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Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA-identical sibling

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Abstract

The clinical manifestations of Fanconi's anemia, an autosomal recessive disorder, include progressive pancytopenia, a predisposition to neoplasia, and nonhematopoietic developmental anomalies [1-3]. Hypersensitivity to the clastogenic effect of DNA-cross-linking agents such as diepoxybutane acts as a diagnostic indicator of the genotype of Fanconi's anemia, both prenatally and postnatally [3-6]. Prenatal HLA typing has made it possible to ascertain whether a fetus is HLA-identical to an affected sibling [7].

We report here on hematopoietic reconstitution in a boy with severe Fanconi's anemia who received cryo-preserved umbilical-cord blood from a sister shown by prenatal testing to be unaffected by the disorder, to have a normal karyotype, and to be HLA-identical to the patient. We used a pretransplantation conditioning procedure developed specifically for the treatment of such patients [8]; this technique makes use of the hypersensitivity of the abnormal cells to alkylating agents that cross-link DNA [9,10] and to irradiation [11] In this case, the availability of cord blood obviated the need for obtaining bone marrow from the infant sibling.

This use of cord blood followed the suggestion of one of us that blood retrieved from umbilical cord at delivery, usually discarded, might restore hematopoiesis – a proposal supported by preparatory studies by some of us [12] and consistent with reports on the presence of hematopoietic stem and multipotential (CFU-GEMM), erythroid (BFU-E), and granulocyte-macrophage (CFU-GM) progenitor cells in human umbilical-cord blood (see the references cited by Broxmeyer et al. [12]).

Keywords: anemia, aplastic therapy, blood preservation, Fanconi anemia, female, fetal blood, HLA antigens, hematopoietic stem cell transplantation, histocompatibility testing, humans, male, pregnancy, prenatal diagnosis, preschool child

Case report

The patient, a five-year-old boy, was first noted to have pancytopenia at 24 months of age. An evaluation (Duke University Medical Center) showed the following hematologic values: hemoglobin concentration, 9.5 g per deciliter; white-cell count, 4.3 x 10^9 per liter, with 22 percent granulocytes, 62 percent lymphocytes, 3 percent monocytes, 1 percent eosinophils, and 8 percent basophils; platelet count, 62 x 10^9 per liter; and reticulocyte level, 2.3 percent. The bone marrow was hypocellular. The patient had the classic malformations of Fanconi's anemia, including retarded growth (5th to 10th percentile), a rudimentary extra thumb on the left hand, the absence of a left kidney, and hypospadias. Testing of the patient's cells with diepoxybutane, performed at 24 months (Rockefeller University), confirmed the diagnosis of Fanconi's anemia. The parents were healthy, and there was no consanguini-

ty or past history of blood disorders in the family. The patient had been treated with danazol (300 mg per day for six months), with a progressive decrease in the blood-cell counts. Before admission, he had received three units of filtered and irradiated packed red cells.

The patient's mother became pregnant in June 1987. Cytogenetic analysis of cultured amniotic-fluid cells (Rockefeller University) showed that the values for chromosome breakage before and after the cells were exposed to diepoxybutane were within the normal range [5]. A girl was delivered vaginally without complications in February 1988. Studies of chromosome breakage in umbilical-cord blood cells indicated that the newborn had a normal karyotype and was not affected by Fanconi's anemia (Table 1).

Source Of Cells	Percent Donor Cells In Sample†		Mean Chromosome Breaks/Cell‡	
	before dab	after dab	before dab	after dab
Patient's peripheral blood on day 0	0	0	0.18	10.6
Donor's cord blood on day 0	100	100	0.00	0.02
Patient's bone marrow on day 120	100	100	0.04	0.30
Patient's peripheral blood Day 50	30	52	0.7	4.0
Day 64	8	32	1.3	5.0
Day 120	12	32	1.9	6.4
Day 204	64	86	0.30	0.49

Table 1: Results of Cytogenetic Analysis.*
*DEB denotes diepoxybutane; its final concentration in the culture medium was 0.1 mg per milliliter in each analysis in this table.
†Analysis performed on quinacrine-stained metaphase preparations.
‡Analysis performed on Giemsa-stained metaphase preparations.

