CHAPTER 15

First human gene therapy trial for haemoglobin disorders

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Summary

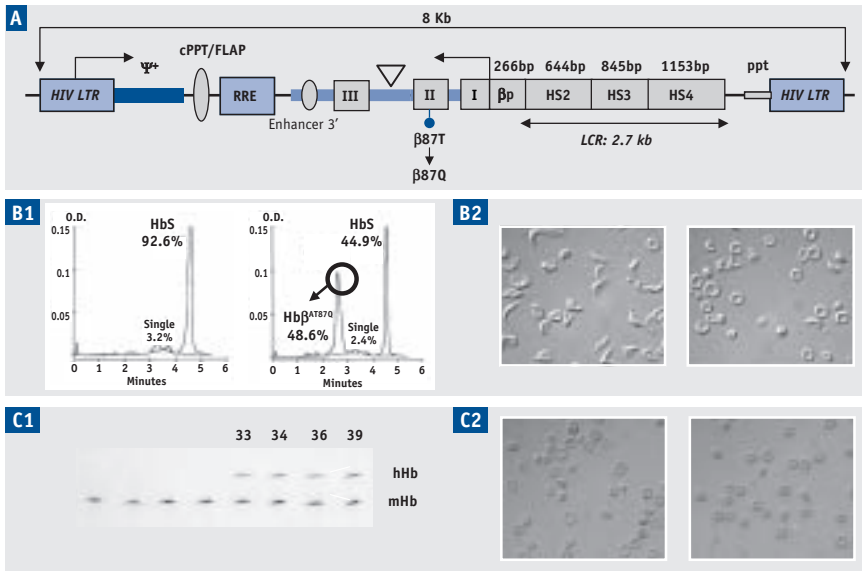
Patients suffering from β-thalassaemia major or severe sickle cell anaemia can be cured by haematopoietic stem cell (HSC) transplantation from HLA-identical familial donors (1). However, the absence of suitable donors for 70-80% of patients, severe complications related to transplantation (increasing with age), and also access and cost limit the use of this cell therapy. For these reasons, *ex vivo* gene therapy has been developed. The approach used involves modifying autologous HSCs with integrating viral vectors containing a therapeutic globin transgene which is specifically expressed in erythroblasts. We and others have demonstrated proof of principle for this approach in mouse models of β-thalassaemia and sickle cell anaemia (2-4), and are now developing a phase I/II clinical trial (5). Clinical results obtained twenty months after gene therapy (GT) with the lenti-globin (LG) vector are very encouraging and open new perspectives. Oncogenic risks related to vector insertion observed in previous clinical trials (6) have been reduced by using: **1**. a self-inactivated (SIN) lentiviral vector; **2.** chromatin-insulating sequences flanking the integrated vector and replacing the viral promoter and enhancer regions; and **3.** an internal erythroid-specific globin promoter and enhancer elements of the globin locus control region (LCR).

Selection and expansion of transduced cells are major future goals to reduce or, if possible, abolish the conditioning required for autologous transplantation. If this can be achieved and if the insertional oncogenesis appears to be minimal, globin gene therapy will be available for all patients including young children with severe disease, ideally using genetically modified HSCs derived from their own cord blood. Alternatively, induced pluripotent stem cells (iPS cells) could be genetically corrected by homologous recombination, and could be used to reduce the risk of insertional mutagenesis. This method has been successful for curing a mouse model of sickle cell anaemia (7).

