Gene therapy for leukodystrophies

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Leukodystrophies (LDs) refer to a group of inherited diseases in which molecular abnormalities of glial cells are responsible for exclusive or predominant defects in myelin formation and/or maintenance within the central and, sometimes, the peripheral nervous system. For three of them [X-linked adrenoleukodystrophy (X-ALD), metachromatic (MLD) and globoid cell LDs], a gene therapy strategy aiming at transferring the disease gene into autologous hematopoietic stem cells (HSCs) using lentiviral vectors has been developed and has already entered into the clinics for X-ALD and MLD. Long-term follow-up has shown that HSCs gene therapy can arrest the devastating progression of X-ALD. Brain gene therapy relying upon intracerebral injections of adeno-associated vectors is also envisaged for MLD. The development of new gene therapy viral vectors allowing targeting of the disease gene into oligodendrocytes or astrocytes should soon benefit other forms of LDs.

INTRODUCTION

The white matter of the brain and spinal cord is a complex structure, which is composed of a vast number of axons, sheathed with a compact, lipid-rich membrane, the myelin. Besides myelinated axons, white matter contains glial cells (astrocytes, microglial cells and oligodendrocytes) that play a major role in structural and trophic support of myelin and axons. In the peripheral nerves, formation and maintenance of myelin sheaths are performed by Schwann cells.

The term leukodystrophies (LDs) (leuko-white, dystrophy-defective nutrition) refers to a group of inherited diseases in which molecular abnormalities of glial cells are responsible for exclusive or predominant defects in myelin sheath formation and/or maintenance within the brain, spinal cord and often also the peripheral nerves. LDs have been initially classified according to the staining characteristics of myelin breakdown products in neuropathological analysis. The identification of enzyme or transporter defects in lysosomes and peroxisomes has allowed to characterize a major group of LDs that are mostly characterized by myelin destruction (de/dysmyelinating LDs) and include X-linked adrenoleukodystrophy (X-ALD) (1,2), metachromatic LD (MLD) (3,4) and globoid cell LD (GLD), also known as Krabbe disease (5,6). More recently, brain magnetic resonance imaging (MRI) that detects with high sensitivity changes in white matter due to its high lipid content has allowed introducing a classification of LDs based on the MRI recognition pattern (7,8). Progresses in LD recognition and molecular biology have accelerated the identification of causative genes of new LDs with an emerging classification that includes the de/dysmyelinating, hypomyelinating and vacuolating forms (9). The high sensitivity of MRI in detecting white matter abnormalities has resulted in the identification of a long list of genetic leukoencephalopathies (10) in which the cause of white matter abnormalities seen at brain MRI is either unknown or secondary to neuronal or axonal defects. This review will focus on LDs as defined above.

Recent data have markedly increased our understanding of LD physiopathology, particularly the role of various glial cells involved in the demyelinating process. If primary central nervous system (CNS) demyelination was, for long, thought to result only from a direct insult to the oligodendrocyte, the cell that makes and maintains the myelin sheath, the identification of genes involved in Alexander disease (11), childhood ataxia with central hypomyelination/vanishing white matter syndrome (12,13) and Canavan disease (14) have highlighted the role of astrocytes in myelin formation and maintenance (15).

Acute or chronic demyelination, regardless of its cause, impairs axonal function (16,17). In particular, the acute loss of myelin at the internode is associated with a conduction
block. Chronic hypomyelination, as seen in patients with Pelizaeus–Merzbacher disease (a hypomyelinating LD) with missense mutations or duplication of the proteolipid protein (*plp*) gene, impairs also the integrity of axons. Studies of autopsy tissues from patients with multiple sclerosis have shown that axon preservation is seen in those areas where remyelination has occurred. This suggests that thin myelin sheaths might be sufficient to maintain axon survival, which is important in respect to therapeutic perspectives in LDs (18). However, axonal function is not only dependent from the integrity of myelin sheaths, but also from a direct function of oligodendrocytes in promoting axon survival (16,17). This important concept comes from observations of genetic mouse models and studies of human pathology. Transgenic mice lacking the oligodendrocyte expressed phosphodiesterase enzyme, or PLP, an integral myelin protein, show long-term axonal degeneration, even in the presence of ultrastructurally normal myelin (19,20). Patients with mutations of the *plp* gene that result in complete absence of PLP develop also axonal degeneration without demyelination (21).

To achieve potential clinical efficiency, any gene therapy strategy for LDs must take into consideration several factors: first, many LDs, particularly those from the demyelinating type such as X-ALD, ML and GLD, result not only in the destruction of myelin sheaths, but also in the loss of oligodendrocytes (and Schwann cells if the peripheral nerve is also affected); secondly, whatever it results from acute/chronic demyelination or oligodendrocyte dysfunction, axonal degeneration ends up in the destruction of axons and therefore in an irreversible damage that will hamper the efficacy of any therapeutic approach. It is thus crucial to determine at which stage of disease progression a gene therapy approach would be clinically relevant, a situation that is not unique to LDs. Moreover, even if this gene therapy strategy will successfully arrest the process leading to myelin destruction, remyelination from oligodendrocyte precursor cells is expected to be less efficacious and harder to promote in a mature brain than in an immature brain (22).

The gene therapy strategies that have been developed so far to tackle three selected LDs rely upon hematopoietic stem cell (HSC) and lentiviral vectors to target microglia precursors, or upon the intracerebral delivery of adeno-associated virus (AAV) vectors targeting mostly neurons. In two of those LDs (MLD and GLD), the gene therapy approach takes also advantage of potential cross-correction of oligodendrocytes by the engineered/genetically corrected cells (microglia or neurons). Despite significant progresses made in the development of new gene therapy vectors, none of them has proved to efficiently and selectively target oligodendrocytes after intracerebral or intravenous injection. Recent screening of new serotypes of AAV vectors or their engineering using high-throughput random diversification and selection have enabled the isolation or creation of new AAV vectors that transduce much better astrocytes in vivo (23,24). However, those new AAV vectors have not yet entered into the clinics.

