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Selective erythroid replacement in murine β-thalassemia using fetal hematopoietic stem cells

(erythropoiesis/transplantation/hemoglobinopathies)

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ABSTRACT **We have explored the application of fetal hematopoietic stem cell (HSC) transplants for cellular replacement in a murine model of β-thalassemia. Liver-derived HSCs from nonthalassemic syngeneic murine fetal donors were transplanted into nonirradiated neonatal β-thalassemic recipients. Significant erythrocyte chimerism (9–27%) was demonstrated in the majority of recipients at 1 month and remained stable or increased (up to 55%) during long-term follow-up in almost all cases. Chimeras had improved phenotypes, as evidenced by decreased reticulocyte counts, increased mean erythrocyte deformability, and decreased iron deposits in comparison to controls. To investigate whether the high degree of peripheral blood chimerism was predominantly a feature of erythroid elements or was a general feature of all hematopoietic elements, chimeras were created using donor HSCs "tagged" with a DNA transgene. Whereas donor hemoglobin comprised >30% of total hemoglobin, nucleated tagged nonerythroid donor cells comprised <1% of peripheral blood elements. Explanations for the observed selective increase in erythroid chimerism include longer survival of normal donor red cells compared to that of thalassemic red cells and the effective maturation of the donor erythroid elements in the bone marrow in chimeric animals. The latter explanation bears consideration because it is consistent with the process of ineffective erythropoiesis, well documented to occur in thalassemia, in which the majority of thalassemic erythroid cells are destroyed during erythropoiesis prior to release from the bone marrow. Overall, these data demonstrate the potential for significant erythroid chimerism and suggest that fetal HSC transplantation may play a significant role in future treatment.**

Fetal-derived hematopoietic stem cells (HSCs) have been suggested as a therapeutic approach to β-thalassemia and a host of other hematopoietic deficiencies (1). There are a number of distinct advantages to the use of the fetus as a HSC donor. The high percentage of hematopoietic progenitors present early in gestation in fetal liver has been suggested to provide a rich potential source of cells that may then repopulate the recipient animal after transplantation. Evidence supporting this has been provided by studies demonstrating a significantly higher replication of fetal liver-derived stem cells than cells obtained from adult bone marrow or blood after transplantation into irradiated animals (2). The markedly decreased generation of cytotoxic T-cell responses in the fetus relative to its adult counterpart also makes the fetus an ideal donor for immunologic reasons, thereby enabling the potential transplantation of HSCs across immunologic barriers without causing an adverse immune response (3).

Fetal HSC transplants have been used for cellular replacement in W mutant (anemic) mice by Fleischman and Mintz (4). In this model, nonirradiated neonatal W mutants with varying degrees of severity of their endogenous defect have maintained long-term *chimerism*, defined by the presence of self-renewing populations of donor HSCs after transplantation. Although this model demonstrates that fetal HSCs can be used to create chimerism in nonirradiated animals with deficiencies in the development of early stem cells, its applicability to the treatment of hematopoietic disorders affecting more differentiated stem cells has not been tested.

The β-thalassemic mouse has a spontaneous mutation at the β-globin locus, and homozygotes are anemic and exhibit many hematologic manifestations of the human disease (5). This model was used to determine whether transplantation of fetal liver-derived HSCs into nonirradiated hosts could be used as a therapeutic approach for this disease. Prior attempts have been made to use stem cell transplantation to cure thalassemia in a similar model, the adult murine α-thalassemic recipient (6). In these experiments, donor cell engraftment was observed only if sublethal total body irradiation of recipients prior to transplantation was performed. We hypothesized that transplantation in the neonatal period, when marrow "space" for donor cell engraftment may still be available, would allow the achievement of significant levels of donor cell engraftment without the need for recipient marrow ablation. We also hypothesized that the increased destruction of thalassemic erythroblasts in the thalassemic bone marrow prior to completion of erythroid maturation (ineffective erythropoiesis), as well as the increased survival of normal versus thalassemic erythrocytes (RBCs), would select for and result in significant levels of circulating RBCs of donor origin in the peripheral circulation. We characterized the phenotype of the thalassemic host animals following transplantation as well as the relative contributions of donor erythroid cells and donor nucleated cells (almost exclusively nonerythroid) to peripheral blood chimerism. Our experiments demonstrated that transplants using fetal liver from normal donors indeed resulted in significant peripheral blood chimerism for donor RBCs and improvement in the phenotype of the recipient thalassemic mice. In addition, we found selective enrichment of the donor erythroid as opposed to donor nonerythroid population in the peripheral circulation.

