Correction of the sickle cell mutation in embryonic stem cells

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Sickle cell anemia is one of the most common genetic disorders worldwide. Patients often suffer from anemia, painful crises, infections, strokes, and cardiopulmonary complications. Although current management has improved the quality of life and survival of patients, cure can be achieved only with bone marrow transplantation when histocompatible donors are available. The ES cell technology suggests that a therapeutic cloning approach may be feasible for treatment of this disease. Using a transgenic/knockout sickle cell anemia mouse model, which harbors 240 kb of human DNA sequences containing the βS-globin gene, we prepared ES cells from blastocysts that had the sickle cell anemia genotype and carried out homologous recombination with DNA constructs that contained the βA-globin gene. We obtained ES cells in which the βS was corrected to the βA sequence. Hematopoietic cells differentiated from these ES cells produced both hemoglobin A and hemoglobin S. This approach can be applied to human ES cells to correct the sickle mutation as well as β-thalassemia mutations.

Correction of the Sickel Mutation

Sickle cell anemia, one of the most common single-gene disorders worldwide, primarily affects people in Africa, the Mediterranean area, the Middle East, and the Indian subcontinent (1). In the United States, the gene frequency among African Americans is ≈0.08, and hence ≈1 in 600 births may be affected by sickle cell anemia (2). The severity of sickle cell anemia varies from a mild clinical course to severe anemia with frequent painful crises, infections, strokes, and cardiopulmonary and renal complications (3). Treatment consists of prevention of infection, blood transfusion for severe anemia and stroke, and treatment of complications. Although the search for antisickling agents is ongoing, no clinically effective agent has yet been found; compounds that increase fetal hemoglobin to inhibit sickling are also being sought (4, 5). The use of hydroxyurea has decreased the frequency of crises and hospitalization and increased the fetal hemoglobin level in some patients (6). At present, sickle cell anemia can be cured by bone marrow transplantation when there is a histocompatible donor (7). However, only a small fraction of patients in the United States have suitable donors (8). The use of cord blood stem cells for transplant has somewhat extended the donor pool (9). However, the management of a group of patients with severe disease is still limited to supportive treatments.

The introduction of ES cell technology suggests that a therapeutic cloning approach can be investigated for the treatment of genetic disorders such as sickle cell anemia. ES cells can be differentiated into hematopoietic cells for transplant into the mouse to cure the immunodeficiency.

Theoretically, such an approach can be used to treat sickle cell anemia for those clinically severe patients who do not have histocompatible donors for transplantation. Skin or other nucleated cells can be cultured from patients, and the nuclei can be transferred to oocytes from donors to make ES cells. The mutation in the βG-globin gene in these ES cells can then be corrected by homologous recombination, and the cells can be differentiated into hematopoietic cells for transplant into the patients.

The availability of mouse models for sickle cell anemia can provide a test for such an approach to treat this disease. There are several mouse models of sickle cell anemia, all carrying the human α-, βS-, and γ-globin transgenes and knockouts of the endogenous mouse α- and βG-globin genes (11–13). Although some of the models were made by injecting truncated βG-globin gene complex under the control of the locus control region (LCR), the one that we have made carries a βG-globin transgene within a 240-kb yeast artificial chromosome that contains the LCR and the α-, 3γ-, 4γ-, δ-, and βA-globin genes in their native context. Therefore, the ES cells from this sickle cell anemia mouse are likely to have the chromatin structure at the βG-globin gene region that resembles that of the human. Hence, this mouse may offer an ideal model to test homologous recombination in ES cells to convert the βG-globin sequence from βS to βA. This model may also be used as a test for the ES cell approach for the treatment of β-thalassemia, because similar corrections are applicable to many of the β-thalassemia mutations. In this study, we made ES cells from the sickle cell anemia mouse, corrected the βS mutation to the normal βA sequence by homologous recombination, differentiated the ES cells to hematopoietic cells, and demonstrated that the corrected ES cells synthesized hemoglobin A as well as hemoglobin S.

Results

Generation of an ES Cell Line That Carries the Sickle Cell Anemia Genotype. The sickle cell anemia mouse line carrying a yeast artificial chromosome containing 240 kb of human βG-globin gene cluster was used in these experiments (13). Female mice carrying homozygous or heterozygous mouse α-globin, heterozygous mouse βG-globin gene knockouts, and homozygous human α- and βG-globin yeast artificial chromosome transgenes were mated with male mice with the same genotype. Blastocysts were isolated and embryonic stem cell lines were prepared according to the standard procedure. We isolated 129 blastocysts and generated 12 ES cell lines from them. The genotypes of the ES cell lines were identified by Southern blot analysis using digoxigenin-labeled mouse α- and mouse βG-globin genes as well as by using human α- and human γ-globin genes as probes (Fig. 1 and 2).