HSC gene therapy for CNS disorders is based on the possibility to engineer in the brain a subset of glial cells, called microglia. Those cells form up to 20% of all glial cells and are ubiquitously distributed throughout the CNS. Resident microglial cells are derived from myeloid precursor cells, which enter the brain and spinal cord during embryogenesis (25,26). Although microglia share resemblance with macrophages and monocytes, recent studies suggest that they are an ontogenically distinct population in the mononuclear phagocyte system (27). Brain microglia sense brain tissue alteration and perform various (innate and adaptative) immunologic functions. However, microglia in the healthy brain do not qualify only as macrophages. Other functions, including the capacity to respond to external threats (pain), to maintain synaptic integrity and to shape neurogenesis are just in the process to be uncovered (28). The factors controlling microglia precursor recruitment from the blood after birth remain elusive and the generation of new microglia in the adult normal brain is still a matter of debate. Following hematopoietic cell transplantation (HCT) in rat, it was shown that a subset of perivascular microglia is replaced by bone marrow-derived cells from the donor (29). In fact, before this important observation was made, allogeneic HCT was utilized to deliver normal lysosomal enzyme to the brain of a patient with Hurler disease (30). Following this first success, allogeneic HCT was used to treat several other CNS lysosomal storage disorders (LSDs), including ML and GLD (31,32). The rationale supporting this approach was that some of the progeny from the engrafted HSCs in the bone marrow could migrate into the blood, cross the blood–brain barrier and differentiate into perivascular microglia producing normal lysosomal enzymes. As lysosomal enzymes can be secreted and taken up by other cells through the mannose 6-phosphate (M6P) and other scavenger receptors, it was expected that such approach could result in the cross-correction of oligodendrocytes and neurons. Allogeneic HCT was also successfully used to treat X-ALD (33) before the cloning of the ALD (ABCD1) gene revealed that it does not encode a secreted enzyme but a non-secreted transporter localized in the peroxisomal membrane (1). The labeling method of bone marrow cells with retroviral vector expressing enhanced green fluorescent protein (EGFP) has confirmed that a significant percentage of newly engrafted microglia-like cells can be observed in the adult mouse brain after HCT (34). It is still debated to which extent true microglia engraftment (defined by the presence of small ramified microglial cells) occurs after HCT, according to the pathology (in particular neuroinflammation) present into the brain at time of HCT and how the integrity or disruption of the blood–brain barrier regulates and/or promotes the engraftment of microglia precursors from the blood (26). For example, no frank microglia engraftment is observed in transplanted mice after administration of cuprizone that induces demyelination without promoting blood–brain-barrier damage (35). Studies in normal non-human primates that have been reconstituted with autologous HSCs transduced with a lentiviral vector expressing EGFP suggests that EGFP expressing engrafted cells in the brain remain mostly in the vicinity of blood vessels, having the shape and markers of perivascular macrophages (36). Clearly, the extent of microglia engraftment depends on the mouse model of neurodegenerative disease and experimental confounds (26,35). Microglia precursors that enter into the adult brain might have properties that are distinct from those of microglial cells that colonize the brain during its development. In particular, myeloid precursors or monocytes that engrat in the brain following
HCT might migrate from perivascular spaces and proliferate under the influence of local clues.

Despite our current limitations in understanding microglia ontogeny in the adult brain, strategies to use microglia precursors aiming at delivering therapeutic genes in LDs have entered into the clinics. With the availability of human immunodeficiency virus type 1 (HIV1)-derived lentiviral vectors that allow more efficient gene transfer into HSCs than murine gamma-retroviruses (γ-RVs) (37), HSC gene therapy has been developed in three LDs to overcome several limitations of allogeneic HCT. First, the allogeneic procedure remains associated with significant mortality and morbidity risk, in particular related to severe graft-versus-host disease. The mortality risk of allogeneic HCT performed with reasonable human leukocyte antigen (HLA)-matched unrelated donor or cord blood remains close to 15–20% in children and 30–40% in adults when using full myeloablative conditioning regimen with busulfan and cyclophosphamide uniformly suffered devastating progression of the disease, patients present with subtle neurocognitive deficits without neurologic deficits. After this initial period, cerebral demyelination progresses rapidly with a devastating course. Patients lose their ability to read, understand language and walk within a few weeks. They develop tetraparesis, cerebellar signs, decreased visual aequity with hemianopsia, central deafness and often seizures. This stage corresponds to the onset of inflammatory lesions with infiltration and accumulation of macrophages and mononuclear cells behind the active edge of demyelinating lesions. At this stage, brain MRI shows marked progression of demyelination and focal disruption of the blood–brain barrier. Most patients enter a vegetative state within 2–4 years of the first symptoms followed at varying intervals thereafter by death in 90% of the cases. Whereas heterozygous women with AMN never develop cerebral demyelination with neuroinflammation (unless they have ABCD1 gene mutation on both alleles), 35% of AMN males develop a cerebral demyelinating form of the disease between 20 and 35 years of age. The outcome of cerebral ALD in adults is the same as in boys: it results in a vegetative stage or death few years after the onset of neurologic symptoms.

Gene transfer in autologous HSCs may offer immunological advantages in relation to graft rejection and graft-versus-host disease which are two serious complications of HCT. Furthermore, allogeneic HCT has rather very limited effects in patients with early onset MLD and GLD. HSC gene therapy with lentiviral vectors may allow expressing the functional lysosomal enzymes at above-normal levels in the transplanted cells resulting in a more robust cross-correction of neurons and glial cells, which was demonstrated of therapeutic relevance in the mouse models of MLD and GLD (39–41).

