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MINI-SYMPOSIUM: X-Linked Adrenoleukodystrophy

Hematopoietic Stem Cell Transplantation and Hematopoietic Stem Cell Gene Therapy in X-Linked Adrenoleukodystrophy

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Abstract

Allogeneic hematopoietic stem cell transplantation (HSCT) is the only therapeutic approach that can arrest cerebral demyelination of X-linked adrenoleukodystrophy (ALD) in boys and results in long-term in a good quality of life, provided the procedure is performed at an early stage of disease. Similar benefits of allogeneic HSCT have been demonstrated in adults with cerebral ALD. However, it is not yet known whether allogeneic HSCT can prevent or rescue adrenomyeloneuropathy. Allogeneic HSCT remains associated with significant morbidity and mortality risks, particularly in adults, and not all ALD patients have donors despite the availability of cord blood. The absence of biological markers that can predict the evolutivity of cerebral disease is a major limitation to propose in due time allogeneic HSCT to ALD patients. Recently, HSC gene therapy using lentiviral vector was shown to have comparable efficacy than allogeneic HSCT in two boys with cerebral ALD who had no Human-leukocyte-antigen (HLA)-matched donor. If these results are confirmed in an extended series of patients, HSC gene therapy may become the first therapeutic option for all ALD male patients who develop cerebral demyelination.

INTRODUCTION

Microglia, the guard cells of the central nervous system (CNS), are the earliest sensors of all forms of pathological incursion. In response to pathogens or damage, they alter their morphology, surface phenotype and gene expression, and their numbers increase markedly, which is termed microgliosis. Microglia are a quite various population of non-neuronal cells that form up to 20% of all glial cells and are ubiquitously distributed throughout the CNS (12, 28, 29, 43). It is clearly established that in contrast to other glial and neural cells, microglia are derived from bone marrow cells (12, 14, 23). The subset of bone marrow cells that are the progenitors of microglia has not been fully characterized, but microglial cells are likely to have a myelomonocytic origin, deriving from myeloid precursors in the bone marrow (12). Before the hematological origin of microglia was demonstrated, allogeneic hematopoietic stem cell transplantation (HSCT) was utilized to deliver normal lysosomal enzyme to the brain of a patient with Hurler's disease (20). It was with the same idea that the first HCSTs were performed in X-adrenoleukodystrophy (4, 37). In fact, this was before the ALD gene was cloned and shown to encode an integral membrane protein localized in peroxisome that could therefore not be secreted (38).

ALLOGENEIC HSCT: LESSONS FROM THE PAST

The first attempt to perform HSCT was not successful (37). Although before transplant, the 13-year-old boy has normal neurologic examination, excepting for a slight spasticity in the right leg, and normal cognitive functions (verbal IQ = 94; performance IQ = 90), he experienced continuous neurological deterioration after HSCT. This patient had a classical parieto-occipital form of cerebral ALD, and retrospectively, the extent of demyelinating lesions could be scored between 10 and 12 (Loes score). The second attempt to perform HCST in ALD was successful (4). Of note, this patient was transplanted at a much early stage of cerebral demyelination. Cerebral demyelination progressed after HCST but eventually completely reversed 18 months after transplant. Twenty-two years later, this patient still has a normal brain magnetic resonance image (MRI) and a normal life. At the age of 30 years, he has not yet developed clinical and electrophysiological signs of adrenomyeloneuropathy (AMN).

Since this initial success, long-term follow-ups have confirmed that HSCT 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 no neurologic and no significant neuropsychological deficits, and limited extension of the demyelinating lesions at brain MRI (5, 22, 26, 27, 30–32, 42, 46, 47). After the transplantation procedure, demyelinating lesions continue usually to extend for 12 months to 18 months and then their progression arrests. This delay in the benefit of HSCT is likely caused by to the slow replacement of brain microglia from bone marrow-derived cells (10, 23, 29).

Initially performed with whole bone marrow from HLA-matched donor or relatives, the clinical benefits of HSCT in ALD have been similar when the transplants were performed with

peripheral CD34+ cells from unrelated donor or cord blood containing also CD34+ cell fraction ((5, 7, 9) G. Michel, A. Fischer and P. Aubourg, personal communication). HSCT has also been performed with the same success in adults with cerebral ALD ((19), A. Buzyn and P. Aubourg, personal communication). Importantly, and in contrast to HSCT performed for CNS lysosomal diseases, there is no cross-correction of mesenchymal stem cells after HSCT in ALD (24).