MATERIALS AND METHODS

Mice. *Donors.* C57BL/6J nonthalassemic fetuses and C57BL/6J nonthalassemic fetuses containing the apolipoprotein A-I (apoA-I) transgene (7). All donors had the "single" hemoglobin haplotype, Hbb^s/Hbb^s.

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Abbreviations: HSC, hematopoietic stem cell; RBC, erythrocyte; apo, apolipoprotein.

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Recipients. C57BL/6J fetuses homozygous for the β-thalassemia allele ("minor" hemoglobin haplotype, Hbb^{th}/Hbb^{th}) and 129/J nonthalassemic fetuses ("diffuse" hemoglobin haplotype, Hbb^{d}/Hbb^{d}).

Donor Cell Preparation and Injection. Hysterotomy was performed on pregnant 14- to 16-day gestation donor C57BL/6J mice. The fetuses were dissected under magnification and the livers were extracted and homogenized in Iscove's modified Dulbecco's medium (GIBCO) using a Dounce homogenizer. Cells were washed three times by centrifugation for 10 min at 1500 rpm. Cell count and viability were determined using trypan blue stain (0.1%) and a suspension of 10^8 cells per ml was made. Five milliliters of tail blood taken from neonates (<12 hr old) from matings between C57BL/6J thalassemic heterozygotes (Hbb^{th}/Hbb^{s}) was screened using cystamine-modified hemoglobin electrophoresis (8). The homozygotes were injected intraperitoneally with 10^6 cells (10 ml) using 50-μm (diameter) glass micropipettes. Injection of newborn 129/J mice was performed in the same manner using the same donor cells.

Screening for Donor Hemoglobin. Three microliters of tail blood was obtained from all injected animals at 30 days and at regular intervals up to 300 days after injection. Lysis of RBCs and cystamine modification of hemoglobin was performed on all samples by adding 3 ml of blood to 3 ml of cystamine stock solution (66.7 μM cystamine dihydrochloride/1.33 μM dithioerythritol) and 20 μl of distilled H_2O (8). Hemoglobin electrophoresis was performed on cellulose acetate membranes, and membranes were scanned in a Beckman R-112 densitometer. Thalassemic homozygotes have hemoglobin containing the β^{minor} polypeptide only, whereas the C57BL/6J donors have hemoglobin containing the β^{single} protein. Heterozygous thalassemic animals contain both β^{minor} and β^{single}, as do transplanted homozygotes in which donor cell engraftment has occurred. The ratio of recipient to donor hemoglobin was calculated for each transplanted animal by densitometric scanning of gels, and the results are expressed as the percentage of total hemoglobin that is of donor origin. For subsequent studies, mice were then divided into chimeras and controls. The control group included nonchimeric thalassemic homozygotes, thalassemic heterozygotes, and normal (nonthalassemic) mice.

Red Cell Indices and Red Cell Deformability. Deformability and cell index determinations were performed on fresh heparinized blood samples obtained by retro-orbital venipuncture. Red cell indices were measured using the H-1 system (Miles Diagnostics, Tarrytown, NY) using special animal software for determining mouse red cell indices. Deformability of intact red cells was measured as described using osmotic gradient ektacytometry, an assay in which whole-cell deformability is measured as a continuous function of suspending medium osmolality according to the methods previously established. The osmolality at which the deformability index reaches a minimum in the hypotonic region of the gradient provides a measure of the average surface area-to-volume ratio of the population of red cells, whereas the hypertonic region of the curve provides information on the state of cell hydration (9, 10).

Hematocrit and Reticulocyte Counts. Hematocrit was measured using microhematocrit tubes that were centrifuged for 10 min at 11,500 rpm in a microhematocrit centrifuge (International Equipment). Reticulocytes were stained using the new methylene blue stain and their percentages were enumerated by counting at least 2000 cells (11).

Histology. Chimeric animals and controls ≈1 year old were euthanized and dissected to obtain liver and spleen. The tissues were fixed using 10% formalin in phosphate-buffered saline. Histologic evaluation of hematopoietic organs followed staining with (*i*) hematoxylin/eosin and (*ii*) iron (12).

