Current status and developments in gene therapy for thalassemia and sickle cell disease

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Abstract

β-thalassemias and sickle cell anemia (SCA) are the most common monogenic diseases worldwide for which curative treatments remain a desired goal. Allogeneic hematopoietic stem cell transplantation (allo-HCT), - the only curative treatment currently available for hemoglobinopaties-, has a narrow application window whereas it incurs several immunological risks. Gene therapy (GT), that is the autologous transplantation of genetically modified hematopoietic stem cells ($CD34^+$), represents a promising new therapeutic strategy which is anticipated to reestablish effective hemoglobin production and render patients transfusion- and drug- independent without the immunological complications that normally accompany allo-HCT. Prior to the application of GT for hemoglobinopathies in the clinic, many years of extensive preclinical research were spent for the optimization of the gene transfer tools and conditions. To date, three GT clinical trials for β-thalassemia and sickle cell disease (SCD) have been conducted or are in progress and 3 cases of transfusion independence in thalassemic β^0/β^E patients have been reported. In the present review, the prerequisites for successful implementation of GT, the tough pathway of GT for hemoglobinopathies towards the clinic and the knowledge gained from the first clinical trials as well as the remaining questions and challenges, will be discussed. Overall, after decades of research including achievements but pitfalls as well, the path to GT of human patients with hemoglobinopathies is currently open and highly promising…

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Introduction

Pathophysiology of hemoglobinopathies

β-thalassemias are the most common monogenetic disorders caused by the co-inheritance of two mutated alleles in the β-globin locus, resulting in partial or complete elimination of β-globin expression. Excess of α-globin chains in the erythroid cells within the bone marrow leads to their intracellular precipitation accompanied by massive intramedullary death of erythroblasts, whereas surviving and maturing erythrocytes display marked abnormalities and significantly reduced life cell span in peripheral blood. Homozygosity, results in disease (thalassemia major or Cooley's anemia), which is characterized by absent (β^0-thalassemia) or severely reduced hemoglobin A (β^+-thalassemia). Consequently, a life-threatening hemolytic anemia develops, which is lethal within the first decade of life should be left untreated.[1] The standard of care consists in lifelong transfusions and iron chelation and has substantially improved the life expectancy of the patients. However, strict compliance to the treatment severely compromises the patients' quality of life, while it constitutes a significant financial burden for national economies. Failing to comply with the conventional treatment, patients are exposed to life-threatening complications.

Sickle cell anemia (SCA) is a severe inherited disorder caused by an abnormal hemoglobin, termed hemoglobin S (HbS), comprised by two α-chains and two mutated β-chains. The mutated β-chains contain a substitution of valine for glutamic acid at position 6 which results in a change in the surface charge thus, predisposing deoxygenated HbS to polymerization, intravascular red cell sickling, painful vaso-occlusive crises and organ damage.[2]

Current curative methods and the need for globin gene therapy

Although the palliative, lifelong transfusions and daily chelation in thalassemic patients as well as Hydroxyurea and transfusions in SCA patients have substantially improved their life expectancy over the last 40 years, these treatments severely compromise their quality of life, while they constitute a significant financial burden for the national economies.[3] Moreover, patients who fail to strictly comply with the treatment, are exposed to life-threatening complications.

The only radical cure available is the allogeneic stem cell transplantation (allo-HCT) from a matched related donor, with high success rates in young patients.[4,5] Nevertheless, this therapeutic option has a narrow application window to young patients without organ damage who have, in addition, a suitable donor whereas it is associated with considerable transplant-related morbidity and mortality especially when patients with advanced disease are transplanted, or unrelated donors are used.[6-9]

Gene therapy, by the introduction of the normal β-globin gene into the patients' own hematopoietic stem cells (HSCs), is anticipated to permanently correct the ineffective erythropoiesis and render the patients treatment-independent. Furthermore, it will be available to all patients within an autologous setting, devoid of the immunological complications which normally accompany allogeneic transplantation.

Prerequisites for gene therapy of hemoglobinopathies

The *in situ* homologous correction of the deficient β-globin gene would be the ideal gene therapy option; this however, is not yet feasible in HSCs. Alternatively, gene transfer strategies have employed "additive gene therapy", by transferring a normal copy of the human β- or γ-globin gene into the HSC genome with viral vectors.

