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CONCISE REVIEW

Gene Therapy with Hematopoietic Stem Cells: The Diseased Bone Marrow's Point of View

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When considering inherited diseases that can be treated by gene transfer into hematopoietic stem cells (HSCs), there are only two in which the HSC and progenitor cell distribution inside the bone marrow and its microenvironment are exactly the same as in a healthy subject: metachromatic leukodystrophy (MLD) and adrenoleukodystrophy (ALD). In all other settings [X-linked severe combined immunodeficiency (X-SCID), adenosine deaminase deficiency, Wiskott-Aldrich syndrome, and β-hemoglobinopathies], the bone marrow content of the different stem and precursor cells and the cells' relationship with the stroma have very specific characteristics. These peculiarities can influence the cells' harvesting and behavior in culture, and the postgraft uptake and further behavior of the gene-modified hematopoietic/precursor cells. In the present mini-review, we shall briefly summarize these characteristics and outline the possible consequences and challenges.

Keywords: bone marrow, gene therapy, HSC

Introduction

Since the description of successful gene therapy for X-linked severe combined immunodeficiency (X-SCID) in 2000, the results of several other trials have confirmed the clinical potential of gene therapy approaches. However, the use of gamma (γ) retroviruses for gene transfer was associated with the occurrence of T-acute lymphoblastic leukemia and myelodysplasia in three clinical trials [in X-SCID, Wiskott-Aldrich syndrome (WAS) and chronic granulomatous disease (CGD)] [1–4]. In fact, the γ-retrovirus's integration sites were concentrated near the 5′ ends of transcription units [5], which facilitated the activation of the nearest oncogenes. Accordingly, most of the efforts in this field have been focused on modifying the vector's characteristics.

The use of self-inactivated (SIN) retroviral vectors has significantly reduced the risk of insertional mutagenesis [6,7]. Ever since a lentiviral-derived vector was first used to treat X-linked adrenoleukodystrophy (ALD) [8], SIN HIV vectors have been the tool of choice for the introduction of a therapeutic gene into autologous hematopoietic stem and progenitor cells (HSPCs). To date, more than 100 patients with various inherited diseases have been transplanted using this strategy, and no causally related adverse events have been reported so far.

Despite these very encouraging results, gene therapy (ie, the long-term correction of a genetic disease via the sustained engraftment of gene-corrected HSPCs) is still not sufficiently effective in some indications. Experience in the field of HSPC transplantation has shown that two factors have a major influence on the long-term engraftment of HSPCs: the conditioning regimen and the quality of the transplanted HSPCs [9]. In gene therapy approaches, autologous HSPCs are sorted from a bone marrow aspirate or from mobilized peripheral blood by using the CD34 surface marker. After in vitro culture in the presence of cytokines and the therapeutic vector, the gene-corrected cells are administered to the patient, who may have previously undergone chemotherapeutic conditioning (to facilitate cell engraftment). As shown for HSPC transplantation, the autologous cells' status and subset composition may have a major impact on both the in vitro gene transfer procedure and the subsequent engraftment. With the exceptions of metachromatic leukodystrophy (MLD) and ALD, the composition of the $CD34^+$ HSPC subset [hematopoietic stem cells (HSCs), myeloid and lymphoid progenitors, and committed precursors] in all other settings [X-SCID, adenosine deaminase (ADA) deficiency, WAS, and β-hemoglobinopathies] presents various biases that can impact the outcome of the transduction procedure.

Other particular features (notably homing properties) can influence the cells' harvesting yield, behavior in culture, and postgraft uptake. In some indications (such as X-SCID), the gene-corrected cells' selective advantage in vivo contributes

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to the success of the procedure. For diseases in which a selective advantage is not expected (CGD, for instance), success relies mostly on the engraftment of high numbers of gene-corrected HSPCs.

In the present mini-review, we shall briefly summarize these success factors and outline possible consequences and challenges.

Severe Combined Immunodeficiencies

Severe combined immunodeficiencies comprise a series of rare, congenital, primary immunodeficiency disorders characterized by a T cell defect, which can be accompanied by defects in natural killer (NK) cells and/or B cells [10,11]. These are life-threatening diseases, in which the patient is extremely susceptibility to infections. X-SCID is caused by mutations in the gene encoding the γ common chain shared by several cytokine receptors, including the interleukin (IL)-2, IL-4, IL-7, IL-9, IL-15, and IL-21 receptors. In view of these receptors' roles in hematopoiesis, X-SCID is characterized by an early block in T and NK cell differentiation, the complete absence of T and NK cells and normal-to-elevated numbers of poorly functional, mature B cells [12,13].

