Sequence-specific modification of a β-thalassemia locus by small DNA fragments in human erythroid progenitor cells

Gene therapy has been proposed as a definitive cure for β-thalassemia. We applied a gene targeting approach, based on the introduction of small DNA fragments (SDF) into erythroid progenitor cells, to specifically modify the β-globin gene sequence at codon 39. The strategy was first tested in normal individuals by delivering mutant SDF that were able to produce the β39 (C→T) mutation. Secondly, wild-type SDF were electroporated into target cells of β39/β39. β-thalassemic patients to correct the endogenous mutation. In both cases, gene modification was assayed by allele-specific polymerase chain reaction of DNA and mRNA, by restriction fragment length polymorphism analysis and by direct sequencing.

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β-thalassemia is an autosomal recessive form of chronic anemia and can be caused by over 200 mutations in the β-globin gene (*HBB*). Since blood transfusions and bone marrow transplantation are limited by shortage of compatible donors and by significant costs and risks, efforts have been focused on the development of alternative clinical treatment, including gene therapy. So far, conventional gene therapy approaches, based on virus-mediated delivery of functional copies of the gene, have been relatively unsuccessful.[1] In contrast, gene targeting methodologies that repair the mutant gene *in situ* seem particularly attractive since theoretically they allow appropriate expression of the target gene, which remains in its native chromosomal environment.[2] In this study, we used a gene targeting approach that employed small DNA fragments (SDF) to directly modify several disease-causing loci.[3-7] We evaluated this approach in terms of its effectiveness in introducing or eliminating the most common Italian *HBB* mutation (β^{39} C→T) in primary erythroid progenitor cells of normal or β-thalassemic individuals, respectively. Differentiation into erythroid progenitor cells was allowed to light-density mononuclear cells (MNC) and enriched hematopoietic $CD34^+$ cells, separated from umbilical cord blood or peripheral blood of normal donors, and from peripheral blood or bone-marrow of β-thalassemic patients, as described elsewhere.[8] Two different SDF of 517-bp and 190-bp were generated by polymerase chain reaction (PCR) using intronic primers and genomic DNA from normal subjects (wild type fragment) or from homozygous β^{39}/β^{39} patients (mutant fragment).

To evaluate the optimal method of intracellular delivery of exogenous DNA into the erythroid progenitor cells, the pEGFP reporter plasmid (Clontech) was used to compare chemical (Lipofectamine, SuperFect) and physical (electroporation) systems assessed by flow cytometric analysis. While both Lipofectamine and Superfect were relatively ineffective at delivering the reporter plasmid to this subset of hematopoietic cells, electroporation (using BTX ECM 830 apparatus) was more efficient with a transfection efficiency of about 10-12%. However, the transfection efficiency decreased to levels below 0.6% when fluoroscein isothiocyanate (FITC)-labeled SDF were electroporated into the target cells. Initially, semi-nested PCR were carried out on gel-purified genomic DNA and cDNA isolated from erythroid progenitor cells

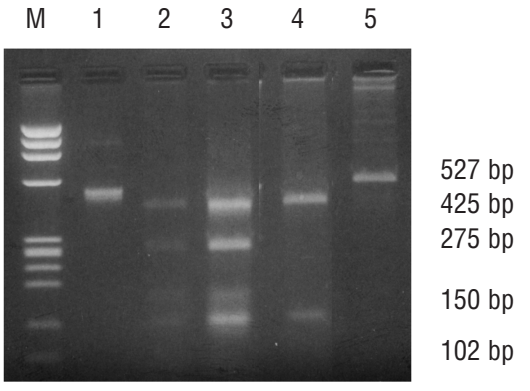

Figure 1. Figure 1. BfaI digestion analysis of PCR products of genomic DNA from normal individuals following the SFHR strategy. BfaI digestion analysis of PCR products of genomic DNA of normal (+/+) cells following SFHR strategy. M=ΦX/HaeIII, molecular marker. 1: undigested PCR product of normal (+/+) cells electroporated with SDF; 2: BfaI-digested PCR product of normal (+/+) cells electroporated with SDF; 3: BfaI-digested PCR product of a heterozygous subject (β39/+); 4: BfaI-digested PCR product of normal (+/+) cells electroporated without SDF; 5: undigested PCR product of normal (+/+) cells electroporated without SDF. 527 bp: undigested product using R37 and R47 primers; 425 bp and 102 bp: BfaI digested products of normal cells; 425 bp, 275 bp, 150 bp and 102 bp: BfaI digested products of heterozygous cells.