Prenatal HLA typing of amniotic-fluid cells (Dr. M.S. Pollack, Methodist Hospital and Baylor Medical Center, Houston) had shown that the fetus was HLA identical to the patient: the first haplotype was Al,B8,DR3, and the second haplotype A29,B44,DR3. Mixed lymphocyte cultures of cells from the patient with cells from the cord blood and peripheral blood of the donor were negative. The cord-blood cells were less reactive against a pool of allogeneic cells than were the peripheral-blood cells (incorporation of [^{3}H]thymidine, 15,437 vs. 112,954 cpm). The ABO blood group of the donor was O Rh+, and that of the recipient B Rh+.

The patient was admitted in September 1988, with the following hematologic values: hemoglobin concentration, 6.8 g per deciliter; white-cell count, 3.1 x 10^9 per liter, with 7 percent granulocytes, 86 percent lymphocytes, and 5 percent monocytes; and platelet count, 18 x 10^9 per liter. The marrow was hypocellular, with 10 percent of normal cellularity, 24 percent myeloid cells, 2 percent erythroblast cells, 67 percent lymphocytes, and no megakaryocytes. There were no hemorrhagic or infectious complications of the patient's illness. His liver and kidney functions were normal. Serologic tests were positive for cytomegalovirus (on enzyme-linked immunosorbent assay) and negative for human immunodeficiency virus, hepatitis B, and toxoplasmosis.

The patient was isolated in a room with laminar airflow, and treatment with oral broad-spectrum nonabsorbable antibiotics (vancomycin, tobramycin, and colistin [Colimycine]) was started nine days before transplantation (on day -8). Fluconazole (50 mg per day) was administered to prevent fungal infection, and oral acyclovir (100 mg per day) to prevent herpes simplex virus infection. For pretransplantation conditioning, cyclophosphamide (5 mg per kilogram of body weight) was given intravenously for four consecutive days (from day -6 to day -3; total, 380 mg) along with hyperhydration. Irradiation was delivered to the thoracoabdominal region by a linear accelerator on day -1 (mean rate, 10.87 cGy per minute), for a total of 5 Gy over a period of 46 minutes; the abdomen received 500 cGy, and the lungs and the right liver lobe, which were shielded, received 67 cGy.

On the day of transplantation (day 0 – October 6, 1988), cryopreserved umbilical-cord blood was thawed and infused without further processing, according to predetermined optimal conditions12 (the surviving whole red cells that had not been hemolyzed belonged to group O). The patient received 0.4 x 10^8 nucleated cells per kilogram, of which a total of 4.37 x 10^5 were CFU-GM (as determined by assay in Paris). Two hours after the infusion, the patient had chills, fever, and hypotension. These symptoms soon resolved, while the patient was receiving the broad-spectrum antibiotics vancomycin and ceftazidime intravenously. For prophylaxis against graft-versus-host disease, cyclosporine was administered intravenously from day – 1 (4.5 mg per kilogram per day), according to a preliminary pharmacokinetic study. All blood products were irradiated (25 Gy).

Ethical and Regulatory Considerations

Written informed consent was obtained from the patient's parents for collecting the umbilical-cord blood and for the transplantation procedure. The treatment plan was reviewed and approved by the institutional review board for clinical investigation of the Duke University Medical Center and by the ethics committee of the Hôpital Saint-Louis. Approval for the receipt, cryopreservation, storage, and release of cord blood was received from the institutional review board of the Indiana University School of Medicine. The Food and Drug Administration considered the procedure equivalent to the storage and transplantation of bone marrow, which are currently not subject to its regulation.