Artemis, RAG1, and RAG2 deficiencies are characterized by the complete blockade of B and T cell differentiation, due to defects in the rearrangement of the B and T cell receptors loci [14–16]. A recent study showed that T cell reconstitution was faster in X-SCID patients treated by gene therapy as compared to haplo-identical hematopoietic stem cell transplantation (HSCT) [17]. Although reconstitution of the B and NK compartments is still poor in both groups, this result suggests that gene therapy might be the best therapeutic option for X-SCID. However, in RAG patients and some X-SCID patients, the very early block in lymphoid differentiation results in an empty thymus and the overrepresentation of $CD34^+CD19^+$ pro-B cells in the bone marrow. This pro-B cell subpopulation can constitute up to 70% of the whole $CD34^+$ harvest (instead of <25%). Although this proportion falls during the culture transduction procedure (our unpublished observations), the pro-B cell subpopulation interferes with and decreases the true dose of HSPCs injected, relative to that calculated from $CD34^+$ counts at the start of the procedure. In our X-SCID study, 3×10^6 $CD34^+$ γc^+ cells/kg were required to reconstitute an immune compartment similar to that found in healthy subjects of the same age [1]. Given that only 50% (on average) of the cells are corrected in conventional gene therapy protocols, the optimal harvest for transplantation should contain at least 6×10^6 $CD34^+$ cells/kg in total (to yield a dose of 3×10^6 genetically modified $CD34^+$ cells/kg). Other European and American groups involved in pediatric gene therapy trials for constitutive immunodeficiencies have since confirmed this dose threshold. The threshold should be increased proportionally if the bone marrow harvest is significantly contaminated by pro-B cells.

ADA deficiency is a metabolic disease in which toxic metabolites affect all the HSPCs. It is characterized by a profound lymphopenia that affects all T, B, and NK lineages. Among the various therapeutic options proposed to ADA patients, gene therapy appeared largely superior to haplo-identical HSCT in terms of survival and immune recovery [18]. When studying the bone marrow of patients with ADA deficiency, Sokolic et al. found clear morphologic evidence of myeloid lineage dysplasia, including marrow hypocellularity, megaloblastic erythropoiesis, and abnormal megakaryocytes [19]. The dysplastic features seen in all hematopoietic lineages reflect adenosine metabolite toxicity at the stem cell level, and thus explain the relative degree of hypocellularity seen in the bone marrow of all patients for whom a core biopsy was performed. As a consequence, the clinical sequelae of myeloid abnormalities may include low absolute neutrophil counts, drug-induced neutropenia, and increased susceptibility to busulfan-induced myeloablation. The existence of this stem cell defect in patients with ADA-SCID may explain the relatively high levels of marking seen in gene therapy trials for this disease after low-dose conditioning [20,21]. To further improve the gene therapy outcome (and especially the number of gene-corrected hematopoietic cells present in the long term), one should increase the yield of the whole $CD34^+$ HSPC harvest. To this end, enzyme replacement therapy with pegylated ADA (PEG-ADA) may be of benefit in ADA-SCID patients because it decreases the levels of adenosine metabolites that are otherwise toxic for hematopoietic progenitor cells.

Chronic Granulomatous Disease

CGD is a rare, inherited, primary immunodeficiency characterized by defective microbicidal activity in phagocytes. This leads to increased susceptibility to recurrent, life-threatening bacterial and fungal infections. CGD is caused by defects in any one of the five subunits of the phagocyte-derived NADPH oxidase [22]. The disease mechanism is highly complex because even when an infection is successfully eliminated, increased production of proinflammatory cytokines, deficient secretion of anti-inflammatory mediators by activated neutrophils, and delayed apoptosis of inflammatory cells often result in a sterile, chronic, granulomatous inflammation. In the past, the transplantation of autologous, gene-modified cells in CGD patients has resulted in poor long-term engraftment. In light of these results, the choice between gene therapy and allogeneic HSCT to treat CGD is still a question of debate.

Several explanations for these problems have been proposed [23]. First, the lack of standard or reduced-intensity conditioning before transplantation of the autologous gene-modified HSPCs may restrict engraftment. Second, the transduced cells do not have a selective advantage. Third, Weisser et al. recently showed that hematopoiesis is dysregulated in patients with CGD [24]. Bone marrow from CGD patients contains a low proportion of primary HSCs and myeloid progenitor cells—even after mobilization with granulocyte colony-stimulating factor (G-CSF). These deficiencies are directly linked to the inflammatory state. Inflammatory signals lead to elevated nuclear factor Kβ activity, transcription of proinflammatory cytokines, and inflammasome activation, and thus further caspase-1-dependent secretion of the proinflammatory cytokines IL-18 and IL-1β. Proinflammatory cytokines (known to induce the proliferation of HSCs and the skewness of HSC toward the myeloid lineage) impair the capacity of transduced HSPCs to engraft and survive over the long term. This link between chronic inflammation, impaired hematopoiesis, and possible engraftment failure has already been reported in mice with mutations in interferon gamma receptor genes and in patients with progressing HIV disease [25,26].