**HSC GENE THERAPY FOR X-ALD**

X-ALD is a metabolic, peroxisomal monogenic disease with an estimated frequency of 1:17,000 males that affect the CNS, adrenal cortex and testis, and results from inactivating mutations in the *ABCD1* gene located in the chromosome Xq28. The *ABCD1* gene encodes a peroxisomal membrane half-ABC transporter, the ALD protein (ALDP), whose loss of function is related to abnormal peroxisomal β-oxidation and accumulation of saturated very-long-chain fatty acids (VLCFAs) in tissues and body fluids (1,2). Several distinct phenotypes occur frequently within the same family and are not correlated with *ABCD1* gene mutations. The most prevalent manifestation of X-ALD is a slowly progressive paraparesis with sphincter disturbances due to the involvement of the long tracts in the spinal cord, referred to as adrenomeyeloneuropathy (AMN). AMN affects adult males after the age of 20 years and frequently heterozygous women after the age of 40 years and is due to an axonal degeneration that is primarily non-inflammatory in nature. Before the onset of AMN in adulthood, X-ALD can also manifest in childhood as an acute cerebral demyelinating disease with neuroinflammation. The affected boys develop normally until 4–12 years of age, then cerebral demyelinating lesions usually start in the splenium of the corpus callosum—less frequently in the genu of corpus callosum, internal capsules or brain stem—and initially show a slow progression over 1–3 years. At this early stage of the disease, patients present with subtle neurocognitive deficits without neurologic deficits. After this initial period, cerebral demyelination progresses rapidly with a devastating course. Patients lose their ability to read, understand language and walk within a few weeks. They develop tetraparesis, cerebellar signs, decreased visual aequity with hemianopsia, central deafness and often seizures. This stage corresponds to the onset of inflammatory lesions with infiltration and accumulation of macrophages and mononuclear cells behind the active edge of demyelinating lesions. At this stage, brain MRI shows marked progression of demyelination and focal disruption of the blood–brain barrier. Most patients enter a vegetative state within 2–4 years of the first symptoms followed at varying intervals thereafter by death in 90% of the cases. Whereas heterozygous women with AMN never develop cerebral demyelination with neuroinflammation (unless they have *ABCD1* gene mutation on both alleles), 35% of AMN males develop a cerebral demyelinating form of the disease between 20 and 35 years of age. The outcome of cerebral ALD in adults is the same as in boys: it results in a vegetative stage or death few years after the onset of neurologic symptoms.

Despite accumulation of VLCFAs in all tissues, including the brain, the ALD mouse does not develop cerebral demyelination. At around 15–16 months of age, the ALD mouse develops moderate motor deficits and neuropathologic lesions in the spinal cord that resembles AMN (42). Oxidative damage can be seen as early as 3 months of age in the spinal cord of ALD mice, before the onset of any neuropathologic or motor abnormalities (43).

Allogenic HCT can arrest the neuroinflammatory demyelinating process of ALD, provided the procedure is performed at an early stage of the disease, in practice when the patients have minimal neurologic and neuropsychological deficits, and limited extension of demyelinating lesions at brain MRI (33,44–46). After the transplantation procedure, demyelinating lesions continue usually to extend for 12–18 months and then they arrest to progress. This delay in the benefit of HCT is likely due to the slow replacement of brain microglia from bone marrow-derived cells. The mechanism by which allogeneic HCT arrests the cerebral demyelinating process in ALD is not known. The conditioning regimen has no effect by itself (47). From the series of 36 ALD patients that were transplanted with allogeneic HCT in France, those four ALD patients showing a failure or a delay to engraft following full myeloablating conditioning regimen with busulfan and cyclophosphamide uniformly suffered devastating progression of cerebral demyelination. HCT in ALD mice does not correct the accumulation of VLCFA in the brain, suggesting there is no metabolic cross-correction, in contrast to CNS lysosomal diseases, such as MLD and GLD. Although yet speculative, it is possible that allogeneic HCT in ALD allows correcting the abnormal function of brain microglia, whether or not this deficiency is related directly to the accumulation of VLCFA. It is indeed intriguing that a subset of microglial cells are lacking at the initial edge of cerebral demyelination in ALD patients (48). Thus, the oligodendrocyte may not be the sole target in X-ALD. Analogous to observations in amyotrophic lateral sclerosis and several other neurodegenerative diseases, the process of demyelination in cerebral ALD might not be...
cell autonomous. The loss of microglia and/or normal microglia function could abrogate the ability to provide neuroprotective factors to deficient oligodendrocytes. Thus, in contrast to MLD and GLD, allogeneic HCT in X-ALD may represent a true form of brain cell therapy and the same applies to HSC gene therapy.

As discussed above, ALD gene transfer into autologous HSCs could represent a valuable alternative to allogeneic HCT. In vitro experiments of ALD gene transfer with lentiviral vectors have shown biochemical correction of monocytes/macrophages derived from transduced ALDP-deficient human CD34+ cells (49). In vivo, the transplantation of lentivirally transduced murine ALD Sca-1+ cells, a functional equivalent of CD34+ cells in humans, into ALD mice resulted in the replacement of 20–25% of brain microglial cells expressing the ALDP 12 months after transplantation (50).

As mentioned before, the ALD mouse does not develop cerebral demyelination, precluding neuropathological and clinical effects of lentiviral gene transfer to be assessed. Xenotransplantation of lentivirally transduced human ALD CD34+ cells into non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice demonstrated in vivo expression of the ALDP in human monocytes and macrophages derived from engrafted human stem cells (51). Human bone marrow-derived cells migrated into the brain of transplanted mice where they differentiated into microglia expressing the human ALDP. Two ALD patients who were candidate for allogeneic HCT but had no HLA-matched donor have been treated using HSC gene therapy with lentiviral vectors more than 4 years ago (50). Two other ALD patients have been treated more recently, but their follow-ups are still too short to be commented in this review.

