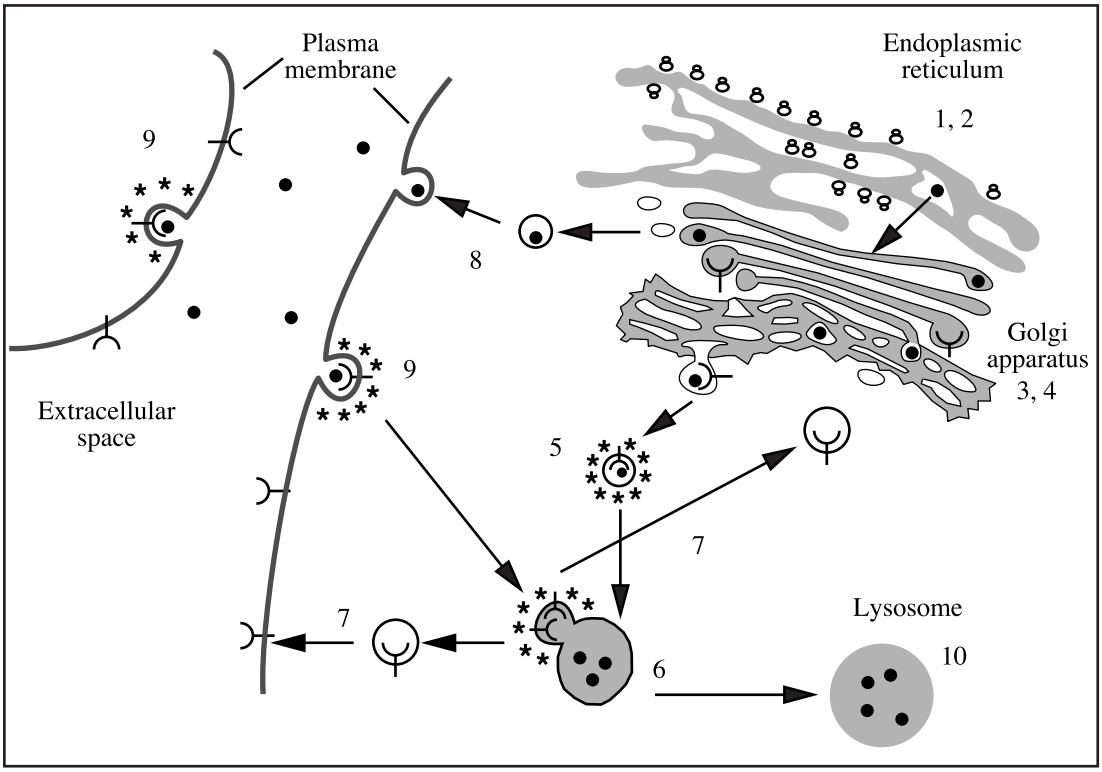

Fig. 1: Lysosomal enzyme transport and recycling of Man-6-P receptors.
- • *lysosomal enzyme*
- ♈ *mannose-6-phosphate receptor (Man-6-P R)*
- *** *intracellular transport in clathrine-coated vesicles*

1) protein synthesis from mRNA; 2) glycosylation and elimination of the signal sequence; 3) synthesis of the Man-6-P recognition marker; 4) association of subunits; 5) transfer to the lysosome via Man-6-P R; 6) release of the enzyme in acidic medium and polarization of receptors; 7) recycling of receptors in the Golgi apparatus and plasma membrane; 8) enzyme secretion vesicles; 9) endocytosis mediated by the Man-6-P R; 10) maturation of enzymes in the lysosome by limited proteolysis.

Although the possibility of a cure of genetic diseases has often been met by scepticism for many decades, strategies with proven efficacy have been proposed over recent years treatment for certain lysosomal storage diseases.

