Fetal Hemoglobin Expression in Transplant Recipients of Placental Blood Hematopoietic Progenitor Cells

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ABSTRACT

Patients who achieved bone marrow engraftment of cord blood-derived progenitor cells provided an opportunity to examine the expression of fetal Hb by neonatal hematopoietic progenitors in a postneonatal host. Cord blood cells from histocompatible siblings were successfully transplanted in two children with the Fanconi anemia syndrome. One of the transplant donors had heterocellular hereditary persistence of fetal Hb, apparently due to γ -globin gene triplication; the other donor was hematologically normal. The $G\gamma/A\gamma$ ratio of the patient who received his transplant from the donor with hereditary persistence of fetal Hb was markedly elevated, similar to that of the transplant donor's cord blood, and this ratio remained elevated in subsequent months. In the other child, the $G\gamma/A\gamma$ ratio immediately after her transplant was typical of the normal newborn, and over the next

several months it reverted to the adult pattern. Globin synthesis studies performed shortly after engraftment demonstrated ratios of fetal Hb/adult Hb synthesis in both patients that were typical of those of normal newborns. Over the next several months, both patients converted to the adult pattern. Fetal Hb to adult Hb switching in these patients seemed to follow a temporal sequence intrinsic to the transplanted neonatal progenitor cells, without discernible influence of postneonatal environmental factors. The program for Hb switching seems to be an inherent feature of neonatal hematopoietic progenitor cells. (*Pediatr Res* 37: 432–436, 1995)

Abbreviations

Hb F, fetal Hb

Blood obtained from the placenta and umbilical cord of newborn infants has been shown to be a rich source of hematopoietic progenitor cells (1). Cord blood cells offer a number of important advantages compared with bone marrow preparations for bone marrow reconstitution (2), and there has been a considerable increase in recent years in the use of cord blood cells as an alternative to bone marrow transplantation (3). Patients who are successfully transplanted with such progenitor cells achieve engraftment with hematopoietic precursors that normally have a pattern of gene expression corresponding to the fetal/neonatal stage of development. Accordingly, these patients provide an opportunity to examine the possible role of postneonatal environmental factors in the developmental expression of these genes. The Hb system is a particularly suitable model for approaching this question.

In the fetus and newborn, most of the Hb in the red cells consists of Hb F, which is composed of α - and γ -globin subunits. By 6 mo of age, nearly all of the Hb F is replaced by the adult form, Hb A, which is made up of α and β subunits. The factors that control this γ to β switch have been a subject

of intense study in recent years, in part because of the therapeutic advantages that might be realized if this process could be controlled or reversed in individuals with β -globin gene abnormalities.

The patients we describe in this report achieved bone marrow reconstitution with hematopoietic progenitor cells obtained at birth from their histocompatible siblings. In each case, the cell donors expressed primarily Hb F at the time the progenitor cells were harvested. The recipients were both well beyond the developmental stage when the γ to β switch is normally completed.

METHODS

Hematologic and Hb measurements. Procedures for erythrocyte and Hb quantification (4), the preparation of Hb solutions for analysis, Hb electrophoresis, and measurements of Hb F and Hb A₂ (5) were as described previously.

Hb studies. The procedures for globin chain fractionation and quantitative estimation used HPLC with a C₄ column (Vydac, The Separations Group, Hesperia, CA) with acetonitrile-water-trifluoroacetic acid gradient elution (6). For globin synthesis studies, washed red cells were incubated for 2 h at 37°C in medium containing L-leucine-³H, as previously de-

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scribed (7). Column chromatography of the labeled globins (8) followed the methods of Clegg *et al.* (9).

DNA analyses. DNA was prepared from blood leukocytes as described by Poncz *et al.* (10). Digests with *XmnI* and/or *PstI* were analyzed by the Southern blotting technique (11) using a γ IVS-II probe.