Peripheral CD34+ cells from patients were collected after granulocyte colony-stimulating factor mobilization and transduced with a HIV1-based lentiviral vector expressing the human ALD cDNA under the control of MND (myeloproliferative sarcoma virus enhancer, negative control region deleted, d587rev primer binding site substituted) promoter (Fig. 1). This lentiviral vector is replication-defective, self-inactivated (SIN) and pseudotyped with the envelope of vesicular stomatitis virus. Transduced CD34+ cells were frozen after transduction to perform on 5% of cells various safety tests that included in particular three replication-competent lentivirus assays. After thawing of transduced CD34+ cells, patients were re-infused with >4.10⁶ transduced CD34+ cells/kg following full myeloablation with busulfan and cyclophosphamide. The patient’s HSCs were ablated to favor engraftment of the gene-corrected HSCs. Hematological recovery occurred between days 13 and 15 and complete immunological reconstitution occurred at 12 months for the first patient and 9 months for the second patient. The efficacy of the HSC transplantation was determined by assessing the number of hematopoietic cells expressing ALDP in bone marrow and peripheral blood cells by immunohistochemistry and the identification of identical insertion sites (IS) of the lentiviral vector in myeloid and lymphoid cells from the same patient. Between 23 and 25% of peripheral blood cells from the two patients expressed the ALDP 30 days after infusion of the transduced CD34+ cells (50). The percentage of corrected peripheral blood cells decreased with time, but stabilized after 16 months. In each patient, the ALDP was expressed in long term at similar percentage in granulocytes, monocytes (that have short half-life) and in B and T lymphocytes (that have longer half-life). This percentage is 10–11% in the two treated patients, 4 years after gene therapy (52).

Identical IS of the lentiviral viral vector were identified in myeloid and lymphoid cells from the two patients in long-term indicating effective gene transfer into term repopulating HSCs. The clonal distribution of gene-modified cells in vivo was studied prospectively by a large-scale analysis of lentivirus IS with high-throughput 454 pyrosequencing of linear amplification-mediated PCR amplicon in CD14+ (monocytes), CD15+ (granulocytes), CD3+ (T lymphocytes), CD19+ (B lymphocytes) and bone marrow CD34+ cells from the two treated patients. The retrieval frequency (sequence count) of identical IS in insertion repertoires obtained by high-throughput sequencing allows a very good estimate of clonal contribution. If the lentiviral integration into the genome of one cell would result in an increased growth rate of this cell, this lentiviral integration site would be retrieved more frequently. Clonal distribution varied, and no frequent clone reappeared with an increasing count. No dominance emerged among active hematopoietic clones in the two treated patients up to now (50,52). Brain MRI analysis showed that progression of demyelination was arrested 14 months after gene therapy in the first patient and 16 months after gene therapy in the second patient. Since and up to the last follow-up (4 years) after gene therapy, demyelinating lesions have not further progressed in both patients (52). The two treated patients have normal neurologic examination 36 months after gene therapy, except for the presence of bilateral quadranopsia in the second patient that appeared 14 months after transplant, and has remained stable thereafter. As a consequence of progression of cerebral demyelination after gene therapy, and in a similar way to that observed after allogeneic HCT, the two patients developed moderate cognitive deficits that have remained stable since 24–30 months after transplant (52). Altogether, results obtained in the first two treated patients indicate that HSC gene therapy results in neurological benefits that are comparable with those seen in patients undergoing successful and non-complicated allogeneic HCT.

The adverse events that have occurred in SCID-X patients treated with γ-RV vector gene therapy (53) have raised serious concerns about retroviral integration-related mutagenesis and leukemogenesis. Transcriptionally active long terminal repeats (LTR) of retroviral vectors are major determinants of genotoxicity (54), whereas the LTR promoter/enhancer of the lentiviral vector used in this study is SIN upon transduction. This and several other differences in lentiviral and oncoretro viral biology suggest that the risk of insertion mutagenesis by a SIN lentiviral vector is likely lower than with γRVs and even SIN-γRVs (55,56). The insertion of lentiviral vectors into or close to genes, including pro-oncogenes, is certainly not entirely neutral (57) and one therefore must remain cautious in term of potential genotoxicity in the long term. This risk of genotoxicity remains a concern but must not to be overemphasized. It must be balanced with the benefit of HSC gene therapy.
As only up to 10–11% of HSCs were corrected in the ALD gene therapy trial in very long term, there is room for improvement. This is particularly important to shorten the period during which cerebral demyelination continues to progress after transplant. More efficient lentiviral vectors, which will become available as lentiviral vector manufacturing improves, will likely boost this figure. Before ALD gene therapy trial was performed, one did not know how many corrected HSCs would need to be infused to achieve clinically relevant neurological benefit in ALD patients, because this crucial issue could not be addressed in the ALD mouse. Gene therapy of ALD provided a benefit similar to that of allogeneic HCT transplantation with full chimerism, despite a relatively low level of HSC correction. This could in part be explained by the fact that the ABCD1 gene was overexpressed in HSCs and myeloid progenitors of microglia. In ALD mice transplanted with lentivirally transduced HSCs, there is no evidence of growth-selective advantage of corrected microglia. The ALD mouse does not develop cerebral demyelination and the situation might be different in human ALD patients with cerebral demyelination. In other terms, corrected microglia precursors might have some growth-selective advantage in the ALD brain environment. It is also possible that brain microglia cells from treated patients might be replaced by infused short-lived myeloid progenitors that contain a higher proportion of gene-corrected cells than HSCs.

Altogether, these preliminary results in the first two treated ALD patients position HSC-based gene therapy as a preferable treatment option for ALD, as it abrogates the morbidity associated with the allogeneic source of HSCs in conventional transplantation. Work is in progress to launch a second ALD gene therapy trial with the aim to correct 2–3-fold more HSCs.