Particular features of lysosomal storage diseases and therapeutic prospects

Lysosomal storage diseases present a number of particularities which must be taken into account in the choice of therapeutic strategy, in order to ensure a maximum chance of success. The first point to be reckoned with is their ubiquitous nature. As lysosomal enzymes are synthesized in all body cells (except erythrocytes) their deficiency is also ubiquitous. However, there is a tissue specificity in the pathological accumulation of non-degraded substrates. There are considerable differences according to the disease, as is illustrated by the fact that the clinical diagnosis is based on the anatomical and functional abnormality of the most severely affected organs (Table II).

Table I: Gene loci of the main lysosomal storage diseases.

Lysosomal storage disease		Enzyme deficiency	Gene locus
Sphingolipidoses			
Wolman		Acid lipase	10q23
Tay-Sachs		β-hexosaminidase A	15q23-24
Sandhoff		β-hexosaminidase A+B	15q13
Hexosaminidase activator deficiency		Hexosaminidase activator	5
Landing		β-galactosidase	3p21
Gaucher		β-glucosidase	1q21
Niemann-Pick A & B		Sphingomyelinase	11p15
Niemann-Pick C		Cholesterol transport	18p
Krabbe		Cerebroside β-galactosidase	14q24-32
Fabry		α-galactosidase	Xq21-22
Metachromatic leucodystrophy		Arylsulfatase A	22q13
Arylsulfatase activator deficiency		Arylsulfatase activator	10
Farber		Acid ceramidase	?
Mucopolysaccharidoses			
Type I	Hurler	α-L-iduronidase	4p16.3
	Scheie	α-L-iduronidase	4p16.3
Type II	Hunter	L-iduronide sulfatase	Xp28
Type III	Sanfilippo A	Sulfamidase	?
	Sanfilippo B	α-glucosaminidase	?
	Sanfilippo C	Acetyl-CoA:α-glucosaminidase acetyltransferase	14 / 21 ?
	Sanfilippo D	N-acetylglucosamine-6-sulfatase	12q14
Type IV	Morquio A	6 sulfate-sulfatase	16q24.3
	Morquio B	N-acetylgalactosamine β-galactosidase	3p21
Type VI	Maroteaux-Lamy	Arylsulfatase B	5p11-q13
Type VII	Sly	β-glucuronidase	7q21-22
Glycoprotein storage disorders			
α-Mannosidosis		α-mannosidase	19cen-q13
β-Mannosidosis		β-mannosidase	?
Fucosidosis		α-fucosidase	1p34-36
Aspartylglucosaminuria		Aspartylglucosaminidase	4p32-33
Mucolipidoses			
Type II and III		N-acetylglucosamine-phosphotransferase	4q21-23
Type I- Sialidosis		N-acetylneuraminidase	6 / 10 ?
Galactosialidosis		Protective protein	20q13.1
Glycogen storage diseases			
Type II Pompe		α-glucosidase	17q23

Secondly, lysosomal enzymes are partially (10% of the total) secreted outside the cells and can be taken up by adjacent or distant cells. This endocytosis depends on several receptor systems [9], each with dual enzymatic and cellular specificity. The Man-6-P receptor, already mentioned, is characteristic for certain cells but its presence in all tissues has not been confirmed. Other receptors, specific for galactose, mannose or fucose, may also play a role. A direct enzyme transfer system, by cell-to-cell contact, has also been described, and its efficacy appears to be superior to that of receptor transfer [10].

In particular, several experimental, pathological and physiological tests indicate that a low level of normal enzymatic activity suffices to ensure normal life. Examples are the attenuated forms of lysosomal storage diseases, compatible with life until adulthood. These types of diseases are associated with an enzyme activity of 5-10%, unlike infantile forms usually due to a total enzyme deficiency. Another example of the functional effi-

Table II: Examples of diseases with organ-specific clinical expression.

