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High Peripheral Blood Progenitor Cell Counts Enable Autologous Backup before Stem Cell Transplantation for Malignant Infantile Osteopetrosis

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ABSTRACT

Autosomal recessive osteopetrosis (OP) is a rare, lethal disorder in which osteoclasts are absent or nonfunctional, resulting in a bone marrow cavity insufficient to support hematopoiesis. Because osteoclasts are derived from hematopoietic precursors, allogeneic hematopoietic cell transplantation can cure the bony manifestations of the disorder. However, high rates of graft failure have been observed in this population. It is not possible to harvest bone marrow from these patients for reinfusion should graft failure be observed. We report that 8 of 10 patients with OP had high numbers of circulating $CD34^+$ cells (3% ± 0.9%). This increased proportion of peripheral $CD34^+$ cells made it possible to harvest 2×10^6 $CD34^+$ cells per kilogram with a total volume of blood ranging from 8.3 to 83.7 mL (1.3-11.6 mL/kg). In addition, colony-forming assays documented significantly more colony-forming unit–granulocyte-macrophage and burst-forming unit–erythroid in the blood of osteopetrotic patients compared with controls; the numbers of colony-forming units approximated those found in control marrow. We conclude that OP patients with high levels of circulating $CD34^+$ are candidates for peripheral blood autologous harvest by limited exchange transfusion. These cells are then available for reinfusion should graft failure be observed in patients for whom retransplantation is impractical.

KEY WORDS

Osteopetrosis • Leukoerythroblastic • Peripheral blood stem cells • Osteoclasts

INTRODUCTION

Malignant infantile osteopetrosis (MIOP) is a rare genetic disease characterized by generalized osteosclerosis, which results from a functional or numeric deficiency of osteoclasts [1]. Major consequences are susceptibility to fractures and nerve compression due to failure of remodeling of the skull base; this results in optic, auditory, and facial nerve compression and hydrocephalus. Obliteration of marrow cavities results in extramedullary hemopoiesis, resulting in hepatosplenomegaly and, frequently, progression to life-threatening pancytopenia. There is also increased susceptibility to infection in some patients because of an unexplained defect of neutrophil superoxide function [2]. This combination of problems results in the death of 70% of affected children by the age of 6 years [3].

Although beneficial effects have been reported in response to a variety of drugs (most notably γ-interferon [4]), stem cell transplantation (SCT) remains the only curative therapy and forms the cornerstone of treatment. After successful engraftment, children normalize their bone density (Figure 1) and hematologic parameters and have stabilization or prevention of

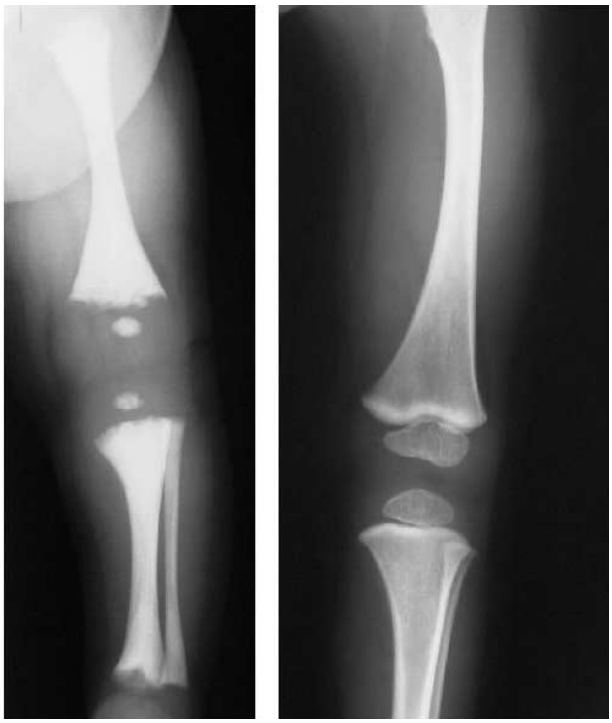

Figure 1. Resolution of osteosclerosis after bone marrow transplantation. Before bone marrow transplantation (left), the leg bones were grossly sclerotic and showed flaring of the distal diaphyses (Ehrlenmeyer flask deformity). By 10 months after bone marrow transplantation (right), there was a marked reduction in bone density and an improvement in bone modeling.

cranial nerve damage and hydrocephalus. Results are excellent after matched sibling allogeneic grafts; 73% of patients are alive and disease free at 5 years after transplantation [5].