Patient 1. This child, currently 8 y of age, was noted at birth to have anomalies of her upper extremities. She was later found to have stenotic ear canals, horseshoe kidneys, café au lait spots, and growth retardation. At 13 mo of age, a chromosomal analysis of her blood mononuclear cells, after incubation with mitomycin C, revealed abnormally increased chromosomal breakages, confirming the diagnosis of Fanconi anemia. At 2.5 y, her Hb concentration was 12 g/dL, and her platelet count 106 000/ μ L. Her blood count values declined steadily thereafter, and at 4 y of age her Hb concentration was 7 g/dL and her platelet count 24 000/ μ L. Six months later, she was conditioned with cyclophosphamide and thoracoabdominal radiation (12) and received a cord blood transplant from her HLA-identical sister. The total volume of the cord blood was 285 mL, with a total nucleated cell count of 9.6×10^8 (0.75×10^8 /kg). The patient achieved successful engraftment; on d 19 posttransplant, her Hb concentration was 10.8 g/dL, her neutrophil count 1150/ μ L, and her platelet count 109 000/ μ L. This child (and patient 2 described below) received cyclosporine as their only posttransplant immunosuppressive treatment.

Patient 2. This boy, who presented with thrombocytopenia at age 4.5 y, was found to have a variety of anomalies including atresia of the auditory canals, thumb abnormalities, café au lait spots, and fused kidneys. The diagnosis of Fanconi anemia was confirmed by chromosomal analysis after incubation of his cells with diepoxybutane. Before transplantation, at the age of 6.5 y, his Hb concentration was 7.2 g/dL, his leukocyte count 1600/ μ L with an absolute neutrophil count of 1000 granulocytes/ μ L, and his platelet count 14 000/ μ L. He received a total of 75 mL of cord blood, containing 5.4×10^8 nucleated cells. His preparative regimen was similar to that of patient 1, with minor modification (13). His posttransplant course was uneventful. The first indications of engraftment were evident by d 14. His last red cell transfusion was given on d 37 posttransplant, and thereafter his Hb concentration remained >8 g/dL. Thirty-six months after his transplant, his blood count was totally normal.

RESULTS

Patient 1. The parents of this child and the sibling who was her transplant donor all exhibited normal hematologic findings. At 14 wk after her transplant, at a point when the graft was well established, her Hb F level was 22.2% with a $G\gamma/A\gamma$ ratio of 1.95; a globin synthesis study demonstrated a γ/β synthesis ratio of 0.22. These findings correspond to those seen in normal infants approximately midway through the γ to β switch.

As controls for this and other globin synthesis studies in these patients, we performed the same determination in three patients shortly following engraftment after allogeneic bone marrow transplants. Their mean γ/β synthesis ratio was 0.13 ± 0.03 .

When the patient was restudied 5 mo later, her Hb F level was 4%, and her $G\gamma/A\gamma$ ratio was 0.58, compatible with a normal early postswitch pattern.

Patient 2. The parents and the transplant donor of this patient also exhibited normal hematologic findings. Analysis of their fetal Hb, however, demonstrated abnormalities in the father and in the sibling whose cord blood served as a source of progenitor cells (Table 1). Both of them had slightly elevated levels of Hb F, accompanied by substantially increased $G\gamma/A\gamma$ ratios. The slide-elution test for Hb F-containing cells (14) showed both of them to have 3 to 6% strongly positively staining cells, with a clearly heterogeneous distribution.

When the patient was studied approximately 3 mo after transplantation, his erythrocytes contained 18.1% Hb F with a $G\gamma/A\gamma$ ratio of 5.8. A globin synthesis study at that time (Fig. 1) demonstrated nearly equal rates of synthesis of the γ and β chains (γ/β synthesis ratio 0.97), a pattern similar to what we have previously observed in newborns (8).

One month later, the Hb F level in his blood increased to 31.5%, but a globin synthesis study at that time showed a significant γ to β transition, with a γ/β synthesis ratio of 0.33. Subsequent levels of Hb F in the patient gradually declined (Fig. 2), but even after 1 y they remained significantly elevated. At approximately 16 mo after his transplant, at which time his Hb F level was 6.6%, a slide-elution test of his erythrocytes demonstrated that 4.4% of the cells had a strongly positive staining reaction for Hb F.

DNA was prepared from blood leukocytes from the patient, his father, and the brother who was his transplant donor. DNA was also prepared from cultured skin fibroblasts from the patient. Digests were made with *XmnI* and with *XmnI* and *PstI*.