Disease	Organ predominantly affected	Symptoms
Fabry	kidney	renal failure, angiokeratomas, acroparesthesiae
Gaucher		
type I	RES[1]	hepatosplenomegaly, haematological disorders, bone lesions
type II	RES-CNS[2]	neurological disorders, hepatosplenomegaly
Pompe		
infantile	heart	cardiomegaly, hypotonia
juvenile	muscle	myopathy
Tay-sachs	CNS	severe psychomotor retardation, blindness, cherry-red spot
Metachromatic leucodystrophy	CNS+ peripheral NS	mental and motor deterioration quadriplegia, optic atrophy
Hurler	generalized	organomegaly, psychomotor retardation

[1] RES: reticulo-endothelial system
[2] CNS: central nervous system

cacy of a low level of lysosomal enzymes is that of enzyme pseudodeficiencies, described over recent years for several lysosomal enzymes. In this case, the enzyme deficiency is profound in particular towards the artificial substrates used in the assay systems, but incomplete towards the natural substrate. Such patients are clinically normal [7].

Current therapeutic possibilities

The three possible levels of intervention are the gene lesion itself, the enzyme deficiency which it induces and the resulting accumulation of substrate. In the context of lysosomal storage diseases, there is little possibility of acting on the quantity of substrate, as in galactosaemia or phenylketonuria. These accumulated molecules are not directly derived from the diet and the metabolic mechanisms governing their synthesis are very complex. However, it is possible to act on the enzyme deficiency.

Replacement therapy

The principle of this modality consists in replacing the missing or inactivated enzyme by the active enzyme. The first trials of replacement therapy, conducted between 1960 and 1970 for several lysosomal storage diseases (Hurler, Hunter, Tay-Sachs, Fabry, Sanfilippo), did not provide any convincing results. These facts were particularly disappointing in diseases affecting the central nervous system (CNS). Trials of enzyme replacement have nevertheless been continued in diseases not affecting the CNS, such as Fabry disease or type I Gaucher disease.

Great progress has been made in our understanding of the structure of lysosomal enzymes and the mechanisms of their transport and transfer from one cell to another [11]. This progress has led to the development of replacement therapy for Gaucher disease, the first success in this field [12, 13]. In this disease, the non-degraded lipids accumulate in macrophages, bone marrow, spleen and liver (Gaucher type I), but also in the CNS (Gaucher type II and III). Glucocerebrosidase, also called β-glucosidase, the deficient enzyme in this disease, has been purified and its carbohydrate structure has been modified to expose the mannose moieties, in order to facilitate uptake of the enzyme by enzyme-deficient macrophages. The modified enzyme is called alglucerase or Ceredase® [13].

More than 1,000 patients suffering from non-neurological forms of Gaucher disease (type I) are currently being treated throughout the world, with variable doses of enzyme according to the protocol. Treatment appears to be effective, because it is associated with varying degrees of remission of symptoms [14-16]. The results are still uncertain in neurological forms of the disease (types II and III), due to the difficulties related to CNS uptake of the replacement enzyme, which is essentially taken up by macrophages and whose secretion appears to be minimal. [17]. Several type II patients are currently receiving treatment and the results should be available in the near future. At the present time, encouraging results have been reported in an 11-month infant treated from birth, in whom progression of the disease was very different from that observed in his untreated brother who died at the age of 10 months [18].

This treatment is not yet available for other lysosomal storage diseases. Its cost is prohibitive, as Ceredase is considered to be the most expensive drug in the world [Wall Street Journal, December 23, 1991]. The recombinant enzyme is now available for Gaucher disease and the first trials of efficacy are currently underway, prior to release onto the market.

Bone marrow transplantation

Bone marrow transplantation is designed to reconstitute the patient's haematopoietic system with stem cells from an immunocompatible healthy donor. The donor's circulating blood cells and the histiocytes which populate the recipient's organs consequently become a life-long source of enzyme. The enzyme secreted into the circulation can be taken up by distant cells *via* endocytosis. Experimental results suggest that direct cell-to-cell transfer is also an important mechanism, which may contribute to the efficacy of transplantations in these instances [11].