Methods

Cytogenetic Studies

Chromosome-breakage studies were performed as described elsewhere [4]. Diepoxybutane was added to bone marrow cultures when they were begun, and the cultured cells were harvested after 24 hours. Peripheral blood was cultured in the presence of phytohemagglutinin for 72 hours; diepoxybutane was present in the medium during the last 48 hours of culture. The frequency of chro-

mosome breakage before and after the addition of diepoxybutane was analyzed in Giemsa-stained metaphase preparations; the ratio of cells from the patient to cells from the donor (male:female) was determined on quinacrine-stained slides to facilitate the identification of the Y chromosome.

DNA Studies: Restriction-Fragment-Length Polymorphism

DNA samples obtained for Southern blotting were digested with Taq1 (New England Biolabs, Beverly, Mass.), separated by gel electrophoresis, transferred to an Immobilon-N filter (Millipore Corp., Bedford, Mass.), hybridized, and washed as previously described [13]. The probe used was CRI-pS232 (DXS278) (Collaborative Research, Boston), which hybridizes with sequences from the X and Y chromosomes.

Collection, Storage, and Shipment of Neonatal Blood

Immediately after the birth of the patient's sister, blood was obtained from her umbilical cord and the placenta as described elsewhere [12] and transported at ambient temperature by overnight express service to a laboratory for cellular analysis, cryopreservation, and storage (Indiana University School of Medicine). A sample of cord blood was also sent elsewhere for cytogenetic analysis (Rockefeller University). After a small sample (<2 ml) had been set aside for laboratory tests, including the determination of the level of nucleated cells and the enumeration of progenitor cells, the blood was frozen without further treatment in dimethyl sulfoxide at a final concentration of 10 percent (vol/vol) as previously described [12] Two bags of cord blood and one bag of placental blood were frozen. Samples (about 1 ml each in Nunc tubes) were similarly frozen for testing of cell recovery after thawing and for confirmation of the HLA types. The volume of blood and the numbers of nucleated and progenitor cells collected are shown in Table 2. These values were within the range associated with successful transplantation of HLA–matched allogeneic bone marrow [12]. Testing of thawed cells, including those in one tube sent from Indiana University to Duke University Medical Center (where the HLA types were confirmed), demonstrated recovery of 79 percent to 90 percent of nucleated cells; on day 14, the mean (±1 SEM) rates of recovery were 100 percent ofCFU-GM, 63±18 percent of BFU-E, and 79±15 percent of CFU-GEMM.

Source of Cells	Progenitor Cells†	
	all densities	low density (<1.077 g/ml)
Donor (cord and placental blood)‡	total cells x 10^{-5}	
Agar culture Day 14 CFU-GM (colonies)	1.52	ND
Day 14 CFU-GM (colonies and clusters)	2.46	ND
Methylcellulose culture (colonies) Day 14 CFU-GM	1.56	ND
BFU-E-2	3.95	ND
BFU-E-1	3.60	ND
CFU-GEMM	0.39	ND
Recipient (bone marrow)	cells/105 cells plated§	
Methylcellulose culture, day 0 Day 14 CFU-GM (colonies)	ND	1
Day 14 CFU-GM (colonies and clusters)	ND	8
BFU-E-1	ND	0
CFU-GEMM	ND	0
Methylcellulose culture, day 30 Day 14 CFU-GM (colonies)	ND	9
BFU-E-1	ND	0
CFU-GEMM	ND	0
Agar culture, day 120 Day 7 CFU-GM (colonies)	22±2	140±12
Day 7 CFU-GM (colonies and clusters)	118±16	416±20
Day 14 CFU-GM (colonies)	36±4	156±8
Day 14 CFU-GM (colonies and clusters)	152±4	228±12
Methylcellulose culture, day 120 Day 14 CFU-GM (colonies)	324±16	416±25
BFU-E-2	77±2	134±6
BFU-E-1	96±8	132±10
CFU-GEMM	4±1	9±1

Table 2. Hematopoietic Progenitor Cells in Blood from the Umbilical Cord and Placenta of the Donor and Bone Marrow from the Recipient.

* The preparation for the assays has been described elsewhere [12]. The assays of the cord blood and the bone marrow on day 120 were performed in Indianapolis, and those of the bone marrow at day 0 and day 30 in Paris.