HSC GENE THERAPY FOR METACHROMATIC AND GLOBOID CELL LDs

MLD is a rare, fatal, inherited autosomal recessive LSD, with an estimated frequency of 1:40,000. The disease is caused by the deficiency of the lysosomal enzyme arylsulfatase A (ARSA), which is involved in sulfatide metabolism through the hydrolysis of the 3-O ester bond of galactosyl and lactosyl sulfatides. The massive accumulation of non-metabolized sulfolipids primarily in white matter cells, but also in neurons and microglia, leads to extensive demyelination and neurodegeneration in the CNS and peripheral nervous system (PNS) (4). Major signs and symptoms are due to the severe and progressive involvement of both the CNS and the PNS. The severity of the clinical syndrome could be variable. Indeed, MLD is classified into four main variants according to the age of onset: late infantile (LI), early and late juveniles (EJ and LJ), and adult (AD). LI and EJ forms being the most common. The LI form manifests most commonly between the first and second year of life. Affected children develop walking difficulties that lead rapidly to tetraplegia, loss of speech and of meaningful contact, blindness and finally decerebration. Death occurs invariably within 5 years from the onset of symptoms. The juvenile variant manifests between 4 and 14 years of age and is further subdivided in early and LJ variants based on onset before or after 6 years of age. EJ and LJ forms are characterized by motor (spastic paraparesis, cerebellar ataxia) and cognitive deficits that result in severe neurological impairment in few years. The prognosis is poor, most patients dying before the age of 20. In the adult form, onset may occur around the age of 15, but often the disorder is not diagnosed until adulthood. Initial symptoms typically affect behavior, manifesting as a change in personality, psychiatric manifestations or in poor school or work performance. Other patients with adult form of MLD may present spastic paraparesis as the main symptom at onset. Most commonly, the duration of the illness varies between 5 and 10 years, but in some patients it extends over decades.

A ‘knockout’ mouse model of MLD has been developed (58). Affected mice have the expected deficiency of ARSA activity and accumulate sulfatides in neuronal and non-neuronal tissues. They develop a disease that resembles MLD, but which is much less severe, lacking in particular overt cerebral demyelination. There is, however, some delay in myelination (59). Behavioral abnormalities appear after 1 year and include impaired motor coordination on the rotarod, abnormal gait, deafness and learning deficits. More recently, ARSA-deficient mice with increased synthesis of sulfatides in neurons or oligodendrocytes and Schwann cells have been generated. The ARSA-deficient mice overexpressing the sulfatide-synthesizing enzymes UDP-galactose:ceramide galactosyltransferase (CGT) and cerebroside sulfotransferase in neurons show a more marked neuronal sulfatide storage and neuromotor coordination deficits than the ARSA-knockout mouse (60). ARSA-deficient mice overexpressing galactose-3-O-sulfotransferase-1 (Gal3st1) under the control of the PLP promoter in oligodendrocytes and Schwann cells develop myelin pathology in the CNS and PNS (61). These transgenic mice represent important models for future testing of conventional and new therapeutic interventions.

GLD, or Krabbe disease, is another autosomal recessive LSD caused by deficiency of the lysosomal enzyme galactocerebrosidase (GALC), which catalyzes the catabolism of galactosylceramide (GalCer) produced by the CGT enzyme in the endoplasmic reticulum and ARSA in the lysosome.
GalCer is implicated in the transduction of signals for oligodendrocytes differentiation and in axon-glia interaction at the paranode level (62). Although GalCer is the first substrate of the GALC enzyme, it does not accumulate in GALC deficient tissues, likely due to the activity of another GALC. GALC deficiency is actually responsible for the accumulation of psychosine, up to 10–20 times normal levels in human infants and animal models. The storage of this toxic molecule has been shown to kill oligodendrocytes by an apoptotic mechanism (63–66). GLD is a rare LSD, with an incidence of 1 in 100 000 live births worldwide. It affects both the CNS and the PNS and the typical neuropathological findings include generalized brain atrophy with the central white matter being replaced by gliotic tissue (67). From a clinical point of view, also GLD is classified according to the patient’s age at onset: early infantile (EI), LI, juvenile (J) and adult forms are described (6). The most severe variant is the one with EI presentation, which accounts for over 90% of the patients. The disease starts around 6 months of age with tonic spasms, nystagmus, muscular rigidity, progressive quadriplegia and progresses to a decerebrate state in few months from onset. The LI variant is much less common and begins between one and half and four years of age. These patients develop mental retardation, ataxia, spasticity and severe peripheral neuropathy. Disease progression is slower than in the EI form leading to death 5–7 years after diagnosis. The late-onset variants have onset between 4 and 19 years or in adulthood (>20 years). These are characterized by a slowly progressive tetraplegia, sensory-motor demyelinating neuropathy and initially preserved mental function; survival into the seventh decade is possible.

Several GLD animal models exist, including naturally occurring ones and large animal models. Before the biochemical defect was identified in human patients in 1970, a canine model of GLD, presenting characteristic pathological features of GLD was described (68). In 1980, a murine model of GLD called twitcher (twi) was identified at the Jackson Laboratory (69). These mice present no detectable GALC activity, psychosine accumulation in the CNS and PNS and develop clinical signs at ~20 days, with very rapid neurological deterioration both in the CNS and PNS, the mice dying at about 40 days of age. In 1989, a non-human primate model of GLD was also described (70). In 2001, a new transgenic mouse model of GLD was obtained by homologous recombination (Trs mouse) (71). These mice develop a phenotype, which is similar to the one observed in twi mice, but with a delayed onset of about 10 days, twitching and tremors starting around day 30 and the life span of these mice being about 50 days.