Bone marrow transplantation in animal models for lysosomal diseases shows a complete biochemical and histological correction in liver, cornea and kidney. Partial correction was also obtained in meninges and in cerebral perivascular cells but the results were totally negative in CNS, even when the enzymatic activity was present.

Several therapeutic transplantations have been performed in patients with Hurler, Hunter, Maroteaux-Lamy, or Gaucher disease, mucolipidosis II, metachromatic leukodystrophy, etc. Variable results have been obtained, but they appear to be encouraging for some of the mucopolysaccharidoses [19]. Visceral symptoms improve and bone lesions stabilise. Excellent results have also been obtained in non-neuropathic forms of Gaucher disease. However, diseases with an early and predominant CNS involvement are not good candidates for bone marrow transplantation, although a correction of the neuronal lesion has been recently demonstrated [20]. Several cases of metachromatic leucodystrophy have received a bone marrow transplantation. No clinical remission has been reported, except for one case described by W. Krivit in 1990 in volume 322 of The New England Journal of Medicine.

Approximately 200 patients with lysosomal storage disease have been treated by bone marrow transplantation. The efficacy of this treatment must be evaluated on a more long-term basis in order to define more clearly the treatment modalities [21]. However, this therapeutic approach is still associated with major problems and risks: the difficulty of finding a compatible donor, the need for immunosuppression, the 10-15% transplant failure rate, the mortality rate of still 10-20% and the risk of graft-versus-host disease [22]. These difficulties can, in principle, be avoided by gene therapy.

Gene therapy

It is logical to assume that in generalized diseases, such as lysosomal storage diseases, gene correction exclusively limited to the most severely affected organ will be insufficient. This is demonstrated by transplantation of a healthy organ, a kidney for example, in patients with Fabry disease. Several months after transplantation, accumulation of substrate in the transplanted organ destroys its function. A generalized enzyme supplement, *via* a peripheral enzyme source, will on its own also probably not be sufficient to effectively restore the function of the most severely affected organ. The ideal therapeutic strategy in lysosomal storage diseases would therefore consist of a combination of generalized enzyme supplement, to ensure a minimal homeostatic threshold, and targeted correction of the most severely affected organ, such as the heart in Pompe disease, the brain in diseases with neurological involvement, etc., in order to obtain a higher enzyme level in the target organ.

Animal models

The many alternatives of gene therapy and their complexity require the use of animal models to verify their efficacy and safety, although animal data can never be entirely extrapolated to man.

Natural models of lysosomal storage diseases have been identified in various animal species. The most typical example and that most extensively used in the development of gene therapy is

β-glucuronidase deficiency (MPS VII) in mice [23]. More than 20 lysosomal enzyme deficiencies have been described in cats, dogs, rats, pigs, calves or other species [24]. Some diseases have been described in a single animal but the model has been lost since the derived line could not be preserved. Some of the diseases exist in species such as cattle, a species difficult to use as a gene therapy model in view of the large quantity of recombinant vector required.

Several teams in various countries are working to develop animal models of lysosomal storage disease by gene knockout. This technique uses homologous recombination to interrupt the gene and totally eliminate its function. However the clinical expression of the model obtained is unpredictable. For example, the glucocerebrosidase-deficient mouse (Gaucher), in contrast with man, dies already a few days after birth [25]. In contrast, the models developed for Tay-Sachs disease [26] and metachromatic leukodystrophy [Gieselmann V: personal communication] involve compensatory metabolic mechanisms not existing in man and without obvious phenotypic features. Recently animal models have been obtained for the deficiency in protective protein, responsible for human galactosialidosis, and for the defect of hexosaminidase B, responsible for Sandhoff disease in humans. These models do reproduce the corresponding human diseases.

