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Gene therapy for Wiskott-Aldrich Syndrome—Long-term reconstitution and clinical benefits, but increased risk for leukemogenesis

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Abbreviations: WAS, Wiskott-Aldrich-Syndrome; HSCT, haematopoietic stem cell transplantation; WASP, WAS protein; HLA, human leukocyte antigen; GT, gene therapy, HSC, haematopoietic stem cell; CGD, chronic granulomatous disease; ADA, adenosine deaminase; SCID, severe combined immunodeficiency; rhG-CSF, recombinant human granulocyte colony-stimulating factor; LTR, long terminal repeat; LAM-PCR, linear amplification-mediated polymerase chain reaction; nr, nonrestrictive; ISs, insertion sites; TSS, transcription start site; T-ALL, T-cell acute lymphoblastic leukemia; AML, acute myeloid leukemia; SIN, self-inativating

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Wiskott-Aldrich-Syndrome (WAS) is a rare X-linked recessive disease caused by mutations of the *WAS* gene. It is characterized by immunodeficiency, autoimmunity, low numbers of small platelets (microthrombocytopenia) and a high risk of cancer, especially B cell lymphoma and leukemia.

Haematopoietic stem cell transplantation (HSCT) is considered the standard curative therapy option – but the procedure can have major side effects and is limited by donor availability. Gene therapy with gammaretroviral vectors is able to overcome some of these shortcomings and lead to (at least a partial and temporary) functional immune system reconstitution, but it is associated with the development of leukemia after viral integration and oncogene transactivation. The high rate of integration-associated oncogene activation underlines the necessity for the development and application of safer genome-engineering technologies with similar efficacy and reduced toxicity.

Wiskott-Aldrich-Syndrome (WAS) is a rare X-linked recessive disease caused by mutations of the *WAS* gene[1] and characterized by autoimmunity, low numbers of small platelets (microthrombocytopenia), immunodeficiency, and a high risk of cancer, especially B cell lymphoma and leukemia.[2] WAS protein (WASP) acts as a key regulator for the polymerization of actin in haematopoietic cells.[3] WASP deficiency, therefore, leads to malfunctions of different leukocyte subsets, including defective T and B cell responses, impaired migration, and significant impairment of NK immunological synapse formation.[4,5] Severe and generalized infections, bleeding and malignancies lead to an early death in severe WAS.[6] The standard therapy is allogeneic HSCT. Although this is usually an effective and curative treatment, it is often associated with significant morbidity and sometimes mortality, especially if no human leukocyte antigen (HLA)-matched HSCT donor is available.[7]

Gene Therapy as an Alternative Treatment for WAS Leads to Molecular Und Functional Correction of Disease

Over the last decades, gammaretrovirus-based HSC gene therapy (GT) has emerged as an alternative therapeutic strategy for the treatment of hereditary diseases of the immune system (reviewed in[8,9]). Patients suffering from chronic granulomatous disease (CGD),[10] adenosine deaminase (ADA)-deficient severe combined immunodeficiency (SCID),[11,12] and X-linked SCID[13-15] experienced clinical improvements and at least partial or temporary correction of immune cell functions. However, severe clinical

side-effects, including acute leukemia secondary to insertional mutagenesis and activation of proto-oncogenes, have raised concerns about therapeutic safety.[16-22]

Ten patients suffering from WAS were treated with haematopoietic GT using classical gammaretroviral vectors between 2006 and 2009.[23,24] Briefly, after stimulation with recombinant human granulocyte colony-stimulating factor (rhG-CSF) and/or plerixafor, peripheral mononuclear cells were harvested and transduced with a replication-incompetent gammaretroviral vector that expressed a healthy copy of *WAS* driven by a murine stem cell virus-derived long terminal repeat (LTR) sequence. Patients were conditioned using myeloablative busulfan therapy (8mg/kg) prior to reinfusion of transduced cells. After GT, we were able to observe a strong and sustained expression of WASP in peripheral nuclear blood cells and in platelets, along with an overall reconstitution of lymphocyte function. Patients also showed remarkable clinical improvements with partial to complete resolution of autoimmunity, bleeding diathesis and susceptibility for infections.