Currently, therapy is mostly supportive for both MLD and GLD patients. As in the case of several other LSDs with severe nervous system involvement, no efficacious treatment is currently available that can stabilize or even reverse the fatal outcome of these devastating diseases. Because of its potential to compensate the deficiency, enzyme replacement therapy (ERT)—parenteral administration of purified recombinant pro-enzyme—has become one of the most promising therapeutic option for several LSDs (72) and it was approved or is being evaluated for the treatment of Pompe disease, mucopolysaccharidoses type I (MPS I), MPS II and MPS VI. However, protein delivery poses serious challenges when the nervous system is the major disease target, as in the case of MLD and GLD, since the blood–brain barrier severely limits access of systemically administered therapeutic molecules to the nervous tissue. Based on preclinical evidence in MLD mice (73), a large-scale manufacturing process of human recombinant ARSA has been developed, and an ERT phase I trial has been recently accomplished. However, results from clinical testing did not provide clear evidence that ERT could have a role in alleviating CNS and/or PNS disease manifestations in MLD patients.

As discussed above, autologous HCT has been developed as a strategy to deliver lysosomal enzymes to the CNS of LSD patients by means of replacement of extra-vascular hematopoietic compartment of the brain (and of other extra-CNS tissues) with cell populations expressing the functional enzyme. Brain macrophages and microglia represent major effectors of the catabolism of the storage material, and their replacement by donor-derived cells has the potential to restore a critical scavenger function. Further, normal donors’ and/or gene-corrected macrophages and microglia represent a local source of functional enzyme for cross-correction of resident neurons and oligodendrocytes. Moreover, replacement of the activated cells with metabolically competent cells may contribute down-regulating neuroinflammation. The therapeutic impact of autologous HCT in LSDs depends on the specific enzymatic deficiency, on the involvement of the CNS and on the stage of the disease at the time of the transplant. Clinical evidences supporting autologous HCT for LSDs with severe CNS involvement have been recently obtained in patients affected by GLD. If performed in the neonatal period, in a largely pre-symptomatic stage, cord blood HCT was shown to alter the natural history of infantile forms of GLD by delaying onset and progression of neurologic symptoms (74). Autologous HCT modifies in a similar way the natural history of the infantile form of MLD when the procedure is performed in the neonatal period (P. Aubourg et al, unpublished data). However, autologous HCT does not result in any substantial neurologic stabilization or improvement if performed in symptomatic patients with infantile forms of MLD and GLD. The main reason for autologous HCT failure/poor therapeutic potential in these conditions is likely the disproportion between the slow pace of cell replacement and defective enzymatic activity reconstitution in the affected brain and the rapid progression of the primary neurologic disease. As discussed above, while the reconstitution of visceral organ macrophages by donor-derived cells has been clearly demonstrated following autologous HCT, substantial debate and poor knowledge exist on the actual occurrence, modalities and timing of CNS microglia replacement by the progeny of the transplanted donor HSCs.

Thus, with the goal of increasing the therapeutic potential of HCT in these severe LSDs, efforts are currently focused on understanding the modalities of microglia replacement in the CNS for optimizing transplantation protocols towards efficient cell reconstitution and on favoring enzyme delivery to the affected tissues, and in particular to the brain, by rendering the tissue infiltrating cells more efficient in enzyme production and secretion, and thus enhancing cross-correction. To this latter goal HSC gene therapy, approaches have been...
employed. Indeed, the use of proficient vectors capable of integrating multiple copies of an expressing cassette containing the cDNA of the lysosomal enzyme of interest under control of a moderate to strong eukaryotic promoter in the majority of the transplanted cells could render autologous HSCs, and their differentiated progeny able to constitutively express supra-normal levels of the therapeutic enzyme for cross-correction. This is of particular relevance at the level of the CNS and of the otherwise hardly accessible PNS manifestations. Despite some initial difficulties, evidences of efficacy of this approach in preventing and/or correcting nervous system manifestations in mouse models of MLD and GLD, as well as of other LSD mice with nervous system involvement, are progressively increasing.

Preliminary experience based on the use of γRVs in the MLD mouse model revealed that unexpectedly high levels of ARSA are required for the correction of the metabolic and functional defects in the CNS (75). Indeed, gene therapy with bone marrow cells expressing the human ARSA cDNA from a γRV resulted in the prevention of sulfatide storage in the liver and kidney, but only in a partial correction of the brain lipid metabolism, of the neuropathology and of the performance of treated animals at behavioral tests. This limitation could be overcome upon use of lentiviral vectors, which enable efficient gene marking and robust, long-term transgene expression in mouse and human HSCs and their progeny with minimal in vitro manipulation and cell perturbation (37). Indeed, by transplanting HSCs transduced with a lentiviral vector carrying the ARSA cDNA, enzyme activity was reconstituted in the hematopoietic system of MLD mice at supra-normal levels and the development of CNS and PNS disease manifestations was prevented (39) and corrected (40) upon pre- and symptomatic treatment, respectively. Remarkably, the expression of the functional ARSA enzyme in HSCs and in their progeny largely above normal donors’ levels proved to be critical for attaining therapeutic benefit. Indeed, gene therapy had a significantly better therapeutic impact than wild-type HSC transplantation and the degree of phenotypic amelioration provided by gene therapy was dependent on the levels of enzyme activity in HSCs and in their progeny. Based on these and additional feasibility and safety data, a phase I/II clinical trial of HSC gene therapy employing lentiviral vectors has recently been launched and is currently open to patients’ recruitment (mldttrial@hsr.it).