† Plus-minus values are means ± 1 SEM; ND denotes not done.

‡ The total volume of cord and placental blood collected was 160 ml and contained 1.19 x 109 nucleated cells.

§ Normal range (Indianapolis laboratory) for low-density cells. 10 to 70 for CFU-GM and 15 to 80 for BFU-E-1 and BFU-E-2.

These were mainly microclusters, with less than 20 cells per cluster.

The two bags of frozen cord blood and the bag of placental blood were sent with an escort by air (with the approval of the airline) to the Hôpital Saint-Louis from Indiana University two weeks before transplantation. The bags were sent in a Dry Shipper container (CMC-3200 wide mouth with platform; Cryomed, New Baltimore, Mich.) maintained at -175°C, a temperature optimal for cryopreservation.

Hematopoietic Progenitor Cells in Vitro

Assays were prepared as previously described [12] In the CFU-GM assay, colonies (>40 cells per aggregate) and clusters (3 to 40 cells per aggregate) were scored after 7 days and 14 days of incubation in agar culture medium. Large-sized colonies formed (>1000 cells). Cell counts were expressed also as colonies plus clusters, for a more accurate estimate of the total CFU-GM compartment. In the assays for BFU-E, CFU-GEMM, and CFU-GM, colonies were scored after 14 days of incubation in methylcellulose culture medium. In the BFU-E-1, CFU-GEMM,

and CFU-GM assays, colonies were scored from the same plates, which included erythropoietin (1 unit per milliliter), hemin (0.1 mM), and 5637 conditioned medium (10 percent vol/vol). BFU-E-2 cells were cultured as BFU-E-1, but without 5637 conditioned medium. Each BFU-E-2 colony contained at least 50 cells or comprised at least three subcolonies, each of which contained at least 10 cells, but were usually much larger. Colonies derived from BFU-E-1 were much larger than those derived from BFU-E-2.

Thawing and Recovery of Cells

Eighty-two percent of nucleated cells of the thawed blood transfused into the patient were viable. Progenitor-cell assays performed in Paris (which differed slightly in technique from assays performed in Indianapolis) indicated that the rate of cell recovery was equal to or greater than the count of progenitor cells recorded before freezing.

Results

Clinical Findings

The clinical course of the patient was uneventful and without complications. He tolerated the pretransplantation conditioning without evident toxic effects. On day 15, a transient skin rash and fever resolved with methylprednisolone treatment (2 mg per kilogram). A skin biopsy revealed few vacuolar basal epidermal cells with a mild lymphoid infiltrate, indicative of grade I graft-versus-host disease as defined according to the Seattle classification [14]. Liver-function tests showed that the serum levels of aspartate and alanine aminotransferases were twice normal, probably as a result of the graft-versus-host disease; values returned to normal on day 47. Cytomegalovirus was repeatedly isolated from the urine, but the patient never had any signs of active infection, and all tests for viremia were negative. Five months after transplantation the patient was discharged with normal clinical and laboratory findings. The doses of cyclosporine and corticosteroids were progressively reduced, and the drugs were discontinued at six months. At present (nine months after transplantation), the patient has no chronic graft-versus-host disease and leads a normal life.

Hematologic Findings

The blood-cell counts began to return to normal (Table 3).

The reticulocyte and granulocyte counts had begun to rise by day 22 after transplantation. The patient received eight transfusions of packed red cells (O Rh+) and 48 units of random platelets. Red cells were last transfused on day 54, and platelets on day 43. Bone marrow aspirated on day 17 was aplastic: an evaluation on day 28 showed 20 percent cellularity, with 19 percent myeloid cells, 73 percent erythroid cells, and few megakaryocytes. On day 120, the marrow had normal cellularity, with 40 percent myeloid cells, 44 percent erythroid cells, and a normal level of megakaryocytes.

Hematopoietic Progenitor Cells

Before transplantation, the marrow contained few or no detectable progenitor cells (Table 2). At 30 days after transplantation, CFU-GM cells were detected but not BFU-E or CFU-GEMM; at 120 days, progenitors were apparent at normal to supranormal frequencies (colonies or clusters per number of cells plated).