The DNA fragments from each of these individuals, identified using the γ IVS-II probe, were indistinguishable from each other but differed from normal controls. The study shown in Figure 3 used DNA from the patient's fibroblasts. The 7-kb and 8-kb fragments in the *XmnI* digest reflect a polymorphic site 5' to the normal $G\gamma-A\gamma$ segment (15). The *XmnI* site 5' to the $G\gamma$ -globin gene also can account for the additional 4.9-kb fragment in the patient's DNA, suggesting that he and the other affected family members have a $G\gamma^G\gamma^A\gamma$ triplication arrangement. The presence of the additional 4.1-kb and 0.8-kb fragments in the *XmnI* + *PstI* digests (Fig. 3) further supports this possibility (15).

DISCUSSION

Findings from a variety of clinical observations have contributed to the characterization of the normal Hb switching

Table 1. Hb F measurements from the parents of patient 2 and from the patient's brother, who was his cord blood transplant donor

	% Hb F	$G\gamma/A\gamma$ ratio	Hb F slide elution test
Father	1.1	9.1	Positive
Mother	0.3	<1.0	Negative
Donor (cord blood)	75.6	6.2	
Donor (2–22-91)	4.8	>10	
Donor (8–9-91)	2.6	>10	Positive

Figure 1. Globin synthesis by blood reticulocytes from patient 2 at approximately 3 mo after his transplant. —, globin protein; ○---○, incorporated L-leucine-³H.

Figure 2. Levels of Hb F in patient 2 after his cord blood transplant. At the time he was transplanted (shown by the arrow), he had received extensive transfusions, and therefore he had very little Hb F in his circulating red cells.

process and also to gaining greater understanding of the role of postneonatal factors in the developmental expression of the Hb system. In accordance with a widely accepted hypothesis that relates Hb F expression to the stage of erythropoietic maturation (16, 17), erythroid cells in earlier stages of maturation have been shown to produce proportionally larger amounts of fetal Hb (18). This change is central to explaining the elevated levels of Hb F associated with "stress erythropoiesis," and it also has been postulated as a potential mechanism for the control of Hb switching.

In studies of Hb expression in baboons, in which erythropoiesis was stimulated by phlebotomy, acute hemolysis, or hypobaric hypoxia (19, 20), high levels of Hb F could be achieved and sustained for extended periods of time by continuation of the erythropoietic stimuli. In human newborn infants with hemolytic disease, in whom erythropoietic regen-

Figure 3. Distribution of DNA fragments in restriction enzyme digests from patient 2 (P) and a normal control (C) using a γ -globin gene (γ IVS-II) probe.

eration was substantially accelerated, Hb switching nevertheless was not observed to be delayed (21, 22), and indeed such infants were shown to have somewhat lower than normal levels of Hb F. On the other hand, infants who suffered hypoxia *in utero* as a result of placental insufficiency were observed to have at least moderately increased levels of Hb F, compatible with delayed Hb switching (23, 24). In infants with postnatal hypoxia due to cyanotic congenital heart disease, however, no evidence was found to suggest any change in the Hb switching program (25).

The apparently inconsistent and generally limited influence of these environmental factors in human infants provided support for an alternative hypothesis, *viz.* that Hb switching might be governed by a "developmental clock" (26) having basically autonomous activity that functions to coordinate Hb switching within the overall developmental program. This hypothesis is consistent with the observation that, at birth, Hb F synthesis by erythroid cells from infants of varying gestational ages correlated closely with the gestational ages of the infants (27). Within this framework, the putative developmen-

tal clock for sequential Hb expression could be an intrinsic feature of the erythroid cell lineage, or alternatively, external factors produced at specific stages of development could act on the erythroid cells to mediate these changes.

Transplantation experiments, in which hematopoietic progenitor cells of fetal or neonatal origin were engrafted into postneonatal recipients, have represented one avenue to approaching this question (28–32). These experiments, however, have yielded unclear and sometimes conflicting results. Moreover, durable transplants were not achieved in any of these studies; none of the transplant recipients survived more than a few weeks. Zanjani *et al.* (28) performed transplantation studies in sheep, which normally undergo a neonatal Hb switch similar to that of the human switching process. When hematopoietic progenitor cells from the livers and bone marrow of sheep fetuses were transplanted into an adult recipient, the engrafted cells produced Hb almost exclusively of the adult type. These observations suggested that factors in the adult hematopoietic environment might have played a significant role in mediating the switch to adult Hb synthesis.