Unfortunately, results are much poorer after nonidentical bone marrow (BM) transplantation. For example, Gerritsen et al. [6] reported nonengraftment in 15 of 45 patients who received non–HLA-identical transplants. In some cases, engraftment can be achieved by proceeding immediately to a second graft from the same or an alternative donor after immune reconditioning with agents such as OKT3 and high-dose steroids. However, this may be contraindicated by severe toxicity, such as veno-occlusive disease, or infection. For these high-risk patients, the usual management would be to attempt rescue of the patient by reinfusing autologous "backup" marrow harvested before BM transplantation. However, this is not possible in MIOP, in which densely sclerotic bones and minimal BM cavities preclude autologous harvest. As a consequence, primary rejection may result in prolonged aplasia and infective death before count recovery.

It has long been recognized that immature myeloid and erythroid precursors circulate in increased numbers in patients with MIOP, producing a leukoerythroblastic blood picture [7,8]. This is thought to be a consequence of the reduced space in the BM cavities (myelophthisis). In 1995, we began to investigate spontaneous peripheral blood stem cell (PBSC) numbers in presenting osteopetrotic patients, reasoning that, if they were markedly increased, this could provide an alternative source of cells for rescue in the event of failed engraftment. We present data on 10 consecutive patients with characteristic presentations of MIOP. We report both unstimulated PBSC counts and colony-forming unit (CFU) assay results, with appropriate controls. Details are also reported on 2 patients who received an autologous reinfusion after failed engraftment.

A further advantage of our work has been to facilitate studies of osteoclast culture from children with both typical and atypical presentations of MIOP [9,10]. In time, this approach may allow better classification of the disease and correlation of the clinical manifestations of the disease with specific genetic defects. It may also allow antenatal diagnosis for families in whom the gene disorder cannot be characterized.

MATERIALS AND METHODS

Patients

Patients were 10 consecutive referrals of infants with MIOP to the BM transplantation unit of the Royal Hospital for Children, Bristol, and the Department of Pediatrics, University of Minnesota. All had typical presentations of severe MIOP, including neonatal hypocalcemic convulsions; nystagmus or blindness; abdominal swelling secondary to hepatosplenomegaly; thrombocytopenia, anemia, or both; and an incidental finding of osteosclerosis on a failure-to-thrive screen. Exceptions were patients 1 and 5, discussed below. Two children were siblings and, for clarity, are presented as subjects 3A and 3B. Full blood counts at presentation and corresponding patient ages are shown in Table 1. Peripheral blood (PB) specimens were obtained for analysis by flow cytometry and clonogenic assays with informed consent.

It is likely that patient 1 had an intermediate form of the disease that is consistent with a longer life expectancy [11]. She was the oldest child, by a considerable margin, to undergo transplantation (at 5 years 4 months). Even though she had neonatal hypocalcemia [12], osteopetrosis (OP) was not diagnosed until 9 months of age, when she lost all vision. Subsequent growth was poor (height less than the 0.4th percentile), and frequent fractures occurred with minimal trauma. However, on referral for transplantation, the patient had minimal hepatomegaly, no splenomegaly, and a well-preserved blood count—atypical findings for severe MIOP.

Patient 5, an Omani of Bedouin ancestry, presented with early severe anemia, thrombocytopenia,

Table 1. *Osteopetrosis Patient Data: Full Blood Counts and Patient Details at the Time of $CD34^+$ Cell Counts and Progenitor Assays*

Variable	Patient No.									
	1	2	3A	3B	4	5	6	7	8	9
Age at first FBC	5 y 2 mo	1 wk	4.5 mo	1 wk	5 mo	3 mo	7.5 mo	7 mo	11 mo	2 y 1 mo
Hemoglobin (g/dL)	9.7	19.6	12.1	21.5	9.7	10.1	10.1	8.4	9.9	30.5
WBC ($\times 10^9$/L)	14.5	19.9	28.2	35.2	12.6	21.9	23.1	20.1	30.7	7.4
% Neutrophils	50	62	38	51	23	50	32	24	31	46
% Lymphocytes	48	30	38	30	63	27	58	71	50	43
% Monocytes	2	8	11	9	2	20	1	5	12	5
% Eosinophils	0	NR	0	0	6	2	2	0	2	0
% Metamyelocytes	0	NR	4	NR	NR	NR	5	0	0	2
% Myelocytes	0	NR	6	NR	5	NR	1	0	5	3
% Basophils	0	NR	3	NR	0	NR	0	0	0	1
Nucleated RBC (per 100 WBC)	1	NR	7	NR	16	NR	2	13	18	36
Platelets ($\times 10^9$/L)	199	300*	151	122	90	30	189	130	60	46

*Patient 2 demonstrates how rapidly thrombocytopenia can evolve, because a pretransplantation platelet count of 22 $\times 10^9$/L was documented just 1 month later.

