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Research Article

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Effect of Human β^{s} -Globin Chains on Cellular Properties of Red Cells from β -Thalassemic Mice

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Abstract

The transgenic mouse system provides an in vivo setting in which to examine the effects on mouse red cells of hemoglobin genes that have been genetically introduced into the animals' genome. In this report, we have analyzed the cellular properties of red cells from homozygous β -thalassemic mice (Hbb^{th-1}/Hbb^{th-1}), homozygous β -thalassemic transgenic mice containing a human β -sickle (β^{*}) gene (Hbb^{th-1}/Hbb^{th-1} + β^{*}), and normal animals. The presence of human β^* -globin chains in red cells from the Hbb^{th-1}/Hbb^{th-1} + β^{s} transgenic animals was noted to have a significant effect on cellular deformability and density distribution, as well as on the degree of anemia in these animals. We conclude from these studies that red cell deformability and density distribution is a sensitive means for assessing at the cellular level the effects of globin genes genetically introduced into whole organisms. In addition, these studies suggest that small decreases in the amount of excess α -globin chains can significantly ameliorate the severity of anemia in the β -thalassemic mouse.

Introduction

The introduction of foreign globin genes into hematopoietic precursor cells provides a means to study the effect of defined globin chains on red cells, as well as to investigate experimental approaches to gene therapy. Two methods have been used to introduce β -globin genes into mouse hematopoietic precursor cells: microinjection of globin sequences into early mouse embryos (transgenic mouse system) (1–4), and infection of mouse bone marrow cells with retroviruses containing globin gene sequences (5–7). Both methods have resulted in the expression of the foreign β -globin gene in the red cells of the mice, albeit the former method at a much higher level than the latter. Because of the relatively high level of tissue-specific expression of foreign β -globin sequences in transgenic mice, these animals, though not a model for gene therapy, are ex-

tremely useful for studying the effect of foreign globin chains on the cellular properties of red cells.

The recent discovery of a murine form of β -thalassemia (8) has added to the usefulness of the transgenic mouse system for investigating the effects of foreign β -globin chains on abnormal red cells. Normal mice of the Hbb^d haplotype produce two β -globin chains, β -major (β^{maj}) and β -minor (β^{min}). Murine β thalassemia results from a spontaneous deletion of the mouse β^{maj} -globin gene (8, 9). Mice homozygous for this mutation make only the β^{min} -globin chain and exhibit clinical signs of β thalassemia, while heterozygous animals are clinically normal. We have previously used the α - to β -globin chain imbalance that exists in these animals in an effort to develop a transgenic mouse model for sickle-cell anemia (10). In that study, β -thalassemic mice were crossed with β^{s} -containing transgenic mice in an attempt to increase the ratio of human β^{s} to the endogenous mouse β -globin chains present in the mouse red cell.

In this study we have analyzed cellular properties of red cells derived from Hbb^{th-1}/Hbb^{th-1} mice, Hbb^{th-1}/Hbb^{th-1} + β^{s} transgenic mice, and normal animals. Results of deformability, density, and hematological studies indicate that the α/β -globin chain imbalance in red cells of thalassemic animals significantly affects all these parameters. The presence of hemoglobin molecules containing human β^{s} -globin chains, making up $\sim 10\%$ of the total hemoglobin in mouse red cells, did not induce sickling of these cells upon deoxygenation. Surprisingly however, this level of human β^{s} expression was sufficient to significantly improve the cellular abnormalities of thalassemic red cells, as well as to decrease the severity of anemia.

Methods

Experimental animals. The Hbb^{th-1}/Hbb^{th-1} mice (8) were the kind gift of Dr. Raymond Popp at the Oak Ridge National Laboratory, Oak Ridge, TN. Mice homozygous for the Hbb^{th-1} allele and also containing the human β^{s} gene were made by crossing β^{s} -containing transgenic mice with Hbb^{th-1}/Hbb^{th-1} animals, selecting the transgenic offspring, and backcrossing them with Hbb^{th-1}/Hbb^{th-1} mice (10). The normal Hbb^d/Hbb^d control animals were BALB/c mice (obtained from The Jackson Laboratories, Bar Harbor, ME).