However, the complexity of globin gene regulation, the need for erythroid- and differentiation stage-specific as well as elevated globin expression along with the high level of safety required for the treatment of chronic diseases with prolonged life expectancy, significantly delayed the initiation of gene therapy clinical trials for hemoglobinopathies, as compared to other genetic diseases.

The tools: lentiviral vectors

The vectors considered most appropriate for gene therapy of hemoglobinopathies are the γ-retroviral and lentiviral vectors, which integrate in the genome of target cells, resulting in long-term and stable transfer of the therapeutic gene.[10]

Despite more than a decade of intensive efforts in the field, potentially therapeutic γ-retroviral globin vectors failed to develop, mainly due to low titers and genetic instability. Lentiviral vectors are considered the best means for gene therapy of hemoglobinopathies and their advantages over γ-retroviral vectors comprise: 1) ability to effectively transduce both dividing and non-dividing cells,[11] 2) higher packaging capacity (~9-10 kb)[12] without compromising genetic stability, 3) safer integration profile[13] and 4) self-inactivating (SIN) design. Self-inactivation is based on the deletion of the U3 element in the 3΄LTR (Long Terminal Repeat), copied, during reverse transcription of the viral RNA, to the 5΄ LTR. Consequently, the viral enhancer is abrogated whereas the therapeutic gene is expressed through an internal, erythroid-specific promoter. These qualities render lentiviral vectors much safer than γ-retroviral, being previously characterized as "high-risk" for insertional mutagenesis resulting in the emergence of leukemias in clinical gene therapy trials for immunodeficiencies (X-SCID and WAS).

Functional and erythroid-specific expression

High-level globin gene expression requires the inclusion of parts of the Locus control region (LCR) of the β-globin locus, in the viral construct. The LCR consists of at least 7 DNase hypersensitive sites (HS), located 48-62 kb upstream of the β-globin gene.[14] It has been shown that the LCR enhances erythroid-specific and differentiation stage-specific expression[15,16] while it prevents gene silencing.[17] Most lentiviral globin vectors constructed to date, express the β- or γ-globin transgene under the control of an internal β-globin promoter, one of the two proximal β-globin enhancers[18] and comprise at least two HS LCR elements.[19-22]

Stable and homogeneous expression

Viral vector expression may be highly differential, since the resulting insertion patterns inevitably subject the incoming vector sequences to a wide array of genomic environments. The variety and variegation of viral vector expression resulting from semi-random integration in chromosomal sites and thus differential exposure to the effects of surrounding chromatin has been described as "position effects". Barrier insulators are naturally occurring DNA elements that help form functional boundaries between adjacent chromatin domains. When genes are to be expressed in ectopic sites, as in gene therapy applications, the new chromosomal environment significantly affects transgene expression. This heterogeneity in expression is usually attributed to adjacency with an endogenous enhancer or silencer or it simply reflects the insertion of the transgene into hetechromatin.[23,24] The best characterized insulator, up to date, is the hypersensitive site 4 from the chicken β-globin locus (cHS4, chicken hypersensitive site 4); flanking viral vectors with cHS4 using a double copy configuration, protects -albeit, not completely- the *in vivo* transgene expression from chromosomal position effects, resulting in stable and homogeneous expression.[25]

Safety

Despite high success of gene therapy in immune deficiencies[26-28] and lysosomal storage diseases,[29,30] insertional mutagenesis provoked by the integrating γ-retroviral vectors, still represents the major procedure-related toxicity. Leukemiogenesis observed in pediatric patients with X-SCID[31] and Wiscott-Aldrich syndrome[32] who have been cured from their disease after treatment with γ-retroviral vectors carrying therapeutic genes, eventually overshadowed initial enthousiasm and directed research towards the reduction of genotoxicity risks. The genotoxic events were associated with the integration of vector provirus near a certain cellular proto-oncogene activating the inappropriate expression of this oncogene via the viral enhancer.

In disorders with short life expectancy, as severe combined immunodeficiency or metachromatic leukodystrophy, a certain degree of procedure-related toxicity could be acceptable should therapeutic benefit counterbalances risk. For instance, 19 out of the 20 children treated with gene therapy for X-SCID are alive and disease-free up to 16 years post treatment, whereas none of them would be living today without gene therapy. However, in patients with chronic and of modest severity disorders, such as treatment-compliant patients with hemoglobinopathies, it is obvious that the expected benefit should highly justify the procedure-associated risk. Irrespectively however, to the severity of the target disease, major research efforts, over the last few years, have focused on the safety of the procedure and the minimization of insertional mutagenesis, through novel viral vectors and rational protocol design.