1. Preclinical studies

The proof of principle and the demonstration of effectiveness *in vivo* of globin gene therapy were obtained in 2000-2002 by using lentiviral vectors derived from HIV-1 (Figure 1A). The expression cassette consists of the β-globin gene (including introns) driven by the β-globin promoter and activated by core regions of the DNAse I hypersensitive sites II, III and IV of the β-globin locus control region (LCR). The threonine codon at position 87 was replaced by a glutamine codon which is present in γ- and δ-globin chains and is thought to be responsible for the inhibition of polymerisation of sickle haemoglobin HbS by foetal (HbF) and adult (HbA_2) haemoglobins. β-thalassaemic and sickle cell mice were myeloablated by a lethal

Figure 1: **Preclinical globin gene therapy studies**



A. Diagram of the human β-globin (β^{A-T87Q}) lentiviral vector (LentiGlobin™, LG). *The 3' β-globin enhancer, the 372bp IVS2 deletion, the β^{A-T87Q} mutation (ACA[Thr] to CAG[Gln]) and DNase I Hypersensitive Sites (HS) 2, HS3 and HS4 of the human β-globin Locus Control Region (LCR) are indicated. HIV LTR, human immunodeficiency type-1 virus long terminal repeat; ψ^{+}, packaging signal; cPPT/flap, central polypurine tract; RRE, Rev-responsive element; βp, human β-globin promoter; ppt, polypurine tract.* ***B. Sickle cell mouse model.*** *B1. HPLC profiles of haemoglobins in mice transplanted with mock- (left) or LG- (right) transduced HSC. B2. Anti-sickling effect of Hbβ^{A-T87Q} in erythrocytes of a mouse transplanted with LG- (right) transduced cells compared to that in erythrocytes of a mouse transplanted with mock- (left) transduced HSC.* ***C. Thalassaemic mouse model.*** *C1. Isoelectrofocusing gel showing the percentage (indicated on top) of haemoglobin that is the transgenic human protein (hHb). C2. Phenotype improvement of erythrocytes from a mouse transplanted with LG- (right) transduced cells compared to that of erythrocytes of a mouse transplanted with mock- (left) transduced HSC.*

dose of irradiation and then grafted with autologous bone marrow cells genetically modified by *ex vivo* incubation with the LG vector. The therapeutic haemoglobin was stably produced in all treated mice, and made up 30-48% of all haemoglobin in both sickle cell mice and β-thalassaemic mice (Figure 1B1 and 1C1). The human transgenic globin was detected in virtually all red cells (not shown). Consequently, the anaemia and specific erythrocyte phenotypes were corrected (Figure 1B2 and 1C2). Secondary transplants demonstrated that long-term repopulating stem cells had been transduced.

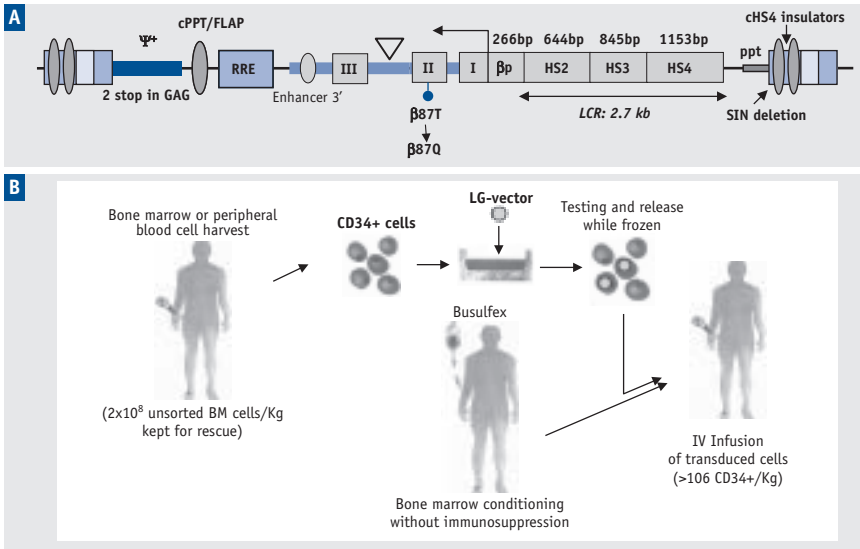
The vector used in mouse experiments (Figure 1A) has been modified in several ways for human gene therapy: i. two potential initiation codons in the 3' part of the packaging signal that overlaps with the beginning of the GAG gene have been mutated into stop codons to prevent translation of GAG-related peptides, ii. both the viral enhancer and the promoter elements have been deleted (400 bp) from the U3 region of the right LTR to generate a SIN vector, and iii. tandem copies of the insulator composed of the chicken β-globin locus DNase I hypersensitive site 4 (core of 250bp) have been inserted into the right LTR U3 region to protect the cells against cis-activation of adjacent genes by enhancer(s) present in the vector. After duplication following reverse transcription the integrated vector is flanked on both sides by insulators. Long-term toxicology studies were performed in primary and secondary transplants, in β-thalassaemic and pre-erythroleukaemic mouse models. The vector was also produced and processed under GMP conditions and tested for efficacy and safety in β-thalassaemic mice following pretransplant conditioning with busulfan. Apart from spleen and liver changes associated with resolution of the β-thalassaemic condition in β-thalassaemic recipients of LG-transduced marrow, we found no remarkable pathology on autopsy or histological examination on the day of sacrifice.