A few specific lessons of this study are outlined below:

Age at GT Might Influence Speed of Haematopoietic Reconstitution

While most of our patients were young children at the time of treatment and mostly reconstituted fast after GT, one patient at the age of 14 at the time of GT experienced only a slow reconstitution of his immune system. A similar observation had already been described in a GT trial for X-SCID,[25] thus pointing to a potentially slower overall reconstitution in older patients.

Gammaretroviral Gene Therapy Vector Integration Favors Certain Genomic Regions

Retroviral insertion site (IS) analysis using standard and nonrestrictive (nr) linear amplification-mediated polymerase chain reaction (LAM-PCR)[26] revealed more than 140,000 unambiguous ISs with an initially highly polyclonal repopulation of the haematopoietic system. A comprehensive analysis of IS patterns demonstrated a typical gammaretroviral insertion pattern with integrations accumulating at transcription start sites (TSS) of gene-coding regions. The majority of most frequently affected genes had previously been described as proto-oncogenes (including *MECOM*[27] and *LMO2*[28]).

Insertional Mutagenesis Leads to Gene Activation and the Development of Haematopoietic Malignancies

Six patients developed T-cell acute lymphoblastic leukemia (T-ALL) between 16 months and 5 y after GT, all of whom carried gammaretroviral insertion within or close to the *LMO2* gene locus. One patient developed acute myeloid leukemia (AML) and LAM-PCR identified an insertion within the *MSD1* gene locus. Insertion site kinetics prior to onset of leukemia were markedly diverse. Whereas all T-ALL patients had a polyclonal IS pattern without indications for a clonal outgrowth, the patient developing AML showed a slow increase of a *MDS1* clone contribution over time. Of note, 2 patients with T-ALL developed AML shortly after or during maintenance therapy, with dominant clones harboring vector ISs close to either *MDS1* or *MN1* gene loci, respectively. In summary, we were able to demonstrate the feasibility of GT for WAS and the sustainability of *WAS* gene expression and functional correction over years, but also that classical gammaretroviral gene therapy is associated with an unacceptably high rate of secondary malignancies in WAS, raising considerable safety concerns.

Treatment Strategy for Leukemic Patients after GT

Patients WAS6, WAS7, WAS9 and WAS10 underwent allogeneic HSCT between 4 and 12 months after their initial diagnosis of T-ALL. Up to date (June 2014) they are in complete remission. Patients WAS1 and WAS8 reached a state of complete clinical, morphological and molecular remission using chemotherapy, but developed AML more than a year after their initial T-ALL diagnosis. They received induction chemotherapy and were treated with allogeneic HSCT. Patient WAS1 is in complete remission (June 2014) whereas patient WAS8 succumbed to transplant-related toxicities. Patient WAS5 had an early leukemia relapse while on consolidation chemotherapy. He has achieved a second state of remission using chemotherapy and was treated by allogeneic HSCT. However, leukemia relapsed and he subsequently succumbed to progressive leukemia.

"Self-inactivating" Vectors as a Novel Tool for Gene Therapy

Over the last years, significant improvements to viral vectors have been proposed and tested experimentally. One of the major advancements is probably the creation of so-called "self-inactivating" (SIN) viruses. By deleting enhancer elements in the LTR region and using internal mammalian promoters, the ability of SIN viruses to transactivate genomic loci in the proximity of the viral IS is dramatically reduced.[29] In contrast to gammaretroviral vectors, lentiviral vectors are characterized by their ability to transduce non-dividing (stem) cells and a genomic integration pattern that does not favor the promoter-region of genes as much as gammaretroviruses do.[30] However, choosing the right internal promoter can be difficult. While strong and ubiquitously active promoters may offer strong expression of the respective gene of interest, concerns have arisen about pathological effects of non-physiological gene expression in defined cellular lineages.[31,32] This may not be a concern in WAS (WAS-protein is physiologically expressed in all nucleated haematopoietic cells and in platelets), yet tissue-specific promoters, like the reconstituted physiological WAS promoter for the first SIN-lenti gene therapy trial for WAS,[33] may promise more physiological lineage-specific gene expression. However, intrinsic promoters are not yet readily available for every gene of interest. In addition to using endogenous promoters, replacing VSV-G pseudotyping with a target-cell-specific

envelope might help to increase specificity and limit potential-side effects.[34,35]