Few data regarding the potential toxicity induced by the over-expression of lysosomal enzymes in different cells and tissues are currently available. To this regard, different aspects should be considered. Enzyme over-expression might per se perturb intracellular homeostasis, thus affecting cell viability and function. In particular, the maintenance of the unique and long-term function of HSCs upon lentiviral-mediated enzyme over-expression requires formal demonstration for each enzyme in adequate models. Further, the metabolic consequences of enzyme over-expression deserve in-depth evaluation. ARSA is a member of the large family of sulfatases, which are enzymes involved in the degradation of sulfated substrates (76). Sulfatase-modifying factor 1 has been identified as common activator of sulfatases (77,78), and it has been suggested to be a rate-limiting factor in the biological activation of these enzymes, the over-expression of one of which may lead to reduced activity of the other sulfatases by a competitive interaction with their common activator. Finally, although the superior proficiency of lentiviral vectors at integrating into HSCs provides a critical advantage over γRV for gene therapy applications, the increase in lentiviral integrated copies in HSCs which is needed to obtain enzyme overexpression may also confer an increased risk of insertional mutagenesis. While the safety of ARSA overexpression as respect to the abovementioned issues was demonstrated, at least in mice (54,55,79) in the case of GALC an unexpected toxicity of lentiviral-mediated enzyme overexpression has been recently described in both murine and human HSCs (80). In particular, the GALC enzyme is involved in the maintenance of a functional HSC niche by contributing to the control of the intracellular content of key sphingolipids and both insufficient and supra-physiologic GALC activity—by inherited genetic deficiency or forced gene expression in patients’ cells and in the disease model—induce alterations of the intracellular content of bioactive downstream products, thus affecting HSC survival and function. Such findings may significantly hamper efforts towards the development of HSC gene therapy for GLD, if it relies on vector-mediated enzyme overexpression. Interestingly enough, it was also recently reported that this peculiar enzyme toxicity does not affect the differentiated HSC progeny, including the effector cells in this HSC gene therapy approach, namely monocytes, macrophages and microglia (41). These data highlighted the need of strategies that upon efficient gene transfer into HSCs could allow repressing enzyme expression in the transduced HSCs and up-regulating transgene expression to above-normal levels, once these cells mature and differentiate. Tissue-specific promoters could be exploited to this goal. However, currently available myeloid promoters are characterized by leakiness and poor strength, significantly affecting the therapeutic potential of the proposed strategy (41). A powerful new therapeutic tool for regulating gene expression in transduced cells is the use of microRNAs, which are small non-coding ribonucleic acids that can either block translation of target messenger RNAs or cause their direct degradation (81,82). HSC-specific microRNAs, which are found in stem cells but not in their differentiated progeny (including microglia), have recently been identified and functionally characterized; one of them (microRNA 126) was successfully employed to regulate GALC expression in hematopoietic cells from a genetically modified construct containing the GALC-encoding gene expressed by a moderate/strong eukaryotic promoter (the phosphoglycerate kinase promoter) and linked to the target sequence of that microRNA (41). This construct, which was compared with the household dimmer switch, which increases or lowers the amount of light simply by regulating the power output (83), allowed maintaining HSC functionality upon GALC gene transfer due to the down-regulation of enzyme expression, and obtaining high levels of GALC expression in the differentiated stem-cell progeny, which, upon infiltration in the affected tissues of GLD mice transplanted neonatally with the transduced HSC, provided a supply of GALC to neural and glial cells. These experiments showed a significant increase in the GALC activity measured in the brain of...
treated mice after transplantation with the genetically modified stem cells, resulting in an amelioration of the overall disease phenotype, including a substantial increase in survival, when compared with untreated mice and to animals receiving normal donors’ HSCs. A clear correlation between vector dose and survival of the reconstituted mice was also observed. Thus, this latter approach may represent an innovative gene therapy strategy, which may not only provide GLD patients with a new treatment option, but also has the potential to augment the safety and efficacy of HSC-based gene therapy for other LSDs.

**BRAIN GENE THERAPY FOR MLD AND OTHER NEURODEGENERATIVE DISORDERS**

Aside from cancer, brain gene therapy has been tested in humans with Parkinson disease (PD) more than in any other disorder. Three approaches to gene therapy for PD are currently in clinical testing. Three use AAV vectors and one lentiviral vectors as the gene delivery vehicle. A phase I study of intracerebral delivery of AAV2-encoding glutamic acid decarboxylase (GAD) for PD has been completed, and detailed results have been reported (84,85). The primary outcome of this study demonstrated that infusion of the AAV2-GAD vector into the subthalamic nucleus appears to be safe at the doses tested (84). The transduction of neurons with very high number of viral copy at and around the sites of injection is likely an important factor that may favor the onset of immune response. While not a blinded or placebo study, there was a significant improvement in clinical ratings as well as in several functional neuroimaging parameters (85). This approach is currently being tested in a phase II trial, which includes a blinded control group. The bilateral putaminal infusion of AAV2 vector encoding neurturin (86) has been tested in patients with advanced PD. In a phase I study, the procedure was well tolerated and seemed to improve motor function of treated patients. In a phase II trial, the intraputaminal injection of AAV2-neurturin was not shown to be superior to sham surgery at 12 months, but benefit could be identified in a longer follow-up (87). Another clinical study utilizes AAV2 vectors to transfer the gene for aromatic acid decarboxylase into the human striatum. The surgical procedure was associated with an increased risk of intracranial hemorrhage in some patients, but the phase I study suggested motor improvement (88). Improvement of functional imaging abnormalities has also been presented at scientific meetings. A last trial in PD involves the use of a tricistronic lentiviral vector derived from the equine infectious anemia virus to encode the critical genes for dopamine synthesis, tyrosine hydroxylase, aromatic L-amino acid decarboxylase and guanosine 5'-triphosphate cyclohydrolase I (89). Safety and preliminary data on motor improvement have been reported at scientific meetings. Following an initial trial in which fibroblasts engineered to express nerve growth factor (NGF) have been transplanted in the brain from patients with Alzheimer disease (90), a phase I trial using AAV2 vectors encoding NGF has also been launched.