2. The first gene therapy trial for haemoglobin disorders

The primary objective of the phase I/II *ex vivo* gene therapy trial (Figure 2), designed for the treatment of severe β-globin disorders, is to determine the tolerance and the safety of the therapy. The secondary objectives are to evaluate the proportion of genetically modified nucleated blood cells, the expression of the therapeutic haemoglobin in blood, and the potential haematological and clinical benefits. Candidates for this experimental *ex vivo* globin gene therapy must have a severe β-globin disorder, thalassaemia major or severe sickle cell anaemia. They must be eligible for stem cell transplantation but without an HLA-identical donor in the family. They have to be free of chronic viral (HIV, HBV, HCV or other) infections. Patients with β-thalassaemia major are eligible if they receive frequent blood transfusions (> 100 mL of packed erythrocytes/kg/year), and have liver evaluation, portal fibrosis and iron chelation of Lucarelli's class I or II (8), with no major complication of iron overload. Sickle cell patients must have the most severe form of the disease either requiring an intensive transfusion regimen for stroke or other complications or having repetitive acute chest syndrome or severe sickle cell crisis with several hospitalisations every year (9), and show no benefit from hydroxycarbamide (hydroxyurea) treatment. Each patient is used as his/her own control, by comparing two year periods, before and after therapy.

Once patients (or the parents for children) have signed the informed consent, they are evaluated for all inclusion and exclusion criteria. Liver biopsy, sperm preservation

Figure 2: **Summary of the clinical trial**



A. Structure of the LG-vector after integration into chromatin *(see figure 1 for legends).* ***B. Overview of the clinical protocol.***

or testis or ovary biopsy are performed. Bone marrow cells (under general anaesthesia) for sickle cell patients or mobilised HSCs for thalassaemic patients are obtained. One sample of the cells is frozen without further manipulation, as a back-up. From the other sample, CD34+ cells are isolated and incubated with cytokines; then the therapeutic vector preparation of clinical grade (GMP) is added and the sample (now called the gene therapy product or GTP) is frozen in liquid nitrogen. Aliquots are used for safety and efficacy controls. The patient is hospitalised in a cell therapy unit, in a sterile environment for bone marrow suppression by treatment with a conditioning agent (Busulfex®) for several days. The GTP is thawed and the cells washed and intravenously infused into the patient. The pathogen-free environment and supportive care are maintained until blood neutrophil counts are above 500/µL. Various markers are tested at intervals during the 2 year follow up, after which the patients are evaluated annually.