Value of Insertion Site Monitoring to Predict Onset of Leukemia and for Treatment Decisions

In contrast to the first 2 patients enrolled in the Paris trial for γc-SCID,[18,19] whose clones harboring *LMO2* gene insertions increased slowly and steadily, IS monitoring could not predict the onset of the fast progressing T-ALL in our patients (regular follow-up analysis was undertaken at intervals of 3 to 6 months). A possible reason for the fast T-ALL progression may be a pre-leukemic clone expansion in a poorly accessible niche like the thymus or lymph nodes, and the acute release of those clones at the onset of leukemia. In contrast, for 2 of the AML patients, an increase of contribution of clones with insertions in close proximity to the *MECOM* gene locus had been detected months prior to onset of leukemia, suggesting a slow expansion of a (pre-)malignant clone. In general, integration site analysis is useful to identify integration clusters in certain gene loci, i.e. *LMO2* and *MECOM*, to reveal whether *in vivo* clonal selection occurs, and whether proto-oncogenes or cell proliferating genes are involved. IS monitoring can help to predict the onset of AML and, depending on donor availability, an early bone marrow transplant can be considered. Even though monitoring clearly did not help to predict the onset of T-ALL by particular clones, the degree of polyclonality reconstitution during treatment can be helpful for the assessment of the risk probability of future malignant transformation and facilitate the decision on the need for an eventual stem cell transplantation (again depending on donor availability).

Population Dynamics and Homeostatic Control–how Human Gene Therapy Trials Significantly Differ from Insertional Mutagenesis Observed *in vitro*

Both the activation of oncogenes and the functional inactivation of tumor-suppressors after viral insertion into the genome are known risk factors for tumor development, and, therefore, have always been a major concern and point of discussion during the development of human gene therapy trials.[36,37] While one single insertional transactivation of an oncogene can, theoretically, be enough to facilitate cellular expansion, malignant transformation typically requires the occurrence of at least a second hit. Furthermore, biological filters may prevent a dominant clonal outgrowth – for example, the activation of strong oncogenes in otherwise normal cells can lead to oncogenic stress and the subsequent activation of tumor-suppressor pathways and cell cycle arrest or cell death.[38] Noteworthy, natural killer (NK) cells, monocytes and certain subsets of T cells (all of which are less functional in severe WAS) have important roles in the physiological anti-tumor immune response.[39] It has been shown that expression of functional WAS protein in a WASP-negative cell can reconstitute the proliferative defects.[40] It is unclear, however, if unphysiologically high levels of transgene expression may render haematopoietic cells more prone to expansion, possibly without dominant mutagenesis, after vector insertion. The use of SIN retroviral vectors with weaker and physiologically active endogenous promoters may prevent this "overcorrection" and make cells less susceptible to undergo clonal dominance.

The first HSCT studies using gammaretroviral vectors have yielded ambiguous results. Ectopic expression of the common gamma chain in γc-SCID patients resulted in T-cell ALL in 5 out of 20 patients, whereas expression of adenosine deaminase in ADA-SCID patients has not led to leukemogenesis. All patients and parents were informed about a risk of leukemogenesis prior to accrual.

Conclusions

We have demonstrated that gene therapy for WAS using classical gammaretroviral vectors is feasible and can lead to long-term correction of the disease, but the rate of leukemogenesis associated with integrational gene activation is very high. New vector designs incorporating self-inactivating LTR configurations and mammalian promoters may improve safety.[33] Even though long-term observations on efficacy and safety are still pending, there is hope that the introduction of these features will reduce side effects while preserving therapeutic efficacy over many years. The development of novel genome-engineering tools may offer new therapeutic strategies for patients with WAS and other primary immunodeficiency disorders.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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