Conflict of interest statement: No conflicts declared.

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We detected the genotypes expected from the mating pairs in these 12 ES cell lines. Among the 12, 10 showed the presence of some mouse α- and β-globin genes. Two cell lines, clone 96 and clone 106, contained complete knockouts of the mouse α- and the mouse β-globin genes and were homozygous for the human α- and βS-globin genes. Thus they had the same genotype of the sickle cell anemia mouse with which we started. We selected ES cell line 96 for subsequent targeting and in vitro differentiation experiments.

**Table 1. Genotypes of ES clones isolated from total of 129 blastocysts**

<table>
<thead>
<tr>
<th>Genotype of ES cell clone</th>
<th>No. of ES clones</th>
<th>ES clone nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>mα mβ hα hβS</td>
<td>3</td>
<td>17, 61, 108</td>
</tr>
<tr>
<td>+/- +/- +/- +/-</td>
<td>1</td>
<td>90</td>
</tr>
<tr>
<td>+/- +/- +/- +/-</td>
<td>1</td>
<td>86</td>
</tr>
<tr>
<td>+/- +/- +/- +/-</td>
<td>5</td>
<td>72, 73, 84, 97, 100</td>
</tr>
<tr>
<td>+/- +/- +/- +/-</td>
<td>2</td>
<td>96, 106</td>
</tr>
</tbody>
</table>

**Identification of Homologous Recombinants.** Gene targeting with the positive/negative selection scheme was used to facilitate homologous recombination (14). Because the sickle cell anemia mice already harbored the neomycin-resistance gene in the mouse α-globin knockout locus, we chose the hygromycin resistance gene as the positive selection marker. We further made use of the cre/lox system by inserting the loxP site on each arm of the targeting construct and flanking the drug-resistance gene.

**Fig. 1.** Genotyping of ES cell clones by Southern blot analysis using mouse (m) α- and β-globin gene probes (A) and human (h) α- and γ-globin gene probes (B). ko, knockout; wt, wild type.

**Fig. 2.** Homologous recombination in ES cells prepared from the sickle cell anemia mice. (A) Targeting construct 1 and 2 and the βS genomic structure. The β-globin gene and flanking sequence of 5.9 kb and 5.0 kb is shown with the three exons indicated by three black boxes for the βS gene and the first exon indicated by the stippled box for the βA gene. The positive selection marker, the hygromycin-resistance gene (Hygr), is flanked by the loxP site (hatched boxes). The negative selectable marker, herpes simplex virus thymidine kinase gene (hsvTK), is at the 3′ end of the constructs. The box Pr is a 400-bp probe used for Southern blot analysis. (B) Two types of recombinants resulting from homologous recombination depending on the site of crossing-over: one correcting βS to βA, the other not correcting βS to βA. (C) Southern blot analysis of DNA of the parental line 96 (P) and the drug-resistant clones (indicated by the numbers). With construct 1, EcoRV digestion produces a 15-kb genomic βS fragment and a 9-kb recombinant fragment. With construct 2, PvuII digestion produces a 12-kb genomic βS and a 14-kb recombinant fragment. The 5-kb band A is the result of star activity of the enzyme PvuII, possibly due to low ionic strength, high pH, or high enzyme concentration in the incubation condition (22).
upstream from the mycin-resistance gene was inserted into the HpaI site 0.8 kb recombination experiments (Fig. 2). Selection in homologous recombination in ES cells. We made between 5.0 kb containing the gene was inserted into the AvrII site after the 3 sequence and 5.9 kb containing the gene was inserted into the HpaI site 0.8 kb upstream from the β^6 codon between 3.3 kb of the 5′ noncoding sequence and 5.9 kb containing the β^6-globin gene. Because we were concerned that the hygromycin-resistance gene might interrupt some yet-unknown control elements at the upstream region, we made construct 2, in which the hygromycin-resistance gene was inserted into the AvrII site after the 3′ enhancer between 5.0 kb containing the β^6-globin gene and 2.5 kb of the 3′ noncoding region.

The ES cell clone 96 was electroporated with DNA of construct 1 or 2 and subjected to selection for hygromycin and ganciclovir. By using a genomic probe outside of the targeting construct, we identified the DNA of the resistant clones with EcoRV yielded a 9-kb recombinant band as well as the 1.4-kb untargeted A DNA would generate a 3.2-kb band instead of a 3.4-kb β^5 band with construct 1 because of the insertion of the hygromycin-resistance gene between these two Bsu36I sites. Of the eight recombinant clones targeted with construct 1, seven gave the 3.2-kb β^5 in addition to the 1.4-kb β^5 band and were therefore corrected from β^5 to β^5 in one allele (Fig. 3). With construct 2, the corrected β^A DNA would generate a 1.2-kb β^A band instead of the 1.4-kb β^5 band. One of the four recombinant clones gave the 1.2-kb in addition to the 1.4-kb β^5 band and therefore was corrected from β^5 to β^A. The higher frequency of correction by construct 1 than by construct 2 appears to agree with the notion that the frequency of crossing-over at the β^6 position increases when the selectable marker is placed closer to the mutation site in construct 1 (0.8 kb) than in construct 2 (2.4 kb).