The first thalassaemia patient treated, at 28 years of age, experienced a period of aplasia that lasted longer than expected. Although no adverse event occurred, untransduced cells were infused out of caution to avoid infectious and haemorrhagic

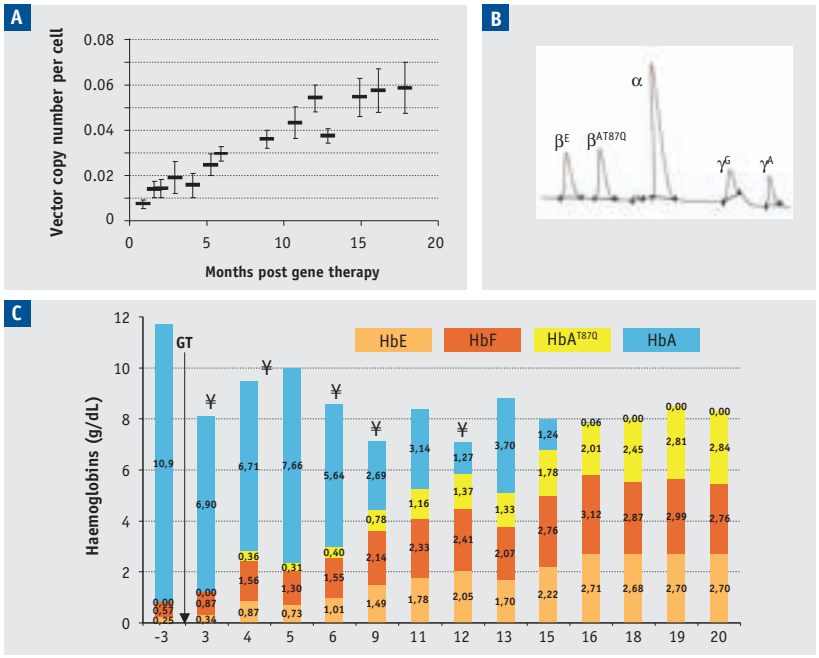
complications during this prolonged high risk period. The increased duration of aplasia was probably related to the limited number of infused haematopoietic cells, genetically modified or not, caused by a change in the quality of the manufactured plastic cell culture bags that was not detected before cell infusion. Autologous cells may have also been affected by the 28 year duration of iron overload, bone marrow alterations associated with thalassaemia major and the *ex vivo* manipulation of cells. Under these conditions of massive dilution with untransduced back-up cells, LG-modified cells were only transiently detected in the peripheral blood as well as the therapeutic haemoglobin in reticulocytes.

The first patient with severe thalassaemia who underwent a complete procedure without injection of back-up cells was given the globin gene therapy at age 18 years. He suffered from severe anaemia due to the β^E/β^0 thalassaemia genotype requiring regular transfusions (about 160 mL of packed erythrocytes/kg/year) from the age of 3 years. Attempts to replace transfusions by hydroxycarbamide (hydroxyurea: Hydrea®) were unsuccessful and there was no suitable familial donor for HSC transplantation. He received $4x10^6$ CD34+ cells/kg. Twenty months after gene therapy, the patient did not suffer any side effects associated with the procedure as a whole. The proportion of LG-modified blood nucleated cells rose progressively (Figure 3A) to 6% after 12 months (assuming not more than one copy of the integrated vector per cell). The requirement for transfusion declined progressively and transfusions were no longer necessary after 12 months. The concentration of therapeutic haemoglobin in blood reached 2.8 g/dL (1/3 of total haemoglobin) 7 months after the last transfusion (19 months after GT) (Figure 3B and 3C).

Anaemia was stabilised (Hb between 8 and 8.5g/dL) following transfusion but the persistent high reticulocyte counts (not shown) indicate that the genetically modified erythroid cells compensated only partially for the deficits of red cells and haemoglobin. The proportion of therapeutic β-globin chain in reticulocytes was low and variable (2-8% of total Hb). Most probably, therapeutic β-globin was distributed among only a small proportion of reticulocytes. The large increase in the proportion of the therapeutic Hb in blood (33% of total Hb) and the low proportion in reticulocytes (mean 3%) suggests that the β-globin-containing reticulocytes generated red cells with an increased survival, probably close to normal life expectancy of healthy red cells. The abundance of foetal Hb (HbF) in reticulocytes increased transiently following patient conditioning and decreased during the recovery from aplasia. Before gene therapy, when the patient was hypertransfused, the proportion of HbF in reticulocytes was 26%, rising to 57% in reticulocytes 2 months after the GT procedure and subsequently progressively decreasing around 20%, 6 months after the last transfusion and 18 months post gene therapy.