from normal donors, using primers external to the fragments (HB3/HB4 for DNA and HB5/HB6 for cDNA), and then allele-specific primers (B39N or B39M). Mutant SDF were able to introduce the β^{39} mutation both at DNA and mRNA levels. Direct sequence analysis of PCR products also showed a heterozygous C to T transition in all samples (*data not shown*). Because the mutation creates a novel *Bfa*I restriction site, site-specific modification could also be assayed by restriction fragment length polymorphism (RFLP) analysis of DNA, showing the appearance of the heterozygous additional bands (275- and 150-bp) after digestion of 527-bp PCR products (Figure 1). To obtain an approximate quantification of the frequency of targeted cells in this model system, we cloned semi-nested PCR products into pGEM vector (Promega) and then screened 123 individual bacterial colonies by allele-specific PCR. The detection of three mutant colonies carrying the β^{39} sequence suggested that the efficiency of short-fragment homologous replacement (SFHR)-mediated modification was approximately 2.4%. Secondly, erythroid precursor cells, expanded from peripheral blood and bone marrow of homozygous patients, were electroporated with wild-type fragments. Targeted correction was detected in eight of ten different experiments, as determined by allele-specific amplification performed on both genomic DNA and mRNA (Figure 2). Direct sequence analysis carried out on positive PCR samples showed the presence of both wild-type and β39 sequences.

Different gene targeting strategies have already shown that site-specific correction of a single point mutation in the *HBB* gene can be achieved, but with variable efficiencies.[7,9] In this study, successful correction was routinely observed when 10^6 cells were electroporated with 1 or 2 μg of 517bp SDF. When the number of cells or the amount of fragment was reduced, no sequence-specific modification was detected, indicating that the ratio of SDF/cellular target is a fundamental factor for successful gene repair.[7] Molecular analysis showed that both the 517- and 190-bp long SDF were able to mediate se-

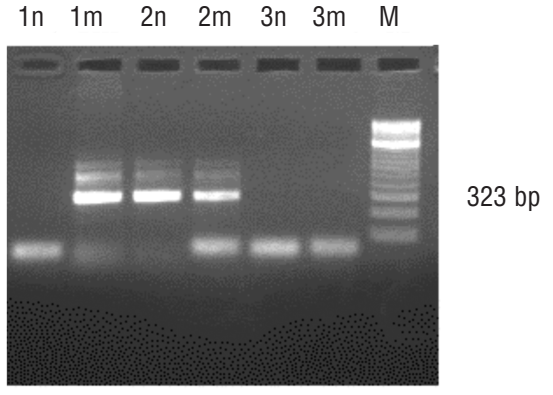

Figure 2. Allele-specific PCR on cDNA from β-thalassemic (β39/β39) patients following the SFHR strategy. The primers used in 1n, 2n, and 3n (HB5 +β39N) are specific for the normal sequence. The primers used in 1m β2m, and 3m (HB5+β39M) are specific for the β39 sequence. 1n-1m: β-thalassemic (β39/β39) cells electroporated without SDF; 2n-2m: β-thalassemic (β39/β39) cells electroporated with SDF; 3n-3m: total RNA of β-thalassemic (β39/β39) cells electroporated with SDF, without reverse transcriptase. M: 100 bp, molecular marker.

quence-specific nucleotide exchange of DNA and mRNA in primary erythroid progenitor cells. The origin of the target cells did not appear to be relevant to the success of the experiments, although samples obtained from bone marrow generally showed better results. The possibility of PCR artifacts, due to residual contaminating SDF, was ruled out using gel-purified genomic DNA, DNase-digested RNA and external primers for PCR analysis, as recently suggested.[7,10] Unfortunately, the number of corrected cells remaining from each experiment was insufficient to carry out protein studies by MALDI-TOF analysis. More importantly, improvements in delivery approaches and/or design of SDF are clearly required to yield sufficient quantities of corrected cells for a significant therapeutic benefit.

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Key words: gene targeting, gene therapy, β-thalassemia, HBB gene.

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Appendix

Sequences of the primers used for the analyses:
HB1L (5'-CACTAGCAACCTCAAACAGAC-3')
HB2L (5'-CCATAGAAAAGAAGGGGAAAG-3') used to generate the 517-bp fragment.
HB1S (5'- TCTGATAGGCACTGACTCTCT-3')
HB2S (5'-AGCCAGGCCATCACTAAAGG-3') used to generate the 190-bp fragment.
HB3 (5'-ATAAAAGTCAGGGCAGAGCCA-3')
HB4 (5'-CCACACTGATGCAATCATTCG-3') used for the first PCR on genomic DNA.
HB 5 (5'-CATTTGCTTCTGACACAACTG-3')
HB6 (5'-AAAGTGATGGGCCAGCACAC-3') used for the first PCR on cDNA
B39N (5'-CAGATCCCCAAAGGACTCAAAGAACCT-GTG-3', normal allele)
B39M (5'- CAGATCCCCAAAGGACTCAAAGAACCT-GTA-3', β-thal allele)
R37 (5'-CCAATCTACTCCCAGGAGCA-3')
R47 (5'-CACTCAGTGTGGCAAAGGTG-3') used for the *Bfa*I digestion.

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