Southern Blot Analysis. Tail DNA (5 μg) was obtained from apoA-I transgenic and nontransgenic animals and digested with restriction enzyme *Pst* I. Serial dilutions of mixtures of transgene-derived DNA to nontransgenic DNA were prepared. DNA was separated on a 1% agarose gel, depurinated, denatured, and transferred to nylon filters. The filters were hybridized with a 2.2-kb *Pst* I fragment of the human apoA-I gene, which had been ^{32}P-labeled by random priming (13). DNA obtained from the blood of chimeric animals for the determination of the amount of chimerism in the nonerythroid nucleated cell population was processed as above.

RESULTS

Demonstration of RBC Chimerism in Peripheral Blood. Injected animals and noninjected controls were screened at regular intervals over a 1-year period after injection for the presence of donor hemoglobin in peripheral blood (Fig. 1). Nine of 14 injected animals are positive for donor hemoglobin on initial screen (Fig. 2). Percentages of donor RBCs increased in all chimeras during the first 45 days after injection and continued to increase or remain stable up to 150 days after injection. Very dramatic increases in percentage of donor RBCs were observed for several animals, most noticeably during the first 1–2 months after injection. The levels of donor hemoglobin began to show a steady decline in three chimeras after 150 days. This decline continued in two chimeras in which no donor hemoglobin could be detected at 280 days. On long-term follow-up screening of the remaining six chimeras 18 months after transplantation, the percentages of donor hemoglobin were found to be maintained or increased. Chimeras a and b (Fig. 2) had 71% and 43% donor hemoglobin at 18 months (540 days), demonstrating a continued increase in their degree of chimerism. At 18 months, chimeras c–f were found to have maintained the levels of chimerism demonstrated at 280 days.

To determine whether the high degree of chimerism seen in these animals was specific for the thalassemic host, a control group of nonthalassemic animals (129/J strain) was injected with the same donor cells as used in the above experiment. No donor hemoglobin was detected in any of 13 injected animals.

Since normal and thalassemic red cells have distinct volume and hemoglobin content distributions, these parameters were assessed in red cells from chimeric and normal animals (Fig. 3). In chimeric animals, two distinct populations of red cells could be seen: a population of donor origin with a mean cell volume of 45 femtoliters (fl) and a mean cell hemoglobin content of 15 pg and a second population of recipient red cells with a mean cell volume of 30 fl and a mean hemoglobin content of 10 pg. The proportion of red cells with indices

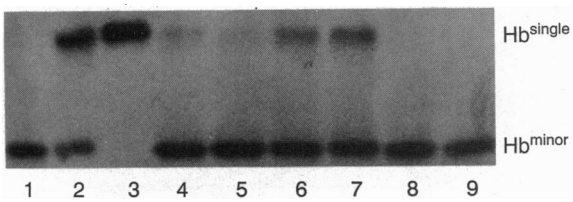

FIG. 1. Hemoglobin electrophoresis performed on peripheral blood from control and transplanted animals. The hemoglobin β^{minor} polypeptide is found in homozygous thalassemic recipients, and hemoglobin β^{single} is found in thalassemic heterozygotes and in the nonthalassemic donors. Bands 1–9 show the hemoglobin profiles of (*i*) a homozygous thalassemic control (band 1), (*ii*) a heterozygous thalassemic control (band 2), (*iii*) a nonthalassemic control (band 3), (*iv*) several homozygous thalassemic animals that demonstrate chimerism after transplantation (bands 4–7), and (*v*) two transplanted but nonchimeric homozygous thalassemic animals (bands 8 and 9).

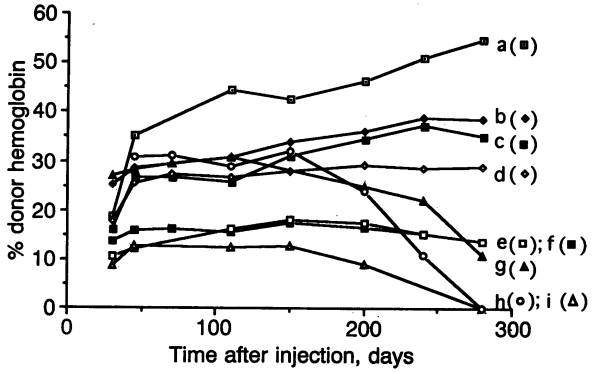

FIG. 2. Degree of chimerism determined by quantitative scanning densitometry performed on hemoglobin electrophoresis gels in nine chimeric animals over 300-day follow-up. Percentages represent the proportion of total hemoglobin that is of donor origin.

corresponding to donor cells increased with increasing chimerism as shown by hemoglobin electrophoresis.