β-globin -encoding lentiviral vectors currently used in clinical trials, have been constructed with safety characteristics that substantially restrict oncogenetic risk with regard to first generation γ-retroviral vectors: the self-inactivation (SIN) design, mentioned above, abolishes the viral LTR, which is the major genotoxic factor.[33,34] No matter the SIN design however, globin vectors additionally carry a very strong Locus Control Region-LCR enhancer, the activation of which should not perturb adjacent genes in the insertion site.[35,36] Enhancer-blocking insulators, which prevent enhancer-mediated transcriptional activation of adjoining regions in the insertion site, have been incorporated into viral vector constructs to reduce the risk of insertional mutagenesis. The chromatin insulator cHS4, has a dual function both as a barrier and enhancer-blocking insulator that could prevent negative insertional effects, however, because of its relatively large size severely compromises vector titers and consequently, transduction efficiency.

Preclinical research

Correction of thalassemia in mice was shown in early 2000 by the group of M. Sadelain with TNS9, the first β-globin vector to correct murine thalassemia intermedia[37] and major[38] by increasing hemoglobin levels to 11-13 g/dl, reducing extramedullary erythropoiesis and hepatic iron accumulation and rescuing thalassemia major mice from

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lethality. Subsequently, other groups including the groups of Leboulch,[39] Persons,[40] Malik[41] and Ferrari[42] also demonstrated long-term correction in β-thalassemia and sickle cell disease mouse models,[43] as well as human thalassemic cells.[44,45]

Clinical protocol design: issues to be addressed

Conditioning

Unlike X-SCID, the disease background of hemoglobinopathies does not provide a selective advantage at the level of genetically modified hematopoietic stem cells (CD34$^+$), which are capable for long-term hematologic reconstitution, but only at the level of gene-corrected erythroblasts and erythrocytes. Consequently, the successful engraftment of transduced CD34$^+$ cells requires the administration of bone marrow conditioning to the patient.

Myeloblative conditioning would warrant predominant vector-derived reconstitution, accompanied, however, by non-negligible transplant-related morbidity and mortality that are particularly undesirable in patients with nonmalignant disease as hemoglobinopathies. On the other hand, nonmyeloablative, low-intensity conditioning would substantially reduce treatment-related toxicity, compromising, however, the engraftment rates and therapeutic benefit.

Although it is widely accepted that a substantial degree of conditioning will be necessary in hemoglobinopathies, prior to therapeutic infusion of genetically modified CD34$^+$ cells, the degree of bone marrow ablation required to ensure adequate engraftment of gene-modified cells without unacceptable toxicity under these settings, still remains a matter of discussion.

Studies involving allogeneic HSC transplantation in patients with β-thalassemia and sickle cell disease, indicated that even low levels of donor chimerism can render recipients transfusion independent, supporting the concept that even a low-level engraftment of gene-modified autologous HSCs could provide significant clinical benefit.[46] However, the threshold level of gene-modified cell chimerism required for clinical improvement of hemoglobinopathies is currently unknown. Studies in thalassemia mouse models suggest that a chimerism level of 20-30%, along with a transgene expression level >15% of the total α-globin will be necessary to ameliorate disease and achieve transfusion independence in patients with β-thalassemia. These prerequisites are generally much higher than any other genetic disorder to be corrected by gene therapy.[47]

Based on the above observations, it is predicted that non-myeloablative doses of Busulfan 8-12 mg/kg or 140 mg/m^2 Melphalan could provide adequate ablation to allow transduced HSCs reach clinically relevant levels, while maintaining an adequate level of safety commensurate with a Phase I clinical trial.[48] However, it would be important in this case, to counterbalance the low engraftment that would be expected under these conditions, by using methods that ensure high levels of gene transfer and high doses of gene-modified cells, as discussed below. On the other hand, there are groups supporting the administration of a fully myeloablative conditioning before the infusion of the genetically-modified cells as a means to maximize the *in vivo* gene transfer, at the expense however, of higher peritransplant toxicity.[49]

Obtaining target cells and defining the optimal autologous graft for gene therapy of hemoglobinopathies

In gene therapy protocols, as for hemoglobinopathies, due to the absence of a selective advantage of gene-corrected stem/progenitor cells and should a reduced intensity conditioning regimen is preferred, it is necessary to infuse considerably high numbers of transplantable HSCs in order to achieve adequate levels of engraftment with gene-modified cells.[48,50] Moreover, for safety purposes, a part of the unpurified graft is stored as "back up" cells, should the genetically modified cells fail to engraft, thus further supporting the need for high CD34$^+$ cell doses for gene therapy of hemoglobinopathies. Consequently, for human gene therapy of hemoglobinopathies, the optimal autologous graft will be defined not only by a high CD34$^+$ cell content, but also by efficient engraftment after infusion.