FBC indicates full blood count; WBC, white blood cell; RBC, red blood cell; NR, not reported.

and gum hypertrophy. Irritability was ascribed to multiple fractures, but spasticity developed during the course of transplantation, suggesting neuronopathic OP (a variant in which osteosclerosis is part of a generalized neurometabolic disease; when recognized early, this form of OP is not a candidate for transplantation because the progression of neurologic disease is unaffected by SCT) [13]. During subsequent SCT, ablative chemotherapy was poorly tolerated, and severe veno-occlusive disease ensued that resulted in death at day 12 after SCT. At postmortem examination, neuronal eosinophilic inclusions were identified, thus confirming the diagnosis of neuronopathic OP.

Progenitor Assays

Mononuclear cells (MNCs) were separated by Ficoll-Hypaque density gradient centrifugation (Gibco; Invitrogen Corporation, Paisley, UK), and residual red blood cells were lysed with an ammonium chloride buffer. Assays were performed on fresh MNC preparations from 3 patients (patients 2, 4, and 6) and on cryopreserved MNCs from the remaining 7 patients. The content of CFU–granulocyte-macrophage (CFU-GM) and burst-forming unit–erythroid (BFU-E) progenitors was evaluated in standard short-term methylcellulose assays [14]. Cultures were initiated in triplicate at 10^4 to 10^6 cells per milliliter and supplemented with erythropoietin (3 IU/mL), interleukin-3 (10 ng/mL), granulocyte-macrophage colony-stimulating factor (10 ng/mL), and stem cell factor (50 ng/mL; all R&D systems, Europe, Ltd., Abingdon, UK). Plates were incubated at 37°C in 5% CO_2 for 14 days, and colonies were then counted by using an inverted microscope. The controls included 2 PB samples from healthy volunteers and 4 normal BM samples from volunteer unrelated donors, collected with consent on the day of allogeneic transplantation.

Fibroblast colony-forming assays (CFU-F) assays were performed in parallel on PB samples from patients 1, 6, 7, and 8 and BM samples from 10 healthy adult volunteer unrelated donors. Red blood cells were lysed with ammonium chloride and resuspended in MesenCult medium (StemCell Technologies Inc., Vancouver, Canada) at concentrations ranging from 1 to 5 $\times$ 10^5/mL (MIOP samples) or 0.5 to 2 $\times$ 10^5/mL (normal BM) in 25-mL tissue culture flasks (Farenheit Lab Supplies, Milton Keynes, UK). After incubation at 37°C for 14 days, medium was removed, and adherent cells were fixed, stained with Giemsa solution (BDH, Poole, UK), and counted.

Presence of Stimulatory Cytokines

To exclude the presence of increased levels of stimulatory cytokines in MIOP serum contributing to high PBSC counts, 100 μL of patient serum from patient 2 (taken just before the commencement of conditioning therapy) was added to a set of clonogenic assay plates initiated with 10^4 normal BM cells. Normal human AB serum (100 μL) was also added in triplicate as a control. The cultures were incubated and scored as described previously.

Immunophenotypic Analysis

Phenotypic analysis was performed by flow cytometry on the Coulter EPICS XL-MCL (Beckman Coulter, High Wycombe, UK) as described previously [15]. The percentage of $CD45^+$ MNCs (fluorescein isothiocyanate–labeled anti-CD45; Sigma Diagnostics, St. Louis, MO) that expressed the CD34 antigen (phycoerythrin-labeled anti-CD34; BD Biosciences, Oxford, UK) was determined by assessing a minimum of 50 000 gated events.

Autologous PBSC Harvests

PBSC backups were obtained in 7 patients either by removal of a sufficient volume of blood (if the

Table 2. *CD34 Content and Progenitor Counts of Peripheral Blood Samples from MIOP Patients*

Patient No.	Age at Time of CD34 Assay (mo)	WBC at Time of CD34 Assay ($\times 10^9$/L)	Degree of Hepatomegaly (L) and Splenomegaly (S) (cm)	Absolute CD34 × 10^7/L (% of MNC)	Total Blood Collected (mL) for 2×10^6 CD34/kg	Required Blood Volume (mL/kg)
2	2.5	13.1	L 2.5, S 4.5	83.8 (6.40%)	9.5	2.1
3A	5	19.3	L 3.5, S 5	156.0 (8.10%)	8.3	1.3
3B	1	14.3	No hepatosplenomegaly	22.9 (1.60%)	35.8	8.7
4	6.5	12.3	L 1, S 4	36.9 (3.00%)	35.2	5.4
6	7.5	15.8	L 2, S 2	43.4 (2.8%)	23.5	4.5
7	6	16.6	L 4, S 3	17.3 (1.04%)	83.2	11.6
8	13	9.7	L.6, S 2.5	31.0 (3.20%)	42.6	6.5
9	26	23.1	L 4, S.4.5	26.3 (1.14%)	83.7	7.6
Mean ± SD		15.5 ± 4		52 ± 17 (3 ± 0.9%)	40 ± 29	6.0 ± 3.4