Hemoglobin A and S Synthesis in Hematopoietic Cells Differentiated from the Correctly Targeted ES Cells. The parental ES cell line 96 and the recombinant clones were cultured in vitro and differentiated into hematopoietic cells by using the two-step differentiation protocol (16, 17). After 14-day culture in the secondary differentiation medium, colonies of erythroid, lymphoid, or mixed cells were seen. Cells in the colonies were displayed on two slides and stained with either the anti-hemoglobin A or the anti-hemoglobin S monoclonal antibody (Fig. 4). The parental cell line 96 was stained positive for hemoglobin S and negative for hemoglobin A, as were the red cells from the peripheral blood of a sickle cell anemia mouse. Of the seven clones corrected with construct 1, five expressed hemoglobin A and S, whereas two expressed only hemoglobin S. Presumably, some rearrangements of the gene at crossing-over might have inhibited β-globin gene expression in these two clones. With construct 2, clone 41, the only one with the corrected sequence, expressed region of the 5.9 kb or the 5.0 kb containing the β-globin gene. The conversion of the β^5 to the β^A sequence during recombination depended on the site of crossing-over. If it occurred before the β^5 codon, the β^5 would remain uncorrected, but if it occurred at or after the β^5 codon, β^5 would be corrected to β^A. To determine if the crossover had corrected the β^5 to the β^5 sequence, the DNAs of the targeted clones were digested with Bsu36I, which cleaves the β^A but not the β^5 sequence at the β^5 position. The corrected β^A DNA would generate a 3.2-kb band instead of a 3.4-kb β^5 band with construct 1 because of the insertion of the hygromycin-resistance gene between these two Bsu36I sites. Of the eight recombinant clones targeted with construct 1, seven gave the 3.2-kb β^5 in addition to the 1.4-kb β^5 band and were therefore corrected from β^5 to β^5 in one allele (Fig. 3). With construct 2, the corrected β^A DNA would generate a 1.2-kb β^A band instead of the 1.4-kb β^5 band. One of the four recombinant clones gave the 1.2-kb in addition to the 1.4-kb β^5 band and therefore was corrected from β^5 to β^A. The higher frequency of correction by construct 1 than by construct 2 appears to agree with the notion that the frequency of crossing-over at the β^6 position increases when the selectable marker is placed closer to the mutation site in construct 1 (0.8 kb) than in construct 2 (2.4 kb).

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hemoglobin S. Corrected recombinant ES cells produced hemoglobin A and the majority of the hematopoietic cells differentiated from the both hemoglobin A and hemoglobin S (Table 2). Hence, the anti-hemoglobin S antibody was used.

For hemoglobin A. Negative indicates no primary anti-hemoglobin A or serves as control for hemoglobin S, and blood from a human serves as a control for hemoglobin A and hemoglobin S. Blood from the sickle cell anemia mouse producing hemoglobin A; 1-6, 1-34, and 2-41, corrected clones producing both ES cells. 96, parental ES cells; 1-7, uncorrected recombinant clone not producing hemoglobin A and hemoglobin S. Blood from the sickle cell anemia mouse. It would be possible to use the hematopoietic cells differentiated from these cells to transplant into recipient sickle cell anemia mice because they have the same genotype. If this technique is to be applied to human diseases, it would be necessary to avoid immune rejection by nuclear transfer. Fibroblasts or other cells could be derived from the patients and their nuclei could be transferred into enucleated oocytes from normal donors. ES cells would then be prepared from them. If ES cells could be prepared from human oocytes after nuclear transfer, homologous recombination with the constructs we describe here could be used to correct the sickle mutation. The possibility of differentiation of ES cells to hematopoietic cells for transplantation to cure the Rag2-deficient mouse also makes it likely that this approach may well be successful in treating sickle cell anemia (10).

The sickle cell anemia mouse that we used had 240 kb of human sequences in the β-globin gene region integrated into the mouse chromosome. Hence, the chromosome conformation in this region is likely to resemble that in human ES cells. Also, the two targeting constructs that we have made can be used to correct mutations of the β-globin gene in human ES cells in diseases other than sickle cell anemia; i.e., they can be applied to correct mutations in β-thalassemia as well. Construct 1 will favor correction of mutations in the promoter region, the first exon, and the first intron, and construct 2 will favor correction of mutations in the third exon and the second intron. Both constructs could be tested with mutations in between.