Vector-bearing cells were not uniformly distributed in all cell types: they made up

Figure 3: **Vector copy number and globin expression**



A. *Mean +/- SD of vector copy number per cell in peripheral blood.* ***B.*** *Reverse-phase HPLC elution profile of globin chains twenty months after GTP infusion.* ***C.*** *Specific and total haemoglobin concentrations (g/dL) with time (in months) after gene therapy (GT). The last transfusion (¥) was performed 12 months post-GT.*

8-12% of granulocytes, 9-13% of early erythroid progenitors, 2-4% of circulating erythroblasts, 6% of B lymphocytes and less than 1% of T lymphocytes. There appeared to be a weak selection for modified common myeloid cells over lymphoid progenitors; alternatively, B lymphocytes may just have a lower turnover than myeloid cells. The low proportion of genetically modified T lymphocytes is very likely due to the fact that cyclophosphamide was not used for conditioning. Busulphan alone is not believed to kill T cells. The reason for the low proportion of circulating erythroblasts carrying the vector as compared to early erythroid progenitors is not known. Interestingly, the proportion of modified early erythroid progenitors (as detected by PCR analysis of colonies) was similar to the proportion of colonies producing the therapeutic haemoglobin (determined by HPLC) suggesting negligible

silencing (position effect variegation) of the therapeutic transgene, protected by the chromatin insulator flanking the integrated vector.

At the most recent follow-up examination, blood and bone marrow cells from this patient had normal morphology other than the thalassaemic features remaining in a large proportion of cells. Importantly, the patient reported good wellbeing. His life is transformed and he is free from doctors, transfusions and frequent blood tests. He is able to perform his full time job without fatigue. Life-long follow-up with periodic evaluation will be required to determine the stability of the corrected phenotype, the long-term survival of repopulating HSCs and oncogenic safety.

In summary, this historical patient, who underwent *ex vivo* globin gene transfer for a frequent and severe genetic disease provides the proof of principle of this therapeutic approach. In particular, the case demonstrates that large amounts of a therapeutic protein (β-globin) can be produced *in vivo* (84 g/L of red cells which is close to the amount of globin produced by the expression of one normal endogenous β gene) and that production can be limited to a single appropriate lineage of cells and differentiation stage by use of a tissue specific transcription system (erythroid promoter and globin LCR) involving a single copy of a "full" gene (containing introns). The case also validates somatic gene transfer using a lentiviral SIN vector with chromatin insulators for transducing long-term repopulating haematopoietic stem cells. It demonstrates that somatic gene transfer, *ex vivo*, can provide transfusion independence for a patient with severe HbE/β^0 thalassaemia disease, the most frequent β-thalassaemia genotype in the world.

For the most severe thalassaemic patients to be included in this trial, we propose to double the proportion of transduced erythroid progenitors to 20%, so as to provide more than 5g of therapeutic Hb/dL of blood, as this should be sufficient to improve the β^0/β^0 thalassaemia major severe phenotype similar to the improvement associated with a stable 20% chimerism provided by an allogeneic haematopoietic transplantation (10).

3. Gene therapy for sickle cell patients

For sickle cell patients, the therapeutic globin gene β^{AT87Q} has been designed to inhibit HbS polymer formation (4). This construct is based on site-directed mutagenesis to replace threonine (T) with glutamine (Q) at position 87 of the β-globin chain. There is a Q residue in this position in the gamma chain of HbF and in the delta chain of HbA_2, known to provide both the main inhibitory effect on HbS polymer formation by HbF and HbA_2, respectively and their antisickling properties. We expect that the presence of 60% modified circulating red cells will greatly improve the severity of sickle cell disease.

Accordingly, 10-20% of reticulocytes expressing the therapeutic haemoglobin will be required. Substantially clinical improvements have been obtained with similar levels of chimerism following allogeneic stem cell transplantation. This initial trial will evaluate our working hypothesis.