Genotyping. The transgenic human β^{s} -containing offspring were identified by the presence of human β^{s} -globin sequences in DNA isolated from a 1-cm tail segment. Polymerase chain reaction (PCR) amplification (11) was used to identify the human sequences. Two 19-nucleotide primers bracketing a 294-basepair fragment of the human β^{s} -globin gene from nucleotide 36 through the first exon to nucleotide 117 of the first intervening sequence were chosen since they selectively amplify only the human β^{s} -globin gene. The reaction mix-

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ture consisted of 50 pmol of each of the oligonucleotide primers mixed with 1 μ g of genomic mouse-tail DNA, 1.5 mM each of dATP, dTTP, dGTP, and dCTP, and 3 U of thermostable Taq polymerase (New England Biolabs, Beverly, MA). Amplification was performed for 30 cycles using a protocol described by Chehab et al. (12). From the 100- μ l volume reaction mix, 10 μ l was loaded on a 10 \times 10 \times 0.1-cm 12% polyacrylamide minigel in Tris-borate, pH 8.0 buffer, and run at 45 mA for 1 h. The gel was stained with 1 μ g/ml ethidium bromide for 5 min and the DNA band visualized under ultraviolet light.

Hemoglobin and red cell indices. Hemoglobin and red cell indices were performed on fresh heparinized mouse blood obtained by retroorbital venipuncture. Hemoglobin separation of cystamine-modified hemolysates was performed by cellulose acetate electrophoresis (13). The samples were run on soaked mylar-backed cellulose acetate plates (Titan III cellulose acetate plates; Helena Laboratories, Beaumont, TX) in a 0.025 M Tris/EDTA/borate pH 8.4 buffer for 20 min at 350 V. The plates were stained with Ponceau S. Hematocrit was measured using microhematocrit tubes that were centrifuged for 10 min at 11,500 rpm in a microhematocrit centrifuge (International Equipment, Needham Heights, MA). Red cell indices were measured using the Technicon H1 system (Technicon Instrument Corporation, Tarrytown, NY).

Cell deformability. Whole-cell deformability was measured using an ektacytometer (14, 15) (Technicon Instruments Corp., Tarrytown, NY). This device imposes a well-defined laminar shear stress field on the cells, while simultaneously monitoring the extent of cell deformation by laser diffractometry. A deformability index (DI) is obtained which is equivalent to the ellipticity of the deforming cells. To measure cell deformability, 20 μ l of a 40% red cell suspension was thoroughly mixed with 4 ml of polyvinylpyrolidone (mol wt 360,000; 4 g%, 32 centipoise at 20°C, 290 mosmol/kg, pH 7.4). The DI was recorded as a function of applied shear stress and the maximum values of DI attained (DI^{max}) were used to compare the deformability of the different samples.

Red cell density. The effect of genotype on red cell density was determined by using eight-step analytic Stractan gradients (17, 18) consisting of 1-ml fractions spanning a density range of 1.084-1.120 g/ml in equal increments. 100 μ l of packed red blood cells from various animals was layered above the gradients as a dilute suspension to fill the 17-ml tube, and the gradients were centrifuged in a SW 27 rotor (Beckman Instruments, Inc., Fullerton, CA) at 20,000 rpm for 30 min at 15°C. To quantify the red cell distribution at the various densities, the cells from each fraction were isolated, washed, and resuspended in defined volumes of cyanomethemoglobin reagent containing 0.01% Triton X-100 to ensure lysis. The percentage of cells in each density fraction was then calculated from spectrophotometric measurement of hemoglobin in each fraction, a histogram of red cell hemoglobin concentration, a histogram of red cell density data.

Results

The transgenic mice used in these studies were derived from the same founder transgenic mouse. The cloned genomic sequence originally injected in creating the founder mouse was a 4.4-kb Pst I fragment. By Southern blot analysis, (data not shown), we estimate that the founder animal and its offspring contain ~ 12 copies of human β^s gene per mouse genome. The Mendelian inheritance of the transgene and the consistency of the β^s -gene copy number among several generations of offspring (10) indicates that the human sequences have inserted into a single region of the mouse genome. The offspring of this human β^s -containing transgenic mouse had previously been crossed and backcrossed with Hbb^{th-1}/Hbb^{th-1} mice, to create a line of Hbb^{th-1}/Hbb^{th-1} + β^s animals.



Figure 1. The upper panel contains the polymerase chain reaction amplification of genomic DNA isolated from mouse tails. The synthetic oligonucleotide primers used to amplify a segment of the human β -globin gene were: 5'-GGGCTGGGCATAAAAGCTA-3' and 5'-AATAGACCAATAGGCAGAG-3'. The 294-bp fragment resulting from the amplification of DNA from four of the six mice indicates that these animals contain the human β -globin gene. The lower panel shows results of cellulose acetate hemoglobin electrophoresis of blood collected from these same six mice.