Chimerism of Nonerythroid Cells. Donor HSCs from mice tagged with the apoA-I transgene were injected into thalassemic recipients under the same conditions as before to determine the degree of chimerism of nonerythroid elements in the peripheral circulation. The apoA-I transgene has been shown to express exclusively in the livers and testes, but not in the bone marrow, and to have no effect on hematologic parameters in transgenic mice (7). Fig. 4 shows the Southern blot analysis using the apoA-I transgene probe of two animals found to be chimeric for peripheral blood RBCs (levels were 26% and 53%). The percentage of peripheral blood cells containing the donor-derived transgene marker is estimated

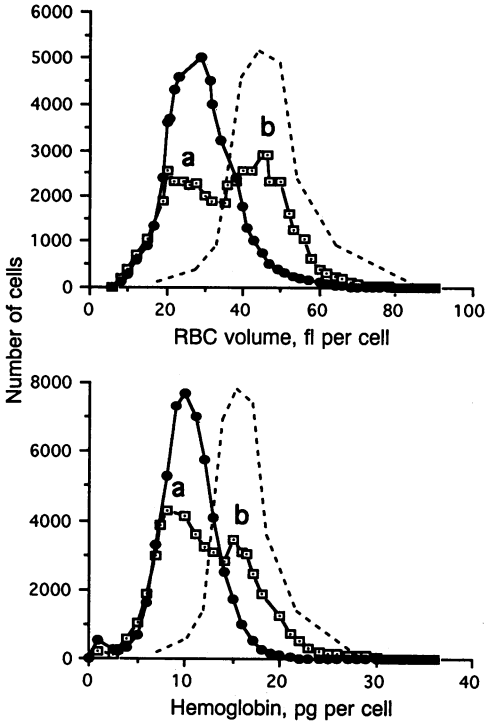

FIG. 3. RBC volume (*Upper*) and hemoglobin content (*Lower*) profiles of a control homozygous thalassemic animal (●) and a representative homozygous animal that demonstrates chimerism after transplantation (⊡). Dashed lines show volume and hemoglobin profiles of nonthalassemic controls. Profiles of the chimera have two peaks, one representing the host population of abnormal thalassemic RBCs (peak a) and the other representing the donor population of normal RBCs (peak b).

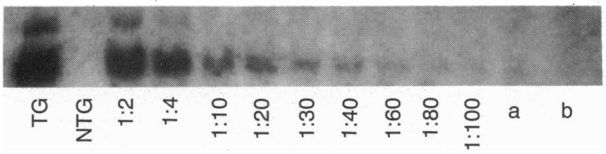

FIG. 4. Southern blot demonstrating a <1:60 ratio of donor-to-host peripheral blood nucleated cells in two chimeric animals. TG and NTG bands represent DNA from control transgenic (apoA-I-containing) and nontransgenic (non-apoA-I-containing) animals, respectively. Bands designated 1:2 through 1:100 are serial dilutions of DNA from animals containing apoA-I transgene in the background of non-transgene-containing DNA. Bands a and b represent DNA from peripheral nucleated cells in two chimeras (with 26% and 53% donor hemoglobin, respectively) that were created using donor HSCs tagged with the apoA-I transgene.

to be <1%. This marked difference in the percentage of donor-derived RBCs and the percentage of donor-derived nonerythroid cells suggest the existence of some mechanism for which the peripheral donor erythroid population is selected.

Correlation of RBC Chimerism and Phenotype. There are statistically significant differences in the reticulocyte counts between control thalassemic mice and chimeras as shown in Fig. 5. Although the reticulocyte counts of chimeric animals were decreased compared to those of thalassemic mice, the counts are still much higher than those of normal mice. This in large part could be the consequence of only partial reconstitution of the red cell mass in chimeric animals, as evidenced by less than normal values of hematocrit in these animals. (Fig. 5).