Bunch *et al.* (29) described experiments in which bone marrow cells from fetal sheep were successfully transplanted into lambs. In these studies, the Hb that was produced postengraftment was found to be primarily fetal. A gradual increase in the synthesis of adult-type Hb of donor origin was then observed; however, this transition occurred earlier than was expected from the gestational age of the transplanted cells, but not as rapidly as would have been anticipated if environmental factors were entirely responsible for producing the Hb switch. A later, more extensive study from the same laboratory (30) resulted in similar observations, leading to the conclusion that “the timing of the switch from fetal to adult hemoglobin in ruminants at least in part reflects inherent programming of the hematopoietic stem cells.”

Human transplantation experiments have been reported by Delfini *et al.* (31) and by Papayannopoulou *et al.* (32). These studies both involved young children with acute leukemia who received transplants of cells obtained from fetal livers. Although these patients survived for only 27–30 d after the transplants, studies of their bone marrow demonstrated that engraftment had taken place. Globin synthesis studies with bone marrow cells and blood reticulocytes from these patients, done between 14 and 28 d posttransplantation, demonstrated γ/β synthesis ratios ranging from approximately 7 to approximately 20. The persistence of fetal patterns of expression in these transplanted cells militated against any significant role of environmental factors in effecting the γ to β switch, suggesting further that the process of Hb switching is likely to be controlled primarily by a mechanism intrinsic to the erythropoietic progenitor cells.

The findings from the two patients we studied, who achieved long-term marrow engraftment, are also compatible with the notion that hematopoietic progenitor cells from the fetus and newborn are intrinsically programmed for γ -globin gene expression. In both of these patients, one whose marrow graft contained an apparent γ -globin gene triplication and the other who presumably had a normal complement of globin genes, the γ to β switching process seemed to follow the temporal

sequence that would have been expected to occur during the first months of infancy.

In the interpretation of these observations, alternative explanations for the transient elevation of Hb F levels in these patients also need to be considered. The Fanconi anemia syndrome, the disorder for which both of these patients received hematopoietic cell transplants, is typically associated with increased levels of Hb F (33), and indeed, before their transplants both of these children had elevated levels of Hb F. Since the time of their engraftment, however, both of these patients have continued to have normal hematologic phenotypes, and there has been no indication of repopulation of their bone marrow by their original cell lines. Neither of the donors of the transplanted cord blood cells had any demonstrable feature of the Fanconi anemia syndrome, although both of them have a high probability of being heterozygous carriers of this abnormality. The parents of these children also had normal hematologic findings. Carriers of the Fanconi anemia syndrome gene typically do not produce elevated levels of Hb F, but it is possible that under conditions of stress reticulocytosis their hematopoiesis might be accompanied by the production of higher than normal levels of Hb F. In any case, inasmuch as Hb F is synthesized in relatively low amounts even in homozygous Fanconi anemia patients, a process of this kind would not likely be sufficient to account for the levels of Hb F we observed in these patients.

Stress reticulocytosis has been shown to be accompanied by a transient increase in Hb F synthesis in patients in marrow regeneration after treatment for iron deficiency (34), in the recovery phase of transient erythroblastopenia of childhood (35), and after phlebotomy (35) or bone marrow transplantation (36). In some patients after engraftment of transplanted bone marrow, their increased rate of synthesis of Hb F was also accompanied by $^G\gamma/A\gamma$ ratios that corresponded to the fetal/newborn pattern (36). Nevertheless, the maximum rates of synthesis of Hb F in the patients recovering from allogeneic bone marrow transplants, who served as “controls” in our study, were substantially less than what we observed after the transplantation of cord blood cells and could not nearly account for the full extent of these changes.

Recent efforts to define the regulatory mechanisms that are responsible for globin switching have used cell hybrids that include part or all of human chromosome 11, which includes the β -globin locus (37, 38), and transgenic mice that express human globins (39, 40). Models that have been developed from studies with these experimental systems have suggested that the regulation of Hb switching may involve processes of both stage-specific autonomous gene silencing (39, 41) and competitive interactions between globin-gene promoter and enhancer elements (38, 42). Evidence from transgenic animal studies also suggests that *trans*-acting “environmental” factors play some role in this process (39).

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