The analysis of the offspring of a typical mating between Hbb^{th-1}/Hbb^{th-1} and Hbb^{th-1}/Hbb^{th-1} + β^{s} -mice is shown in Fig. 1. The PCR amplification of mouse DNA with human β -globin-specific primers and cellulose acetate electrophoresis of blood from these same animals is shown in the upper and lower panels. As predicted, only those animals containing the human β^{s} gene (indicated by the presence of the 294 bp amplified fragment) also contained an additional hemoglobin species (labeled HbX) in their red cells. Quantitative scanning of this cellulose acetate hemoglobin electrophoresis plate demonstrated that the hybrid hemoglobin, HbX, makes up ~ 10% of the total hemoglobin produced by these animals. Previously we have shown that a similar percentage of the total β -globin chains present in these red blood cells is the human β^{s} -globin chain (10).

The effect of the human-mouse hybrid hemoglobin on red cell deformability and density was evaluated. The red cell deformability profiles of normal, thalassemic, and thalassemic/ transgenic mice are illustrated in Fig. 2. The red cells of the thalassemic mice are significantly less deformable than those from the normal mice at all values of applied shear stress (mean maximum DI of 0.11 versus 0.52 for normal). The magnitude of the reduction in cellular deformability seen for the thalassemic mouse red cells is similar to that seen for human red cells obtained from individuals with β -thalassemia intermedia (19, 20). In comparison with thalassemic cells, red cells from the thalassemic/transgenic mice were significantly more deformable (mean maximum DI of 0.25). Thus, the presence of human β^{s} -globin chains in the thalassemic animals' red cells results in a marked improvement in cellular deformability.

In addition to their decreased cellular deformability, another distinguishing feature of human thalassemic red cells is an altered density distribution profile, which reflects deranged cell volume regulation (13). To determine whether exogenous β -globin chains can affect the cell density distribution, density profiles of red cells from the various mice were evaluated (Fig.



Figure 2. Deformability index versus shear stress curves for red cells from normal Hbb^d/Hbb^d mice, thalassemic Hbb^{th-1}/Hbb^{th-1} mice, and thalassemic Hbb^{th-1}/Hbb^{th-1} + human β^{s} transgenic mice. Each curve is derived from an individual animal.

3). The cell hemoglobin concentration profile of normal mouse red cells was tightly distributed. In contrast, in blood of thalassemic mice, the number of red cells with cell hemoglobin concentrations in the low end of the normal range increased markedly, and a small increase in the number of red cells with high cell hemoglobin concentrations was also seen. The cell hemoglobin concentration distribution profiles of red cells from thalassemic/transgenic mice was normalized compared with the profile of the thalassemic animals. This normalization is reflected by a decrease in the number of red cells with low cell hemoglobin concentration and a subsequent narrowing of the width of the distribution profile.

The difference in red cell hemoglobin density distribution between the thalassemic and thalassemic/transgenic mice is further illustrated by a decrease in the hemoglobin concentration distribution width (HDW)¹ of 3.8 ± 0.16 versus 3.1 ± 0.07 (Table I). HDW, which is a measure of variations in individual red cell hemoglobin concentration, is derived as the standard deviation of the hemoglobin concentration histogram. It provides information equivalent to cell density distribution profiles. A smaller value of HDW indicates a narrower cell density distribution. The improvement in deformability and normalization of the density distribution profile of red cells from thalassemic/transgenic mice as compared with the thalassemic animals was also accompanied by improvements in a variety of other hematologic indices (Table I). The increased hemoglobin levels in the transgenic animals suggests lengthened survival of its red cells.

The red cell morphology in the oxygenated and deoxygenated state was examined by phase-contrast microscopy. While significant improvements in deformability indices and red cell hydration status could be induced by synthesis of small amounts of human β^{s} -globin chain in transgenic mouse red cells, no red cell sickling resulted from the presence of this globin chain following deoxygenation.



Cell hemoglobin concentration (g/dL)

Figure 3. Histograms of cell hemoglobin concentration distribution profiles of red cells from normal, thalassemic, and transgenic mice. The distribution is obtained from individual animals of the different phenotypes.