Ex vivo *expression of specific transgenes in patients' cells*

One of the essential preliminary steps in gene therapy is the control of the expression of the "corrector" gene transduced into deficient cells cultured from patients. Several human genes involved in lysosomal storage diseases, carried by various recombinant vectors, have demonstrated good expression or even significant cellular overexpression. For example, the expression of the iduronate-2-sulfatase gene in a retroviral vector is 10 to 70 times greater than normal in the lymphoblasts of patients with Hunter syndrome [27]. The gene for steroid sulfatase transduced with an Epstein-Barr viral vector into keratinocytes from patients with X-linked ichthyosis has an overexpression 100 times greater than normal [28]. The genes for glucocerebrosidase and arylsulfatase A, transduced by an adeno-associated virus (AAV) in fibroblasts from Gaucher disease and from metachromatic leukodystrophy, respectively, also have a marked expression. In our laboratory, Saïd Akli, using an adenoviral recombinant vector for the HEX A gene, observed an overexpression of hexosaminidase A in the fibroblasts of patients with Tay-Sachs disease. Considerable secretion of the enzyme into the culture medium was also demonstrated.

Experimental correction of murine MPS VII (β-glucuronidase deficiency)

The existence of a natural murine model of MPS VII, homologous to human β-glucuronidase deficiency, enabled the team led by Olivier Danos and Jean Michel Heard at the Institut Pasteur in Paris, among other teams elsewhere, to obtain remarkable results *in vivo* by using implants of genetically modified cells. A retroviral vector containing human cDNA for β-glucuronidase was used to transfer the gene into cutaneous fibroblasts, bone marrow cells and myoblasts, which were then reimplanted into MPS VII mice. Fibroblasts introduced into collagen gel were used to constitute an "organoid" which, implanted into the animal's peritoneal cavity, expressed and secreted β-glucuronidase for the following 150 days. Considerable enzymatic activity was demonstrated in several organs and the signs of lysosomal overload, typical of the disease, disappeared in the liver and spleen [23, 29].

In the same laboratory, treated haematopoietic cells were used for autologous bone marrow transplantation in irradiated animals. Only 5% of haematopoietic cells contained the vector, but the enzyme produced was sufficient to reduce the organ overload. Genetically modified myoblasts, transplanted into the muscles of mice, secreted the enzyme, which was demonstrated in the liver one month later.

Other recombinant vectors are currently being evaluated for the correction of mucopolysaccharidoses (Hunter syndrome, MPS VI). Some of these experiments are currently being performed in large animals, prior to the development of human clinical protocols.

In vivo *targeted gene transfer*

A large number of studies have been conducted in order to demonstrate the possibility of targeted gene transfer to specific organs. Most of these experiments have been performed with various vectors carrying a nonspecific gene, usually the *Escherichia coli* β-galactosidase gene (Lac Z), due to the simple demonstration of its expression by staining. The results of these experiments should theoretically be applicable to all diseases affecting the organ in question.

Gene transfer into the CNS has been demonstrated using viral vectors: adenovirus [30, 31] retrovirus [32], herpes virus [33], or liposomes [34]. In our laboratory, Catherine Caillaud and Saïd Akli showed that 100% of neuronal and glial cells in primary culture, transfected with recombinant adenovirus carrying the Lac Z gene, expressed β-galactosidase, with no cytopathogenic effects. Several teams, including ours, have also shown that this same recombinant adenovirus, injected *in vivo* into various nuclei or cerebral structures or by intraventricular injection, can transfect both neurons and glial cells (astrocytes, microglia, ependymal cells) in the rat. Gene expression is rapid and intense, and persists for at least two months. The synthesized enzyme, taken up by