Reconstitution by Donor Cells

Blood- Type Studies
The patient‘s red cells (B Rh+) disappeared progressively and became undetectable on day 90 after transplantation; 46 days after the last transfusion, 100 percent of red cells were of the donor‘s

Day	Hemoglobin	Leukocytes	Granulocytes	Lymphocytes	Platelets	Reticuloytes
	g/dl	no. of cells x 10^{-9} per liter				
-20	6.8	3.1	0.25	2.8	18	10
0	9.7	0.8	0.0	0.8	120	0
8	10.9	0.4	0.0	0.4	80	0
15	11.6	0.4	0.0	0.4	39	0
22	7.8	0.9	0.3	0.6	50	5
29	8.5	1.0	0.3	0.5	105	17
36	9.4	1.7	0.6	0.5	55	36
43	11.3	5.1	2.4	1.9	31	90
50	8.9	3.4	1.5	0.7	62	162
57	8.9	5.6	3.2	1.0	174	63
90	11.3	5.1	4.0	1.1	296	50
120	13.0	3.9	2.3	1.1	265	40
160	12.0	3.7	1.4	1.6	293	45
240	12.3	5.2	2.7	1.6	354	50
282	12.2	4.8	2.3	1.2	315	---

Table 3. Blood-Cell Counts before and after Transplantation.

blood type (O Rh+) and were found to have remained so at 240 days after transplantation.

Cytogenetic Studies
Table 1 shows the results of cytogenetic studies of bone marrow aspirated on day 120 and peripheral blood obtained on days 50, 64, 120, and 204. The chromosomal complement of the bone marrow was 46,XX. No cells of the patient (male cells) in metaphase were seen among 50 quinacrine-stained or 100 Giemsa-stained cells analyzed. The chromosomebreakage frequencies were 0.04 and 0.30 breaks per cell in the base-line and diepoxybutane-treated cultures, respectively.

Through day 120 after transplantation, more than half the metaphases seen in cytogenetic preparations of phytohemagglutinin-stimulated peripheral-blood cultures were of host origin, as indicated by the presence of a Y chromosome. These cells, of lymphoid origin, had greatly elevated levels of chromosome breakage at base line and severe radiation damage, manifested by the presence of multiple dicentrics, rings, and chromosomal fragments; they were also hypersensitive to the clastogenic effect of diepoxybutane, showing multiple chromatid breaks and exchanges typical of the cells characterizing Fanconi's anemia. The cells of donor origin (female) did not have elevated levels of chromosome breakage. By day 204 ($6^{1/}2$ months after transplantation), the majority of peripheral-blood lymphocytes were of donor origin.

These findings reflected engraftment with the donor cells and survival of a minor population of radiationdamaged host cells in the blood – an outcome similar to that in reports of bone marrow transplantation by others [15].

DNA Studies
The CRI-pS232 probe recognizes a complex set of fragments at a highly polymorphic locus on the X chromosome, as well as a polymorphic locus on the Y chromosome [16]. In the present case, all the variable bands present in the DNA of the donor were seen in the DNA extracted from the peripheral blood of the recipient after transplantation (Fig. 1). In addition, the X and Y alleles of the recipient were seen as faint bands, indicating some chimerism in the peripheral blood, with primarily donor cells present.

DNA was extracted from the peripheral blood of the father (lane 1), the mother (lane 2), the patient before transplantation (lane 3), the patient's HI.A-identical sister (lane 4), the patient's HA non-identical sister (lane 5), and the patient on post-transplantation day 50 (lane 6), day 64 (lane 7), and day 120 (lane 8).

The DNA obtained from the patient after transplantation was primarily of donor origin. Each DNA sample was digested with the restriction enzyme Taq1 and examined with the probe CRI-pS232, which recognizes a complex set of fragments at a highly polymorphic locus on the X chromosome as well as a locus on the Y chromosome. The thick arrows indicate X-specific bands from the donor, and the thin arrows denote those from the recipient. Y-specific bands are seen at 4.9 and 3.8 kb. A constant band at 3.0 kb is seen in the DNA of all the subjects.