The osmotic deformability profiles of red cells from animals with a high degree of chimerism were significantly closer to those of normal red cells compared to red cells of thalassemic animals (Fig. 6). The higher the degree of chimerism, the greater was the improvement in cellular deformability.

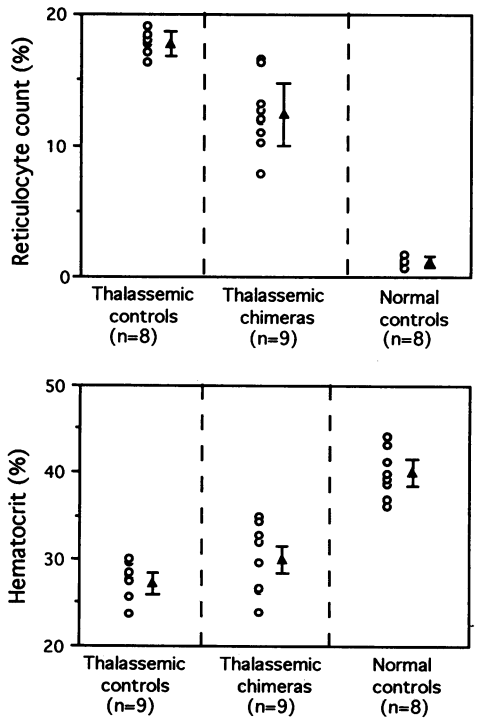

FIG. 5. Reticulocyte counts (*Upper*) and hematocrits (*Lower*) for each mouse screened (○) and mean ± SEM (⊼) for each group. The reticulocyte counts in chimeras were significantly lower than in untreated homozygous controls.

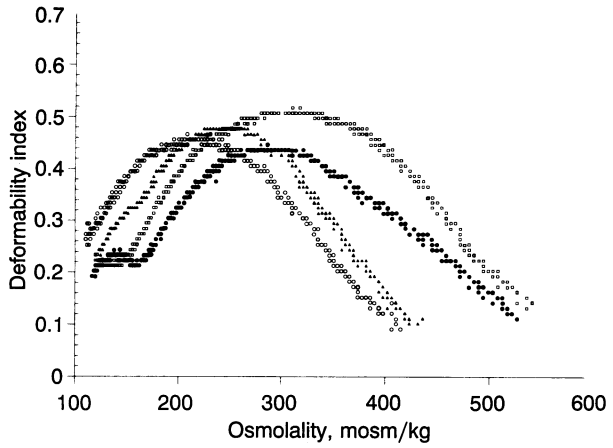

FIG. 6. Osmotic deformability profiles of RBCs in (*i*) a normal nonthalassemic animal (□), (*ii*) a homozygous thalassemic control (○), (*iii*) a chimera with 27% donor hemoglobin (●), and (*iv*) a chimera with 16% donor hemoglobin (▲). As the peripheral blood chimerism increased, the osmotic deformability profiles of red cells in these chimeric animals were closer to the normal deformability profiles. The extent of loss of deformability in the hypotonic region for red cells in chimeric animals improved as the levels of red cell chimerism increased.

Fig. 7 demonstrates the histologic evaluation of livers and spleens from chimeric and thalassemic animals. A significant decrease in the amount of iron deposits was noted in the organs of the chimeras compared to that observed in organs of thalassemic animals. In chimeric animals, the decreased iron deposition is likely to be secondary to the presence of a large population of donor-derived RBCs with a longer half-life than those of thalassemic animals. This in turn causes a decrease in the rate of turnover of the total red cell mass in chimeras and decreases the build-up of heme iron. Fig. 7 also demonstrates that the spleens of chimeric animals had decreased numbers of hematopoietic cells in comparison to the hypercellularity observed in the spleens of thalassemic controls. This suggests a significant decrease in extramedullary hematopoiesis in chimeric animals compared to thalassemic controls.