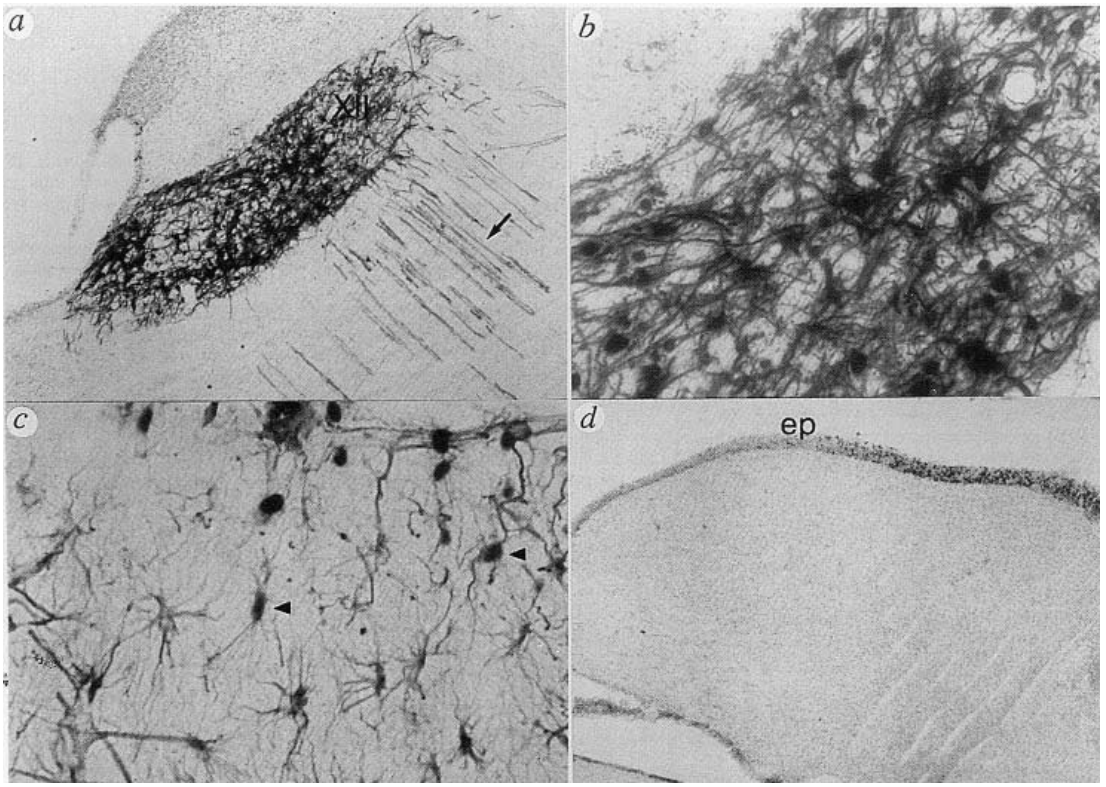

Fig. 2: Histochemical staining of β-galactosidase in various cerebral cells 4 days after injection of adenovirus (Ad Lac Z). (a) and (b): massive labelling of neurons in the nucleus of the vagus nerve after stereotactic injection (a, x 100; b, x 400); the arrow in "a" shows retrograde transport along the axons; (c): transfected astrocytes (arrows) identified by double staining specific for the fibrillary acid protein (x 400); (d): staining of ependymal cells by intraventricular injection (x 100).

nerve endings, is transported in retrograde fashion and can be detected at a distance from the site of injection (Fig. 2). This last result encouraged us to investigate other routes of access to the brain. We obtained positive results following nasal instillation of the adenoviral vector and demonstrated that gene transfer to the brain is possible *via* this route, but only at a low degree [35]. Trials of intracerebral transplantation of normal embryonic neuronal cells or genetically corrected fibroblasts, as well as *ex vivo* genetically corrected autologous bone marrow transplantation [36], also demonstrate the possibility of indirect gene transfer in the brain, as the transplanted cells represented 20% of microglial cells. This result raises hope for successful treatment of neuronopathic lysosomal storage diseases.

Other organs have also been studied for gene transfer: heart, liver, skin, spleen, blood vessels. Interesting results have been obtained and a large number of papers have been published on this subject.