In summary, we have shown that it is possible to correct the mutation in sickle cell anemia in ES cells that contains 240 kb of human DNA sequence. This approach can potentially be applied to human ES cells to correct mutations in the β-globin gene in sickle cell anemia and β-thalassemia.

Materials and Methods

Generation of ES Cell Line. Mice homozygous for human α- and β-thalassemia transgenes, heterozygous for mouse α-globin, and heterozygous for mouse β-globin gene knockouts were mated. ES cell lines were isolated according to the standard protocol (18, 19). Briefly, blastocysts were harvested from 3.5-day-old embryos and placed individually on mitomycin-treated primary
targeted clones were identified by Southern blot analysis using medium and expanded, and DNA was extracted from them. Colonies were picked after 2 weeks of culture in the selective construct 2, in which the hygromycin-resistance gene was inserted after the 3’ position without interfering with globin gene expression. Because the sequences 5’ to the β-globin gene contain elements that control gene expression, we inserted the hygromycin-resistance gene in construct 1 at the HpaII site 0.8 kb 5’ to the ββ position, flanked 5’ by 3.3 kb of homologous noncoding sequences and 3’ by 5.9 kb containing the β-globin gene. Because this location of the hygromycin-resistance gene might still disrupt some as-yet-undefined control elements in the upstream region, we made construct 2, in which the hygromycin-resistance gene was inserted after the 3’ enhancer of the β-globin gene, at the AvrII site 2.4 kb 3’ to the ββ codon. The two arms were 5.0 kb containing the β-globin 5’ and 2.5 kb of homologous noncoding sequence 3’ to the hygromycin-resistance gene, which was also flanked by loxP sites (Fig. 2 A).

Gene Targeting and Identification of Homologous Recombinants. Gene targeting in the ES cells carrying the sickle cell genotype was carried out as described in ref. 13. Briefly, 20 μg of construct was linearized at the NotI site and electroporated into 3 × 10⁶ ES cells in 0.8 ml of Hepes-buffered saline in a 0.4-cm gap cuvette with a single pulse of 240 V and 125 μF in a Gene Pulser (Bio-Rad). Hygromycin (150 μg/ml) and ganciclovir (2 μM final concentration) were added 24 h after electroporation. Resistant colonies were picked after 2 weeks of culture in the selective medium and expanded, and DNA was extracted from them. Targeted clones were identified by Southern blot analysis using a 5’ probe upstream from the sequence present in the targeting constructs.

Identification of the ES Cell Clones Corrected from ββ to βA. To identify whether the crossover had corrected the ββ to the βA sequence, the DNAs of the targeted clones were digested with Bsu36I, which cleaves the βA but not the ββ sequence at the ββ position. The corrected βA DNA would generate a 3.2-kb band instead of a 3.4-kb ββ band in construct 1 and a 1.2-kb βA band instead of a 1.4-kb ββ band in construct 2 (Fig. 3).

In Vitro Hematopoietic Differentiation of ES Cells. ES cell lines carrying the sickle cell anemia or corrected βA genotype were differentiated into hematopoietic cells in vitro by using the two-step differentiation procedure in semisolid methylcellulose-based medium according to the manufacturer’s recommendation (StemCell Technologies, Vancouver). In the first step, single ES cells were suspended in methylcellulose-based medium for 10 days to promote their primary differentiation into embryoid bodies (EBs). In the second step, EBs were disrupted by collagenase and single cells were replated in methylcellulose-based medium containing erythropoietin, mouse IL-3, mouse IL-6, and stem cell factor. After 10–14 days of incubation, colonies were counted, harvested, washed with PBS, and immunostained.

Immunostaining of Hemoglobins. Hematopoietic colonies from in vitro differentiation were collected in PBS and cytospun onto glass slides, followed by fixation with 100% methanol for 20 min at room temperature. The cells were rehydrated in PBS and sequentially incubated in blocking solution of PBS containing 5% skim milk, PBS containing 10% goat serum, and PBS containing 0.5% human γ-globulin and 1 mM EDTA. The cells were then stained with the primary monoclonal antibodies, anti-hemoglobin A, or anti-hemoglobin S (PerkinElmer) for 40 min at 37°C, washed three times with PBS, and incubated with Alexa Fluor 488- or Alexa Fluor 555-conjugated secondary anti-mouse antibodies (Molecular Probes) in PBS for 30 min at 37°C. The cells were then washed three times in PBS, mounted with mounting medium with DAPI (Vector Laboratories), and observed under fluorescence microscopy.

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