DISCUSSION

Transplants of fetal liver-derived HSCs into nonirradiated homozygous murine β-thalassemic recipients have been shown here to be capable of producing long-term chimerism as well as improvements in phenotype. The levels of engraftment in some chimeric animals approach 50–60% after 10 months of follow-up, making their hemoglobin profiles similar to those of heterozygotes. High levels of chimerism are maintained in most animals up to 18 months of age, indicating that donor RBC numbers may be sustained, and in some cases increased, over very long periods of time after transplantation. In contrast to our findings, adult α-thalassemic recipients transplanted using adult bone marrow donor cells failed to demonstrate donor cell engraftment unless sublethal total body irradiation was performed prior to transplantation (6). Therefore, the success of these transplants suggests that fetal HSC transplantation into the neonatal β-thalassemic host may provide a new approach for the treatment of this disease without the need for bone marrow ablation.

Since studies of red cell survival in the β-thalassemic murine host using ^{51}Cr-labeled erythrocytes have demonstrated that the half-life of normal RBCs is 23.5 days while that of thalassemic cells is 8 days (5), the high levels of peripheral RBC chimerism may in part be explained by the longer half-life of normal versus thalassemic RBCs. The efficient maturation of donor-derived erythroid cells in the bone marrow compared to that of host-derived erythroid cells is another factor contributing to high levels of peripheral red cell chimerism. This mechanism merits serious consideration since *ineffective erythropoiesis*, the destruction of abnormal erythroid cells prior to their release from the bone marrow, has been well documented to occur in thalassemia (14, 15). In the current model, after the transplantation of normal HSCs into the thalassemic host, the selective destruction of many host erythroid cells prior to the completion of maturation could confer an advantage to the smaller numbers of donor erythroid cells that exhibit normal maturation and efficient release of their progeny into the peripheral circulation. By contrast, we expected no selective maturational advantage to be conferred to nonerythroid donor cells since these cells mature normally in thalassemic animals. Indeed, our experiments using "tagged" donor HSCs confirmed our prediction by showing that the percentage of donor-derived erythroid cells was at least 30 times that of the donor-derived nucleated (mostly granulocytic/lymphoid) cells in the peripheral circulation and provided supportive evidence for the occurrence of ineffective erythropoiesis and for its potential contribution to the development of high levels of peripheral blood red cell chimerism. The advantage conferred to erythroid donor cells by the process of ineffective erythropoiesis may mean that

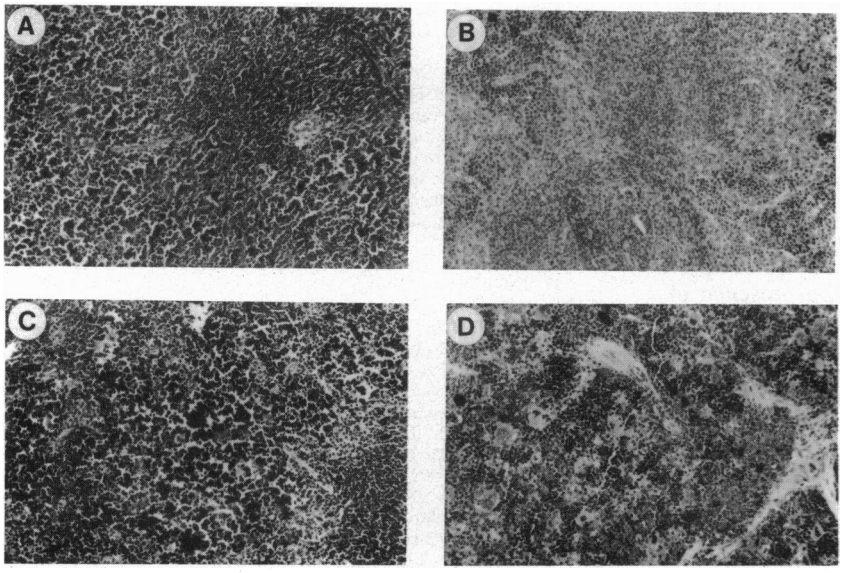

FIG. 7. Histologic evaluation of spleen in chimeras and controls. (*A*) Hematoxylin/eosin-stained preparation in a control thalassemic mouse. (*B*) Same as *A* except for a chimeric mouse. (*C*) Section of spleen in a control thalassemic animal that has been stained for iron. The iron deposits are reduced in the chimeric animal (*D*). (×160)

the level of engraftment of donor HSCs in bone marrow that is required to ameliorate anemia may be lower than for other hemoglobinopathies, such as sickle cell anemia, in which the extent of ineffective erythropoiesis is much lower.