Taken as a whole, the literature data provide us with a rationale for (1) treating CGD patients with anti-inflammatory drugs before HSC harvesting and (2) collecting large numbers of $CD34^+$ cells, to circumvent the low HSC count. It remains to be seen whether these changes will improve the outcomes—especially the long-term engraftment of gene-corrected cells—of ongoing or future gene therapy trials in the clinic.

Wiskott-Aldrich Syndrome

WAS is an X-linked, inherited immunodeficiency characterized by the association of recurrent infections, thrombocytopenia, eczema, and a high risk of lymphoid malignancy and autoimmune disease. Mutations in the *WAS* gene are responsible for the disease, and result in defective or absent expression of the WAS protein (WASp) and, ultimately, a loss of function. Expression of WASp is restricted to hematopoietic cells. WASp is a key multi-adapter protein involved in the transduction of signals from a broad range of membrane receptors to the actin cytoskeleton. Interaction between incoming signals and specific WASp domains results in induction of the actin polarization required for directed motility, adhesion, and phagocytosis. Studies of HSCs from WAS patients have shown that the disease mechanism is related to dysregulation of the actin cytoskeleton in response to stimuli [27–29]. WASp-deficient B lymphocytes are impaired in their ability to migrate, adhere, and form long protrusions [30]. In immature, WASp-deficient dendritic cells, podosomes are absent, residual dysmorphic lamellipodia and filopodia are not polarized, and migration is severely compromised [31,32]. Furthermore, T cells from WAS patients contain few, small surface microvilli and respond poorly to stroma cell-derived factor-1 (SDF-1) in migration assays [33] and to antigen receptor-induced stimulation [34]. Taken as a whole, the data suggest that a defect in cell migration and membrane motility is the common denominator in this complex immunodeficiency [32]. The alteration of B cell homeostasis concerns both the central and peripheral compartments, and it has recently been described in detail [35]. As mentioned above for RAG SCID patients, we observed over-representation of the bone marrow B cell precursor cell population in patients with WAS, which might affect the transduction procedure (unpublished results). Hence, it might be useful to either deplete this compartment before harvesting or (if this is not technically feasible) infuse a greater quantity (8–9 million cells/kg) of transduced $CD34^+$ HSPCs.

Lacout et al. [36] revealed that WAS is even more complex than first suspected; they used three different approaches to demonstrate that WASp-deficient HSPCs (and not only mature WASp-deficient hematopoietic cells) have impaired migratory and homing capacities. In particular, they showed that WASp-deficient $CD34^+$ cells display a twofold decrease in the SDF-1 chemotactic response and in their ability to repopulate secondary recipients. These characteristics may explain the skewed X-chromosomal inactivation pattern reported in female carriers of WAS, and also agree with observations of skewed X-chromosome inactivation in marrow $CD34^+$ cells. Two groups have already speculated that this a consequence of a reduction in homing capacity of the WASp-deficient HSCs during ontogeny; indeed, this has now been demonstrated in a murine model of WAS [36,37]. It is not known to what extent this homing impairment is responsible for the variable myeloid cell and platelet engraftment reported in WAS gene therapy trials [38]; this issue is under investigation. Intra-bone injection might be of value in this setting [39].

Beside the biased composition and the migratory defects of WASP HSPCs, this impairment in cytoskeleton function and membrane characteristics is probably also responsible for the very low recovery of WAS-deficient HSCs after cryopreservation (Hacein-Bey-Abina, unpublished results). The transduced cells must therefore be transplanted immediately after the end of the in vitro procedure. Furthermore, the duration of the conditioning regimen must be shortened accordingly, which influences the choice of the regimen.

Up to now, only 10 patients have been treated by gene therapy using a lentivirus strategy and the follow-up is still limited. It is thus very difficult at this stage of development to draw any conclusion on the superiority of gene therapy over allogeneic HSCT. All the parameters described above should be taken into account in ongoing clinical trials, given that the goal is to increase the engraftment of transduced HSPCs.

Hemoglobinopathies

β-thalassemia is an inherited autosomal hemoglobinopathy in which β-globin chain synthesis is absent (β0 thalassemia) or reduced (β+ thalassemia) in erythroid cells. The imbalance of alpha- and beta-globin chain synthesis is responsible for the accumulation of aberrant free alpha-globin chains, which form highly toxic aggregates in erythroid progenitors and red blood cells (RBCs). Gene therapy has been tested in 10 patients. While limited, the preliminary results of gene therapy for β-thalassemia appear in favor of this approach when no human leucocyte antigen-genoidentical donor is available. However, there is room for improvement.