Mobilized peripheral blood has been demonstrated to provide 3-4 fold higher numbers of CD34$^+$ cells with faster engrafting capacity over conventional bone marrow harvest.[50,51] The cytokine G-CSF (Granulocyte colony-stimulating factor) was thus far the sole HSC mobilizing agent available for clinical use and is widely administered in autologous or allogeneic HSC transplantation[52] as well as stem cell gene therapy applications.[29,45,53] The use of G-CSF however, has been associated with certain morbitities;[54,55] furthermore, a number of patients fail to mobilize efficiently and some normal donors need to undergo extended aphereses.[56] Limitations regarding the use of G-CSF also apply in hemoglobinopathies including the precipitation of severe, or even fatal, sickling crises in patients with SCA[57] and the development of hyperleukocytosis in splenectomized patients with thalassemia.[58]

Plerixafor (former AMD3100), a reversible inhibitor of the CXCR4/SDF-1 axis, has recently been launched in the market and was shown to rapidly mobilize CD34$^+$ cells and to boost the mobilization potential by several fold in combination with G-CSF.[59,60]

In view of a gene therapy clinical trial for thalassemia and in order to address safety and efficacy issues governing stem cell mobilization, we have previously investigated in two clinical trials, various CD34$^+$ cell mobilization strategies using G-CSF-alone, Hydroxyurea (HU)+G-CSF, Plerixafor-alone and Plerixafor+G-CSF.

The first trial (THAL001) enrolled 26 patients and demonstrated that mobilization with G-CSF is safe and effective in non-splenectomized patients. However, in splenectomized patients, G-CSF triggered hyperleukocytosis that necessitated a significant dose reduction leading to suboptimal CD34$^+$ cell yields. One-month HU-pretreatment prevented hyperleukocytosis and allowed successful CD34$^+$ cell collections, when an optimal 2-week washout period was maintained before G-CSF administration, significantly prolonging, however, the mobilization procedure.[58]

Due to the limitations of G-CSF mobilization in splenectomized patients with thalassemia and also in other patients where gene therapy could serve as an alternative therapeutic option (sickle cell disease, Fanconi anemia),[57,61] we investigated, in a second trial (THAL002), Plerixafor (Mozobil) as a single mobilizing agent and in combination with G-CSF, when failure to collect sufficient HSC numbers by single-agent mobilization (≥6x10^6/kg) was encountered.[62] The THAL002 trial enrolled 20 patients and showed that Plerixafor can rapidly and effectively mobilize CD34$^+$ cells, without causing hyperleukocytosis in splenectomized patients. As such, its use could be also considered for gene therapy of SCD where documented complications preclude G-CSF mobilization. Patients with primary mobilization failure by G-CSF or Plerixafor were remobilized with Plerixafor+G-CSF. The combination of the two agents was well-tolerated and increased the per apheresis CD34$^+$ yield by 3-14 times and in all cases, resulted in single-apheresis collections. The great synergism of the combination was particularly evident in the splenectomized cohort of patients, in which, despite the up to 75% G-CSF dose reduction to avoid hyperleukocytosis, it still provided high cell yields (6-12.7x10^6/kg) by single apheresis. In conlusion, the combination of Plerixafor+G-CSF is the optimal method for obtaining vast numbers of CD34$^+$ cells by single collection from adults with thalassemia, or when single-agent mobilization failure is highly likely.

The superiority of Plerixafor+G-CSF graft over the differently mobi-

lized grafts was not only quantitative but qualitative as well, since both in competitive transplantation models using thalassemic mouse cells,[63] and in partially myeloablated xenografts of human thalassemic $CD34^+$ cells transduced with the TNS9.3.55 β-globin lentiviral vector, Plerixafor+G-CSF-mobilized cells exhibited superior multilineage engraftment over single-agent-mobilized cells while they produced the highest β-globin output per vector copy (Karponi G et al, submitted).