The favorable phenotypic improvements observed after transplantation provide further encouraging evidence that fetal HSC transplantation may be used to treat β-thalassemia. The first of these favorable changes was a decrease in reticulocytosis and extramedullary hematopoiesis in chimeric animals compared to controls. Since high reticulocyte counts and extramedullary hematopoiesis occur as compensatory responses to the decreased red cell mass in thalassemic animals, the decrease in both of these parameters demonstrates that transplantation resulted in some degree of compensation for the hemolytic anemia in thalassemic animals. Further phenotypic improvements included the reduction in iron deposits after HSC transplantation. Heme iron deposition in numerous end-organs occurs as a result of the increased destruction of thalassemic RBCs, which in turn is thought to result from membrane fragility, abnormal morphology, and decreased deformability of thalassemic RBCs (16). The reduction in iron deposits seen after HSC transplantation in these experiments reflects the decrease in the percentage of abnormal thalassemic RBCs in the peripheral circulation in chimeras and represents a significant improvement in phenotype in comparison to control thalassemic animals. We have shown a direct effect of HSC transplantation on RBC characteristics by demonstrating that the mean deformability profiles of the RBC population are being normalized in chimeras. RBC rigidity has been shown to cause, in addition to early cell destruction, decreased small vessel perfusion in a number of animal models (17). The increased mean RBC deformability may therefore play a critical role in the reduction of hemolysis and the amelioration of end-organ disease.

The reasons for the failure of engraftment in some animals are unclear, but we suggest that the performance of transplants in the nonirradiated host in which a relatively small number of donor HSCs are present in the background of significantly larger numbers of host HSCs may help to account for this observation. It has been shown that donor HSCs must be given in significantly larger numbers to the nonirradiated host in comparison to the irradiated host to allow donor cell detection in the peripheral circulation (18). Perhaps in those animals that did not demonstrate engraftment after transplantation, the numbers of donor HSCs reaching the host bone marrow from the peritoneal cavity were insufficient to give rise to detectable donor lineages in the periphery. It is also important to consider why no donor erythroid cells were ever detected after transplantation into the nonthalassemic 129/J host, despite transplantation using the same numbers of donor cells under identical conditions. We suggest that the presence of the decreased RBC mass in thalassemic animals may provide a "driving force" for the proliferation and differentiation of donor erythroid progenitors, resulting in the presence of donor erythroid cells in the periphery after transplantation. This driving force would not have been present in the nonanemic 129/J host, resulting in failure to observe donor erythroid lineages in the peripheral circulation. Studies by Fleischman and Mintz (4) have provided some support for the concept that the presence of anemia may be favorable for the engraftment and proliferation of donor HSCs by demonstrating that fetal HSCs, when transplanted into W/W^v mice that have varying degrees of anemia resulted in higher levels of engraftment in animals in which the severity of the anemia was greatest.

The reasons for decreased donor hemoglobin detection after 150 days in some chimeras are also unclear. The presence of large numbers of short-term repopulating cells (STRCs) as opposed to long-term repopulating cells (LTRCs) in the donor fetal liver may be important in explaining the decrease in the levels of engraftment in a few chimeras. LTRC and STRC have been demonstrated in adult marrow. The former have been shown to provide permanent repopulation of the entire marrow after transplantation, whereas the latter disappear within 6 months (19). Since it has been demonstrated that the frequency of LTRCs is five to six times less in fetal marrow compared to adult marrow, the presence of large numbers of STRCs and fewer numbers of LTRCs in the donor fetal liver may account for the temporary engraftment in some animals (20).

Although the precise mechanisms by which erythroid chimerism is maintained in the thalassemic host require further investigation, these studies in postnatal nonirradiated recipients provide encouraging evidence for the potential therapy of β-thalassemia by fetal HSC transplantation. We suggest that this approach may be applicable to the *in utero* transplantation of HSCs using allogeneic or even xenogeneic donor cells. With the advent of first-trimester diagnosis in human subjects, a window during which fetal HSCs could be delivered to the thalassemic fetus prior to the onset of disease may become available. This would provide the advantage of allowing transplantation at a time when the bone marrow is most receptive to HSC engraftment and while the fetus remains immunotolerant of foreign antigen (21, 22).

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