In β-thalassemia, anemia is due to both peripheral hemolysis and the bone marrow's impaired ability to produce terminally differentiated erythrocytes—a defect referred as dyserythropoiesis or ineffective erythropoiesis. The key steps in dyserythropoiesis have been now well characterized in vitro and in vivo [40–42]. The bone marrow of patients with β-thalassemia is characterized by (1) accelerated erythroid differentiation, (2) a maturation block at the polychromatophilic stage, and (3) elevated death of erythroid precursors [41]. The first consequence of dyserythropoiesis is the accumulation of erythroid progenitors; the bone marrow of patients suffering from β-thalassemia contains five to six times more erythroid precursors (primarily basophilic and polychromatophilic erythroblasts) than normal. The highly altered composition of HSPCs in this disease explains the initial failure of patients' bone marrow to provide an appropriate HSC harvest and thus the requirement for mobilization for gene transfer strategies [43,44]. The optimum regimen for restoring the balance between bone marrow HSPCs before harvesting has not been yet determined, and research on this topic is essential.

Even more problematic is sickle cell disease (SCD), where the bone marrow alterations described below are combined with a systemic endothelial dysfunction and chronic activation, which might influence the homeostasis

and egress of $CD34^+$ cells [45]. Furthermore, some studies have highlighted the mechanisms of dyserythropoiesis in SCD. The ferrokinetic measurement of erythropoiesis and the ultrastructural study of a bone marrow aspirate have revealed the presence of erythroid hyperplasia, an abnormally low reticulocyte response, the presence of hemoglobin S polymers in reticulocytes, sickling of nucleated erythroblasts, and extensive marrow erythrophagocytosis [46,47]. Circulating immature erythrocytes in peripheral blood samples from SCD patients show substantial annexin-V staining, suggesting abnormally elevated apoptotic activity in the context of SCD [48,49]. The best evidence of dyserythropoiesis in SCD has come from studies of allotransplanted SCD patients. The results suggested that just 10% of donor chimerism may be enough to reduce the symptoms associated with severe SCD [50]. The development of mixed hematopoietic chimerism in SCD patients following nonmyeloablative transplants has been used by Wu et al. as a model system for the side-by-side comparison of recipient hemoglobin S (SS) and donor heterozygous hemoglobin S/hemoglobin A (SA) erythropoiesis in vivo [51]. Direct in vivo evidence showed that ineffective erythropoiesis in patients with SCD occurs even earlier in erythroid development than first realized [50–52]. Since mature erythrocytes can be fully replaced by relatively low numbers of total donor-derived mononuclear cells, these studies strongly support the hypothesis whereby ineffective erythropoiesis is an important disease mechanism in SCD.

The particular sedimentation properties of RBCs from patients with SCD interfere with white blood cell collections and strongly affect the yield of recovery of $CD34^+$ cells. The combination of ineffective erythropoiesis and sedimentation anomalies explains why two to three bone marrow harvests are needed to collect enough HSPCs in SCD patients. It is noteworthy that vaso-occlusive crises (potentially leading to acute thoracic syndrome, multi-organ failure, and death) have been reported following attempts to mobilize $CD34^+$ with G-CSF in patients with SCD. This growth factor is therefore strongly contraindicated in patients with SCD [53–56]. Hence, there is an urgent need for protocols that reduce bone marrow dyserythropoiesis, increase the efficacy and safely of HSC mobilization, and optimize isolation of the mononuclear cell compartment (limiting the loss of $CD34^+$ HSPCs). All these modifications could help ensure the transplantation of an optimal number of gene-corrected HSPCs in SCD patients, with a view to achieving a sustained and complete cure for this disease.

Conclusions

In view of the above examples, it is clear that the optimization of gene therapy requires better characterization or identification of the features of bone marrow homeostasis in disease settings. Recent progress has been achieved in the harvesting and expansion of healthy HSPCs [57]. Although this progress is of great value, caution is required when translating these findings into a diseased HSPC setting [58]. Furthermore, cord blood cells (often used as a source of healthy HSPCs) do not have exactly the same biological characteristics as their adult counterparts, and HSPCs derived from children under the age of 10 behave differently in culture [59]. The GMP-standard industrial production of gene-corrected $CD34^+$ HSPCs is not feasible unless we cannot correct their homeostasis and state in each disease setting. A few well-performed clinical studies of patients' HSPCs should be undertaken before gene therapy trials are initiated. Although this approach might be feasible in adult patients, it is highly debatable in children. The patients' inflammatory state is an important biological parameter; it must always be evaluated and treated on a case-by-case basis before HSPC harvesting. In this respect, CGD and SCD are very particular settings with specific challenges. Close collaboration between fundamental research and clinical research is still essential in this complex field.

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