Other approaches to gene transfer, allowing generalized enzyme correction of the whole body, have also been tested. For example, fibroblasts, genetically corrected in culture and reintroduced into the body by implantation in an organ (liver, spleen, etc.) or in the form of an organoid, in order to distribute the missing enzyme, have given encouraging results [37 and personal results]. Also muscle constitutes an advantageous tissue for peripheral expression of a transgene within a strategy for gene therapy of lysosomal diseases.

Gene therapy protocols for human lysosomal storage diseases

Gaucher disease

The reasons which make Gaucher disease a leading candidate for gene therapy include cloning of the human glucocerebrosidase gene, tangible clinical benefits of allogeneic bone marrow transplantation, positive results obtained with replacement therapy and the discovery of the essential role played by macrophages.

Several strategies, targeted towards the macrophage cell line, have been proposed. Since 1992, John Barranger's team has used a recombinant retroviral vector to transduce the glucocerebrosidase gene in murine haematopoietic stem cells and have demonstrated the feasibility of transfer of this gene into haematopoietic cells, with an efficacy lasting several months. Other teams from Los Angeles Children's Hospital have demonstrated that 3 months after transplantation the corrected haematopoietic stem cells are found in liver, lung, brain and spinal cord, and they express the glucocerebrosidase 4 months later [36]. This result suggests once more that gene therapy may also be beneficial for the neuropathic lysosomal diseases.

More recently, human blood cells were used to demonstrate effective transfer of the glucocerebrosidase gene in order to propose clinical protocols [38, 39]. Haematopoietic stem cells (CD34+) were 10-fold enriched by immunoabsorption methods from bone marrow, peripheral blood or cord blood. Approximately 10-30% of stem cells were able to be transduced *in vitro* by the recombinant retroviral vector. The ensuing gene expression of these cells was 2- to 3-fold higher than in non-transfected cells. Haematopoietic cells from patients with Gaucher disease were also effectively transduced and the enzyme deficiency was corrected in cultured stem cells.

In June 1993, the RAC (Recombinant DNA Advisory Committee, USA) approved the clinical application of the protocol using a retroviral vector to transfer the glucocerebrosidase gene *ex vivo* into the haematopoietic stem cells of 15 patients. The subsequent autologous transplantation of these cells may provide the patients with macrophages capable of synthesizing the normal enzyme for life [40].

Hurler Disease

A clinical protocol for gene therapy of Hurler disease in man, by implantation of *ex vivo* transfected fibroblasts, using a retrovirus carrying the cDNA for α-L-iduronidase, the deficient enzyme in this disease, is currently being examined by French health authorities. The approach will be the intraperitoneal implantation of an "organoid" obtained *in vitro* [29].

Hunter Disease

Hunter disease is particular by its X linked inheritance. At the University of Minnesota, USA, a protocol consisting in the *ex vivo* transduction of peripheral blood stem cells by a retroviral vector containing iduronate-2-sulfatase gene, has been conceived by CB Whitley. Two adults and two children are planned to receive repeated infusions with transduced cells.

Future prospects

The experimental success of gene therapy in lysosomal storage diseases raises hopes for clinical applications in the near future. We have seen that clinical protocols are already in progress.

The heterogeneity of expression of these diseases in man requires the development of various strategies designed to induce generalized or targeted correction, and to determine the most appropriate vector and the most inoffensive route of administration, according to the severity of the disease and the patient's age at the time of introduction of treatment.

A large number of aspects still need to be clarified and others will no doubt be encountered in the future. Several steps have to be improved: i) the percentage of haematopoietic stem cells infected and maintaining a stable expression of the transgene; ii) the long term expression of the transgene *in vivo*; iii) the immunological reactions of treated organisms : firstly against the vector, particularly when it is an adenovirus; secondly, against the synthesised enzyme under control of the therapeutic gene. New generations of vectors have already been obtained, but it is too soon to estimate their efficacy and innocuity.

Several teams in various parts of the world, including our own, have focused their research efforts on this subject. Their results are to be published in the near future.

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