Based on the above, it is implied that Plerixafor+G-CSF cells represent the optimal graft source for gene therapy of thalassemia. Utilization of such a graft with favorable transplantation characteristics, may allow for the implementation of a reduced intensity conditioning for gene therapy, potentially reaching clinically relevant gene transfer and expression rates, under minimal transplant-related toxicity. Gene therapy for SCD may require bone marrow as a graft source or the exploration of tolerability and efficacy of Plerixafor-alone mobilization in SCD patients.

Clinical trials

The first gene therapy clinical trial for patients with β-thalassemia was initiated in Paris by the group of P. Leboulch using a SIN β-globin lentiviral vector containing the cHS4 chromatin insulator (SIN-LentiGlobin®HPV569). Three β^0/β^E thalassemic patients were treated with gene-corrected autologous HSCs, after a full-myeloablative conditioning (Busulfan 14 mg/kg).[64,65] The first patient received his unmanipulated back up cells due to engraftment failure of the gene-modified cells and long-lasting aplasia and the third patient, two years post gene therapy, shows low gene marking *in vivo* and remains transfusion-dependent. The second patient however, is transfusion-independent for over 6 year with stable hemoglobin levels at 8-8.5g/dl. A fraction of the patient's hemoglobin however, was derived from a dominant hematopoietic clone (which has began to recede) harboring a vector insertion in the HMGA2 proto-oncogene and another fraction from an unexpected increase of HbF that emerged post transplant.[49]

The second clinical trial is currently running at the Memorial Sloan-Kettering Cancer Center in New York, by the group of M. Sadelain. As announced at the ASGCT Annual Meeting in May 2014, three β^0/β^+ thalassemic patients received their gene-corrected G-CSF-mobilized $CD34^+$ cell grafts, after a reduced intensity conditioning with 8 mg/kg Busulfan. The first two patients who completed the one-year follow-up remain transfusion-dependent but with increasing intervals between transfusions.

A third international clinical trial using a modified LentiGlobin® vector (BB305 SIN, uninsulated) is in progress and currently open in France and USA, sponsored by Blue Bird BIO. So far, four β^0/β^E thalassemic patients and one patient with SCA have been treated with genetically modified HSCs after a full myeloablative conditioning with 14 mg/kg Busulfan. Early results have been announced for two patients in France (EHA 2014, Milan), who are transfusion-independent 2 and 4.5 months post gene therapy with hemoglobin levels at 10.1 and 11.6 g/dl from which 4.4 and 6.6 g/dl are derived from the gene-corrected cells (the rest b mainly comes from the endogenous bE and to a lesser extent, bF production).

The knowledge gained from the clinical trials

Despite the proof of principle that gene therapy can cure thalassemia and the unequivocal progress achieved in the field, the knowledge gained from the clinical trials so far, underlines the need for additional improvements towards safety and efficacy. Although the SIN configuration and lentiviral integrating pattern, along with the erythroid specificity of expression, rendered globin lentiviral vectors as "low-risk" for insertional mutagenesis after genetic correction of target cells, the observed clonal dominance in the first French trial has raised safety concerns. In addition, higher in vivo globin gene transfer is needed to ensure that the β^0/β^0 genotype will also be cured or/and allow the administration of a partially myeloablative conditioning to patients.

Efforts are made to minimize the risk of insertional oncogenesis and increase the efficacy of lentiviral globin vectors by genome-wide identification and functional characterization of human, novel, powerful and small-sized enhancer-blocking insulators to be incorporated in globin vectors and erythroid-specific enhancers to substitute for the conventional β-globin microLCR.

Alternative approaches

Genome editing, as well as the development of induced Pluripotent Stem Cells (iPS), are expected to provide future therapeutic perspectives for thalassemia, either by targeted, *in situ* correction of deficient genes, or by selecting "safe harbors" of vector integration in the genome.

Conclusions

After decades of research, which were marked by pitfalls but significant preclinical success as well, the initiation of clinical trials outlined a new era in the field of thalassemia gene therapy. Irrespectively to the emerging need of surplus protocol and viral construct refinements, which continuously challenge the field of gene therapy for thalassemia, the proof of principle has already been demonstrated and soon more, reproducible therapeutic outcomes are to come.

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