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.

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. 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. In this study, we used a gene targeting approach that employed small DNA fragments (SDF) to directly modify several disease-causing loci. We evaluated this approach in terms of its effectiveness in introducing or eliminating the most common Italian HBB mutation (β39 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 βthalassemic patients, as described elsewhere. Two different SDF of 517-bp and 190-bp were generated by polymerase chain reaction (PCR) using intron primers and genomic DNA from normal subjects (wild type fragment) or from homozygous β39/β39 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, seminested PCR were carried out on gel-purified genomic DNA and cDNA isolated from erythroid progenitor cells from normal donors, using primers external to the fragments (HBB/HB4 for DNA and HB5/HB6 for cDNA), and then allele-specific primers (B39N or B39M). Mutant SDF were able to introduce the β39 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 BfaI 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 seminested PCR products into pGEM vector (Promega) and screened 123 individual bacterial colonies by allele-specific PCR. The detection of three mutant colonies carrying the β39 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. In this study, successful correction was routinely observed when 106 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. 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 + B39M) are specific for the normal sequence. The primers used in 1m, 2m, and 3m (HB5 + B39N) are specific for the β-thalassemic (β39/β39) sequence. 1n-1m: β-thalassemic (β39/β39) cells electroporated with SDF; 1n-2m: total RNA of β-thalassemic (β39/β39) cells electroporated with SDF; without reverse transcriptase. M: 100 bp, molecular marker.

Appendix

Sequences of the primers used for the analyses:

HB1L (5’-CCTAGCCTAATCAAAGAGC-3’)

HB2L (5’-CCATGAGAGAGGGGAAGG-3’) used for the first PCR on genomic DNA.

HB3 (5’-TCTGATAGGACCTGACTCTTCT-3’)

HB2S (5’-AGCCAGGCCATCCTAAGG-3’) used to generate the 190-bp fragment.

HB4 (5’-GCCACTGATCACCTCATTG-3’) used for the first PCR on cDNA.

B39N (5’-CAGATCCCAAGGACTCAAAGACCTG-3’, normal allele)

B39M (5’-CAGATCCCCAAAGGACTCAAAGACCTGTA-3’, β-thal allele)

References