All those trials aimed at targeting a therapeutic gene in a relatively small brain volume. It is now well known that the diffusion of AAV serotype 2 (and this is also true for lentiviral vectors) is limited from the injection sites within the brain. Targeting a larger brain volume with AAV2 would require to perform many intracerebral injections of the vector. This is technically possible but would increase the risk of the neurosurgical procedure. Intracerebral injection of the AAV2 vector has nevertheless been used to treat children with LI neuronal ceroid lipofuscinosis (LINCL) (91). LINCL (also known as Jansky–Bielschowsky disease) is a form of Batten disease caused by mutations in the ceroid lipofuscinosis neuronal 2 (CNL2) gene that encodes tripeptidyl-peptidase I (TPP1). TPP1 is a lysosomal hydrolase that removes tripeptides from the N-terminus of small polypeptides. As for several other lysosomal enzymes, TPP1 can be secreted and taken up by other cells through the M6P and other scavenger receptor pathways. Deficiency of TPP-1 results in prominent and widespread neuronal loss in the cortex, cerebellum and hippocampus of affected patients. The vector was administrated safely to 12 different locations in the CNS. The trial was not matched, randomized or blinded and lacked a placebo/sham control group. Most patients were at a relatively advanced stage of disease at time of treatment and the chronic and variable evolution of LINCL disease complicated the evaluation of clinical benefits. Nevertheless, assessment of the primary outcome variable suggested a slowing of progression of CNL2 disease in the treated children (91).

Treating patients with LI form of MLD, the most frequent MLD phenotype, represents a huge challenge because they develop between 14 and 24 months of age a devastating motor and cognitive degradation leading to vegetative stage in few months. Intracerebral delivery of viral vectors encoding ARSA has potentially the advantage to allow, at least in the short-term, more rapid and significant expression of ARSA in the brain than HSC gene therapy with lentiviral vectors. Both strategies rely upon ARSA overexpression and cross-correction of oligodendrocytes. The correlate is that none of these two strategies targets directly oligodendrocytes in vivo. Importantly, HSC gene therapy will not change the slow pace at which ARSA-deficient brain microglia will be replaced by myeloid precursors expressing lentiviral-encoded ARSA after transplant. Significant expression of therapeutic transgene occurs 3 weeks after intracerebral injection of single-stranded AAV vectors. It occurs even sooner with double-stranded AAV (92). AAV and lentiviral vector transport along neuronal connections can expand the zone of correction beyond the injection areas (93). The risk of the neurosurgical procedure for brain gene therapy (94) can easily be balanced with the risk of the myeloablating conditioning regimen which is still mandatory to perform HSC gene therapy. The risk of immune reaction against the transgene must not, however, be underestimated for in vivo gene therapy. Immune reaction against the ARSA transgene can ultimately result in the destruction of transduced neurons and the development of encephalomyelitis-like disease. Of note, nearly all MLD patients express ARSA enzyme (although it is enzymatically deficient) lowering this risk. The transduction of neurons with very high number of viral vector copy at and around the sites of injection is likely an important factor that may favor the onset of immune response. Besides improvement in the method of vector delivery within the brain to avoid back-flow of infused viral particles along the cannula and achieve more widespread and even diffusion of the vector from injection sites (95), the optimal dose of the
vector to be injected in patients can be determined by performing toxicological studies in non-human primates injected with higher vector dose than scheduled for patients.

Proof of concept that intracerebral delivery of AAV5 or lentiviral vectors encoding ARSA is efficient in MLD mice has been achieved (96–98). In normal non-human primates, the cerebral injection of AAV5 vector encoding human ARSA showed the presence of ARSA genome in 37 to 46% of injected hemisphere and significant overexpression of ARSA enzyme (above normal baseline values in normal monkeys) in a significant (50–65%) volume of the injected hemisphere (99). Changing the capsid of the vector might enhance the distribution of the therapeutic gene in the CNS. AAVrh10, isolated from the CNS is among the leading organ systems for which innovative gene therapy is technically very complex and the progress of an intractable and devastating disease. Accomplished for two LDs. For one of them, X-ALD, long-term safety studies in non-human primates demonstrated a better spread beyond the intracerebral site of injection in rats, in comparison with AAV2, AAV5 and AAV8 vectors and provided the highest level of TPP1 lysosomal enzyme in the CNL2 mouse model (100). Preliminary experiments in MLD mice indicate that AAVrh10 encoding ARSA corrects more rapidly oligodendrocyte damage than AAV5-ARSA vector (Cartier and Aubourg, unpublished data). This increased efficacy is both mediated by higher ability of the AAVrh10 vector to transduce high number of neurons at significant distance from injection sites and better axonal transport of the vector. Ongoing experiments aim at evaluating the safety and efficacy of AAVrh10 vector encoding ARSA in normal non-human primates.

Although a viral vector targeting in vivo oligodendrocytes is still lacking, these results indicate that brain gene therapy with new serotypes of AAV vectors could be a valuable alternative to HSC gene therapy in MLD, particularly to treat patients who develop very rapidly progressive forms of the disease. Brain gene therapy will, however, not correct the peripheral neuropathy that may remain a major issue in treated MLD patients in long term.

Concomitant intrathecal administration of the AAV vector could possibly correct neurons in the spinal cord but also the peripheral nerve through the transport of the viral vector and ARSA enzyme along axons and then cross-correction of myelinating Schwann cells through the M6P receptor pathway (101,102).

CONCLUSIONS

Gene therapy has evolved substantially in recent years. The CNS is among the leading organ systems for which innovative gene therapy clinical trials are launched. The methodology for performing gene therapy is technically very complex and difficult but translation to human clinical trials has been accomplished for two LDs. For one of them, X-ALD, long-term results in two treated patients support the view that gene therapy is a cutting-edge technology that allows arresting the progression of an intractable and devastating disease. Among the increasing list of LDs, only a few discussed in this review will benefit from the HSC gene therapy strategy. If gene therapy for lysosomal LDs can take advantage of a cross-correction strategy facilitated by overexpression of the therapeutic enzyme, most other LDs will require to target the therapeutic gene either to oligodendrocytes or astrocytes. Recent advances to engineer AAV vectors for targeted delivery in specific glial cell types (24,103) should allow treating in a near future many other LDs for which there is currently no treatment option.

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