Discussion

Bone marrow and, under certain circumstances, the blood of adults (see references cited by Broxmeyer et al [12]) and the liver of fetuses [17] have been used in the transplantation of hematopoietic cells. The clinical and biologic data presented above show that the umbilical-cord blood of a single newborn is sufficient to induce hematopoietic reconstitution. The virtually complete occupation of the patient's myeloid system by cells from his sibling was indicated by the cytogenetic studies, blood typing, and studies of DNA polymorphisms and by the absence of undue chromosomal fragility. At present, a small percentage of lymphoid cells with chromosome damage remains in the circulation, but there has been a trend toward the elimination of these damaged cells.

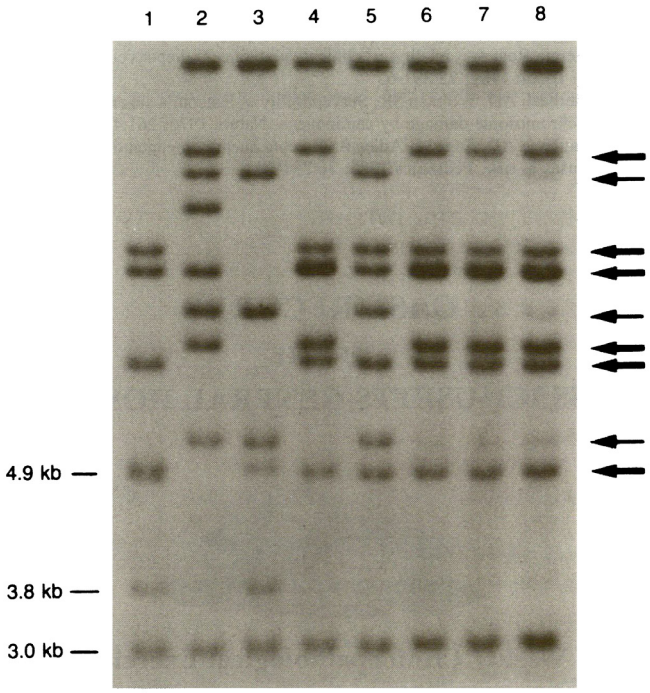

Figure 1. Southern Blot Analysis of DNA from the Patient and His Family.

DNA was extracted from the peripheral blood of the father (lane 1), the mother (lane 2), the patient before transplantation (lane 3), the patient's HI.A-identical sister (lane 4), the patient's HA non-identical sister (lane 5), and the patient on post-transplantation day 50 (lane 6), day 64 (lane 7), and day 120 (lane 8).

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There may be several reasons why cellular repopulation was somewhat delayed in our patient as compared with patients who have undergone transplantation with HLA-matched allogeneic (sibling) bone marrow. For instance, cells that have been frozen and thawed may take longer than fresh cells to seed, respond, and differentiate in their respective inducing microenvironments. Also, consistent with the relative paucity of CFU-GM on day 7 as compared with the more immature CFU-GM on day 14 [12], umbilical-cord blood may typically have a profile with immaturity and less differentiation of hematopoietic stem and progenitor cells. The modified conditioning regimen was adequate to permit complete engraftment with 0.4×10^8 total nucleated cord-blood cells per kilogram, as compared with the mean of 3×10^8 total nucleated bone marrow cells per kilogram that is usually given for bone marrow transplantation.

The use of cord blood in the treatment of Fanconi's anemia may be extended to the treatment of other conditions for which bone marrow transplantation is indicated, and demonstrates the potential of cryopreservation for prospective use of autologous blood.

In the latter respect, our success with allogeneic cord blood is particularly important, because studies in animals have shown that fewer reconstituting cells are required to restore hematopoietic function when these are syngeneic rather than allogeneic [18].

Thus, umbilical-cord blood can be considered an efficacious source of sufficient cells for clinical hematopoietic reconstitution.

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