Discussion

Alterations in red cell deformability and density distribution characteristics have previously been documented in red cells from humans with β thalassemia (19, 20). The change in these parameters seen in murine red cells in this study were similar to those noted in red cells from humans with β thalassemia. These results support the authenticity of the β -thalassemic mouse red cell as a model for studying alterations in red cells of humans with β thalassemia.

Deformability characteristics of red cells are an extremely sensitive measure of membrane damage. By altering the cellular composition of mouse β -thalassemic red cells and comparing changes in the deformability of these cells, we were able to gain insights into the cellular pathobiology of this condition. The α/β synthesis ratio in the thalassemic mouse red cell is significantly greater than one (22). The introduction of human β^{s} -globin chains in the thalassemic mouse red cells was associated with a significant improvement in the deformability of these cells. An explanation for this effect is that excess α -globin chains, which likely are reduced in the thalassemic/transgenic red cell, play a major role in the severity of membrane damage. Another explanation, though less probable, is that properties of the human/mouse hybrid hemoglobin molecule are directly associated with improvements in red cell deformability.

^{1.} Abbreviation used in this paper: HDW, hemoglobin distribution width.

	Hemoglobin	Hematocrit	RBC	MCV	RDW	МСН	MCHC	HDW
	g/dl	%	×10 ⁶ /µl	ſl	· · · · · · · · · · · · · · · · · · ·	Pg	g/dl	
Normal mice								
Hbb ^d /Hbb ^d	16.3±0.3	47.4±1.0	9.8±0.2	48.0±0.2	13.2±0.2	16.4±0.1	34.1±0.2	1.95±0.01
Transgenic mice								
Hbb ^{th-1} /Hbb ^{th-1} + β^{s}	13.4±0.4	40.3±0.8	11.1±0.2	36.4±0.7	23.6±0.7	12.1±0.3	33.3±0.4	3.1±0.07
Thalassemic mice								
Hbb ^{th-1} /Hbb ^{th-1}	9.7±0.6	28.8±1.3	8.6±0.7	33.7±1.3	24.6±1.8	11.3±0.3	33.8±0.6	3.8±0.16

Table I. Red Cell Indices in Normal, Thalassemic, and Thalassemic/transgenic Mice

A distinguishing feature of human (19, 20) and, as we have shown in this study, mouse thalassemic red cells is their broad density distribution profile. The wide cell hemoglobin concentration profile is believed to reflect deranged cell volume regulation as a result of membrane oxidative damage induced by excess α -globin chains (19). Normalization of the density distribution profile of red cells from thalassemic/transgenic mice supports the hypothesis that a decrease in the number of excess α -globin chains is primarily responsible for the improvements in cellular properties of red cells from these animals.

Our original goal in constructing this line of transgenic mice was to create animals that would phenotypically mimic the human disease sickle-cell anemia. Though the murine red cells containing the human β^{s} -globin chain did not sickle, presence of this molecule significantly improved the anemia of these animals. Not only was there a normalization of red cell density and deformability, but most of the measured hematologic indices also improved. Though we have not directly measured red cell survival, the increase in both hemoglobin and hematocrit clearly imply a decrease in the hemolytic component of the disease in thalassemic/transgenic mice. Near complete hematologic corrections were observed by Costantini et al. (21) after the introduction of either the normal human β or mouse β^{maj} -globin genes into thalassemic mice. In that study, hemoglobin molecules containing β -globin chains coded for by the transgene made up over half the total hemoglobin produced by the thalassemic/transgenic animals. Although human β - β ^s-, and mouse β ^{maj}-globin chains may have different properties in mouse red cells, the partial correction of cellular and hematologic consequences of mouse β thalassemia demonstrated in this present study is significant due to the low level of β^{s} expression. It suggests that if the number of excess α -globin chains in human β -thalassemic red cells could be reduced even slightly, a lessening in the clinical severity of the disease might occur.

The transgenic animals used in this study were homozygous for the mouse β -thalassemic allele at the mouse β -globin locus, and hemizygous for the β^{s} -globin sequences at a separate unlinked chromosomal location. We are now breeding mice that are homozygous for both the mouse β -thalassemic allele and the human β^{s} -globin sequences. We expect that animals with this genotype will have increased synthesis of β^{s} -globin chains and a concomitant improvement in hematologic parameters compared with the hemizygous transgenic mice of the present study.

Red cell density distribution and membrane deformability appear to be extremely sensitive and clinically important measurements of red cell function in both mice and humans. Results from this study indicate that the analysis of these properties may be useful in the future development and evaluation of genetic approaches for treating disorders of hemoglobin synthesis.

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