These positive results must not underestimate the serious limitations of HSCT in ALD, even today with the important medical progresses made in HSC transplantation and the availability of cord blood as a source of donor cells. It is our experience that for nearly all boys who were an index case in the ALD family and presented with evident cognitive deficits because of cerebral demyelination at time of diagnosis, it was generally too late to propose safely an HSCT. At this stage, we and others unfortunately experienced many times that the HSCT procedure could accelerate markedly the neurologic decline, sometimes only in a few weeks. In practice, for the childhood period, and at least up to 10 years to 12 years of age, HSCT is mostly limited to ALD patients who presents first Addison's disease as clinical sign of ALD or who are identified during the genetic counseling of a family, and in whom and both cases repeated brain MRI can detect the appearance of first signs of cerebral demyelination in due time for HSCT. For adults, and despite the fact that cerebral demyelination progresses initially at this age more slowly, most transplanted patients were adults who initially had AMN and in whom repeated brain MRI detected early signs of cerebral demyelination. The indication of HSCT in ALD remains difficult, given that the prediction of disease evolutivity still entirely relies upon clinical features: the age of the patient, the extent and recent evolutivity of brain lesions. There is therefore an urgent need for biological markers that can help neurologists and hematologists in transplant units to select more easily and more appropriately the patients who could be good candidate for HSCT. In our experience, magnetic resonance spectroscopy and even diffusion tensor imaging have been of little help on an individual basis.

In addition, HSCT remained associated with significant risks of severe graft vs. host disease and prolonged immune deficiency (11, 21). For many of the boys who survived those complications, cerebral demyelinating lesions continued to progress significantly, and even though they eventually arrested, the patients remained in long-term with significant motor, visual, audial and cognitive deficits resulting in a poor quality of life. At last, one must remind that the mortality risk of HSCST performed with (reasonable) HLA-matched unrelated donor or cord blood remains close to 15% to 20% in children and 30% to 40% in adults when using full myeloablation with cyclophosphamide and busulfan, the most common conditioning regimen used up to now in ALD.

With or without the use of antioxidant treatment (48), reduced-intensity conditioning (RIC) regimen could possibly allow to propose HSCT to ALD patients at a slightly more advanced stage of cerebral demyelination (41, 45, 49). RIC regimens have indeed a lower risk of toxicity. In addition, HSC gene therapy (see next section) indicates that 100% correction of HSC is not necessary to achieve clinical benefits in ALD, ie, to arrest the cerebral demyelination. The RIC regimen deserves strong consideration, particularly in adults in whom a full myeloablation is associated with significant toxicity and mortality risks. One must, however, balance the fact that if the RIC regimens result in partial chimerism that could be sufficient to arrest to the cerebral demyelination of ALD, they are also associated with a significant risk of engraftment failure.

The mechanism by which HSCT arrests the neuroinflammatory demyelinating process in ALD is not known. The conditioning regimen has no effect by itself (40). From the series of 36 ALD patients that were transplanted with allogeneic HCST in France, those four ALD patients showing a failure or a delay to engraft the allogeneic HCT after the same full conditioning regimen uniformly suffered devastating progression of cerebral demyelination (A. Fischer and P. Aubourg, personal communication). HSCT in ALD mice does not correct the accumulation of very-long-chain-fatty acids (VLCFA) in brain. Although, yet speculative, it is possible that HSCT in ALD allows to correct abnormal function of brain microglia, whether or not this deficiency is related 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 (15). Thus oligodendrocyte and axon may not be the sole target in ALD. Analogous to observations in amyotrophic lateral sclerosis and other neurodegenerative diseases, the process of demyelination in cerebral ALD might not be cell autonomous. The loss of microglia could abrogate their ability to provide neuroprotective factors for oligodendrocyte at risk. Interestingly, also, bone marrow-resident HSC and progenitor cells are known to enter the blood and to home back to the bone marrow (33). Under physiological conditions, migratory HSCs and progenitors contribute to the continuous restoration of specialized hematopoietic cells that reside in peripheral tissues. Whether HSC and/or hematopoietic progenitors can enter into the brain is not known. If yes, and upon exposure to toll-like receptor agonists, migratory HSCs and/or myeloid progenitors could proliferate locally within the brain and generate normal innate immune effector microglial cells. This could be an important issue in ALD if we consider the possibility that microglial cells might have a deficient function, possibly involving innate immune functions.