4. Oncogenic safety

Insertional oncogenesis was of great concern following the first case of leukaemia, in August 2002, during the successful SCID-X1 gene therapy trial performed at Necker hospital in Paris (11). To prevent the activation of a cellular proto-oncogene in different cell types, the vector has been modified in various ways: **1.** the murine γ retroviral vector has been replaced with a lentiviral vector; **2.** the viral promoter and enhancer have been deleted from the long terminal repeat of the virus; **3.** a chromatin insulator has been added; **4.** the copy number of the vector used has been reduced to one per transduced cell; **5.** the use of a late erythroid-specific promoter and enhancer; **6.** the use of a non oncogenic therapeutic gene, the β-globin gene; and **7.** the avoidance of selective advantage of modified HSCs over unmodified cells. Analyses of oncogenic safety in two appropriate mouse models did not show any oncogenic risk for the LG vector.

However, during integration of the vector into open chromatin there is the theoretical risk of disrupting a site responsible for the repression of a potential cellular oncogene or abolishing the expression of a tumour suppressor gene. Insertion of the vector into a sequence repressing a gene that increases the proliferation of haematopoietic cells could theoretically provide an advantage to genetically modified cells. Furthermore, the erythroid-specific promoter used in this trial may activate proto-oncogenes in erythroid cells or supply a proliferative advantage to those cells if the chromatin insulator is "leaky". Finally, recent studies have shown that genes encoding micro RNAs (miRNAs) constitute around 4% of the genome and that miRNAs inhibit the post transcriptional expression of many genes coding for proteins. Disrupting one miR gene may therefore result in increased production of one or several oncogenes (12). Integration site analysis, gene expression profile and long-term follow up will be necessary to evaluate the safety of the whole procedure.

5. Perspectives for therapy by globin gene addition

To reduce, and if possible, to abolish the need for cytotoxic conditioning of the patient before autologous transplantation, there are two major directions of intensive exploration: **i.** the *ex vivo* selection of transduced cells so that transplants contain a majority of corrected cells (13) and **ii.** the expansion of the modified cells. Expansion can theoretically be done *ex vivo* (14) to increase the number of modified

HSC or *in vivo*, to expand the number of erythroid cells expressing the therapeutic haemoglobin. In both cases the expansion must be "benign" without oncogenic risk and transient or under a tight control.

These improvements will be made available within the next few years and will have to be tested first in large animals, primates if possible; subsequently it will be possible to evaluate the partial conditioning of the patients. In case of positive results, out-patient gene therapy should be possible and complications, such as sterility or an increased risk of cancer, will be substantially reduced. In addition, the cost of the overall procedure should decline. These expected developments may make it possible to treat very young patients, soon after the appearance of severity factors of their disease, and before the appearance of complications. The development of cord blood banking for patients with severe Hb disorders would be ideal to provide a non-invasive source of autologous stem cells.

6. Alternative to therapy by globin gene addition: homologous recombination in iPS cells

Homologous recombination between exogenous and genomic DNA, *i.e.* gene targeting, would provide means to correct mutant genes in the context of their physiological regulation and may circumvent problems associated with insertional mutagenesis. However, a drawback of this concept is the inefficiency of the methods available to transfect and to modify large numbers of primary cells by homologous recombination. Consequently, this approach was until recently irrelevant for genetic haematopoietic disorders. The discovery of iPS cells, derived from adult differentiated tissues, and capable of proliferation and differentiation into HSC has partially solved this problem (15). Indeed, iPS cells derived from skin fibroblasts of adult sickle mice have been obtained and corrected for the HbS mutation with a normal β-globin DNA. Genetically restored iPS cells were selected and guided in culture to form HSCs, in turn used successfully as HSC therapy for mouse sickle cell anaemia (7). However, non specific integration of the correcting DNA, generation of teratoma (a landmark of iPS cells) and oncogenesis are major concerns and currently limit considerably the use of this approach. Further work is warranted, in particular to ensure safety.

Aknowledgements

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