An important issue with respect to the efficiency and effectiveness of HSCT in ALD is the integrity of the blood–brain barrier. Recent data in mice suggest that disruption of the blood–brain barrier could be necessary in order for myeloid progenitors to penetrate into the brain and differentiate into microglia (2, 34, 35). The question remains open in ALD given that most and maybe all successfully transplanted patients had enhanced contrast of cerebral demyelinating lesions after intravenous injection of gadolinium reflecting abnormal blood–brain barrier. We have, however, the personal experience of one boy transplanted at a really asymptomatic stage (with normal brain MRI) at 3 years in whom demyelinating lesion appeared 1 year after transplant in the splenium of corpus callosum and then remained unchanged up to now, 6 years later. This observation might be important for the development of HSC gene therapy at an asymptomatic stage (see next section).

At last, and this question is very often raised by the patients themselves, can HSCT have beneficial effects in AMN? The answer is clearly: we do not know. The very long term follow-up of ALD patients transplanted more than 15 years ago is unlikely to give a clear answer, at least in the short term. Our personal experience for the few patients transplanted in France more than 15 years ago is that none have yet developed clinical and even electrophysiological signs of AMN. However, these patients are too few and far to have reached the age of 50 years. We are currently addressing this

important issue in the ALD mice that presents a late-onset of AMN-like symptoms. However, in this model, and given the slow replacement of brain microglia after HSCT, it will only be possible to determine whether HSCT can prevent AMN, not rescue AMN, once symptoms have occurred. Currently, HSCT is not recommended in AMN patients without cerebral involvement given the risk of the procedure. HSCT with an RIC regimen could potentially be proposed to AMN, provided again that the procedure is performed at an early stage of spinal cord involvement. Indeed, it is unlikely that HSCT can reverse severe and likely irreversible axonal damage.

HSC GENE THERAPY WITH LENTIVIRAL VECTOR

Overall, 65% of ALD male patients are at risk of developing fatal cerebral demyelination in childhood or adulthood, and allogeneic HSCT has serious limitations. Transplantation of autologous HSCs genetically modified to express the missing protein may circumvent the majority of the problems associated with allogeneic HSCT. In 1994, transplantation of murine bone marrow cells, which had been transduced with a murine retroviral vector that expressed the glucocerebrosidase gene (which is deficient in Gaucher's disease), resulted in the replacement of 20% of microglia with donor-derived microglia that expressed the glucocerebrosidase gene for 3 months to 4 months after transplantation (25). In 2001, an human immunodeficiency virus type 1 (HIV-1)-derived lentivirus vector was used to transduce *ex vivo* murine bone marrow cells, which were reinjected into irradiated mice (44); this study demonstrated that bone marrow-derived microglia that expressed the green fluorescent protein reporter gene was specifically attracted to sites of neuronal damage. Up to the first gene therapy in ALD, HSC gene therapy was shown to provide clinical benefits only in two severe inherited immune deficiency: the adenosine deaminase deficiency and the severe combined immunodeficiency-X1 (1, 6, 16). In those trials, autologous HSCs were genetically corrected *ex vivo* before reinjection using a murine gammaretrovirus taking advantage that despite this viral vector transduces with little efficacy HSCs, there is a marked selective growth advantage of corrected cells that favors their engraftment and expansion *in vivo*.

For diseases, such as ALD, in which transgene expression does not confer a selective growth advantage, lentiviral vectors have generated great hopes. Lentiviral vectors, such as those derived from HIV-1, can indeed transduce nondividing cells and were shown, *in vitro* and in mice, to allow more efficient gene transfer into HSCs than murine gammaretrovirus vectors (36, 39). *In vitro* experiments of ALD gene transfer with lentiviral vector have shown biochemical correction of monocytes/macrophages derived from transduced ALD protein-deficient human CD34+ cells (8). *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% to 25% of brain microglial cells expressing the ALD protein 12 months after transplantation. Unfortunately, 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 ALD protein in human monocytes and macrophages derived from engrafted human stem cells (3). Human bone marrow-derived cells migrated into the brain of transplanted mice where they differentiated into microglia expressing the human ALD protein.

Two patients who were candidates for HSCT but had no HLA-matched donor have been treated using HSC gene therapy with lentiviral vector. Peripheral CD34+ cells were collected after granulocyte-colony stimulating factor (G-CSF) mobilization and transduced with an myeloproliferative sarcoma virus enhancer, negative control region deleted, dl587rev primer binding site substituted (MND)-ALD lentiviral vector. This vector is replication-defective, self-inactivated (SIN) and VSV-G envelope-pseudotyped. CD34+ cells from the patients were transduced with the MND-ALD vector during 16 h in the presence of cytokines at low concentrations. Transduced CD34+ cells were frozen after transduction to perform on 5% of cells various safety tests that included in particular three replication-competent lentivirus (RCL) assays. Transduction efficacy of CD34+ cells ranged from 35% to 50% with a mean number of lentiviral integrated copy between 0.65 and 0.70. After the thawing of transduced CD34+ cells, the patients were reinfused with more than 4.10^6 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 for the two patients. Nearly complete immunological recovery occurred at 12 months for the first patient and 9 months for the second patient. Bone marrow aspirates were normal at 12 months and 24 months post-gene therapy and all RCL tests have been negative up to the last follow-up.

The efficacy of the HSC transduction was determined by assessing the number of hematopoietic cells expressing ALD protein in bone marrow and peripheral blood cells by immunohistochemistry and the identification of identical insertion sites (IS) of lentiviral vector in myeloid and lymphoid cells from the same patient. Between 23% and 25% of peripheral blood cells from the three patients expressed the transgene 30 days after infusion of the transduced CD34+ cells. The percentage of corrected peripheral blood cells decreased with time but stabilized between 12 months and 16 months in the two treated patients. In each patient, ALD protein 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). Thirty-six months after gene therapy, this percentage is approximately 10% for the first treated patient and 14% for the second treated patient. Identical IS of lentiviral viral vector were identified in myeloid and lymphoid cells from the two patients in the long term, indicating effective gene transfer into HSC. Based on mRNA expression of the transgene in peripheral blood cells, there was no indication of inactivation (variegation) of the MND promoter *in vivo*.

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), C19+ (B-lymphocytes) and bone marrow CD34+ 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. 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. Brain MRI analysis showed that progression of demyelination was arrested 14 months after gene therapy in the first patient and 20 months after gene therapy in the second patient. Since and up to the last follow-up 36 months after gene therapy, demyelinating lesions have not further progressed in both patients. The first 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 HSCT, the two patients developed moderate cognitive deficits that has remained stable since 24 months to 30 months after the transplant. Altogether, the results obtained in the first two treated patients indicate that HSC gene therapy results in neurological benefits that are comparable to those seen in patients undergoing successful and noncomplicated allogeneic HCT.

The adverse events that have occurred in SCID-X1 patients treated with gammaretrovirus vector gene therapy have raised serious concerns about retroviral integration-related mutagenesis and leukemogenesis (17, 18). Transcriptionally, active long terminal repeats (LTR) of retroviral vectors are major determinants of genotoxicity (13), whereas the LTR promoter/enhancer of lentiviral vector used in this study is SIN upon transduction. This and several other differences in lentiviral and oncoretroviral biology suggest that the risk of insertional mutagenesis by a SIN lentiviral vector is likely lower than with gammaretrovirus and even SIN gammaretrovirus. The insertion of lentiviral vector into or close to genes, including pro-oncogenes, is certainly not entirely neutral, 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 be overemphasized and balanced with the benefit of HSC gene therapy. Five out of the 20 SCID-X1 patients treated by HSC gene therapy indeed developed leukemia because of retroviral insertion, but only one died from this complication. Overall, the mortality risk was 5%, far below that of allogeneic HSCT in this disease. From the remaining 19 SCID-X1 patients, gene therapy has failed in two older patients, but 17 patients remained cured from their SCID-X1 disease and leukemia.

As only up to 14% of HSCs were corrected in the ALD gene therapy trial, there is room for improvement. This is particularly important to shorten the period during which cerebral demyelination continues to progress after transplant. Higher vector titers, which will become available as lentiviral vector manufacturing improves, will likely boost this figure. Before this gene therapy trial was performed, we 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 phenotypically normal ALD mouse. Gene therapy of ALD provided a benefit similar to that of allogeneic HSCT transplantation with full chimerism, despite a relatively low level of gene 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. However, 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 progenitors that contain a higher proportion of gene-corrected cells than HSCs.

Altogether, these preliminary results in the first two 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. In particular, HSC gene therapy might be considered as a first therapeutic option for adult ALD patients who develop cerebral demyelination, and for who the mortality risk of allogeneic HSCT is close to 40%. In the future, improved HSC transduction protocols may overcome the need for bone marrow conditioning, and HSC gene therapy could potentially be envisaged at an asymptomatic stage in all ALD males and with the setting of neonatal ALD screening. If HSC gene therapy may prove to be of benefit in ALD mice and if allogeneic HSCT with RIC regimen provides encouraging results in AMN patients, one may even envisage the possibility that HSC gene therapy could be performed at an asymptomatic stage in all ALD boys and girls. However, the road is still very long before this figure becomes reality.

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