CD34 content is expressed as a percentage of mononuclear cells. Patients 3A and 3B were siblings. Defects in the freeze/thaw process may have affected viability in the sample from patient 3A (assessed by trypan blue exclusion as 25%), thus explaining the marked differences in progenitor cell content between siblings. The volume of PB required to produce an autologous harvest containing 2×10^6 CD34 cells per kilogram of recipient weight was estimated; where a harvest was performed, the actual volume required to achieve this yield is also shown. The amount of blood required for a harvest of 2×10^6 CD34 cells per kilogram is presented on a per-kilogram basis.

WBC indicates white blood cells.

predicted volume to achieve a yield of 2×10^6 $CD34^+$ cells per kilogram was <7 mL/kg) or by limited exchange. Exchanges were performed against fresh whole blood (collected in citrate phosphate dextrose and transfused through a blood warmer) in 10-mL aliquots with the aim of exchanging a maximum of 100 mL per 15-minute period. Serum calcium was checked at the mid and end points of the procedure, and temperature, pulse, respiration rate, and blood temperature were recorded at the same times. No adverse events occurred during any procedure.

PB $CD34^+$ cell counts were low just before harvest (1.2 and 4.34×10^7/L, respectively) in patients 1 and 5, children with intermediate and neuronopathic forms of OP, respectively. Granulocyte colony-stimulating factor (G-CSF; 10 μg/kg/day) was therefore administered to these children for 1 and 5 days, respectively, before exchange transfusion.

RESULTS

Progenitor Cell Counts

The results of $CD34^+$ cell assays and CFU assays in PB samples from MIOP patients are shown in Table 2, excluding results from the 2 patients (patients 1 and 5) with atypical OP. When first investigated at 4 years, patient 1 had a $CD34^+$ cell count of 0.1% and correspondingly low progenitor assay results. Patient 5 had a $CD34^+$ cell count of 40.2×10^7/L when first studied at 2 months of age, but this decreased spontaneously to 4.3×10^7/L just 2 weeks later without therapy. Progenitor studies in this patient were conducted on a sample taken at autologous PBSC collection taken after 5 days of G-CSF stimulation. By this stage, the absolute $CD34^+$ cell count had increased from 4.3×10^7/L to 23.0×10^7/L, and the CFU-GM and BFU-E precursor frequencies were 202 ± 10 and 621 ± 31 per 10^5 cells, respectively. The BFU-E from patient 5 were also unusually large and well hemoglobinized.

One patient (patient 3A), the sister of patient 3B, had a 10-fold lower frequency of progenitors. Extended storage of the sample may have contributed to this discrepancy (after thawing, the contents of the ampoule showed only 25% viability by trypan blue exclusion), although this seems unlikely to be the sole cause. The relatively low progenitor potential of cells from patient 9 is unexplained, although it was noteworthy that most CFU-GM colonies derived from this child were particularly large (up to 10^6 cells per colony).

Assay results for normal PB and BM samples are shown in Table 3. As expected, the $CD34^+$ percentages of normal (unstimulated) blood were very low,

Table 3. *Progenitor Assays from Normal PB and BM Samples and OP Patients*

Patient No.	CFU-GM/10^5 Cells	BFU-E/10^5 Cells
2	780 ± 75	460 ± 10
3A	19 ± 3	12 ± 2
3B	310 ± 60	110 ± 7
4	750 ± 38	227 ± 9
6	555 ± 45	290 ± 90
7	660 ± 10	360 ± 80
8	157 ± 9	145 ± 20
9	65 ± 7	39 ± 4
OP patients (mean ± SD)	412 ± 312	205 ± 158
Control PBMC (n = 2)	0.7 ± 0.15	1.8 ± 0.2
Control BM (n = 8)	341 ± 150	277 ± 166

The CFU-GM and BFU-E progenitor cell content in peripheral blood from OP patients was comparable to that of marrow from volunteer unrelated adult bone marrow donors but was significantly different from assays from normal adult PB ($P \leq .02$).

WBC indicates white blood cells; PBMC, peripheral blood mononuclear cells.

and their ability to produce progenitors was limited. These differences contrasted with those of the MIOP PB, for which the $CD34^+$ cell percentages ranged from 1.04% to 8.1% in the remaining 8 children, a result very similar to that obtained from normal BM (1.5%-3.2% $CD34^+$ cells after an MNC enrichment process). The proliferative capacity of MIOP PB was comparable to that of normal BM. The addition of serum taken at the time of exchange transfusion from an MIOP patient (patient 2) to one of the normal BM samples neither enhanced nor inhibited progenitor proliferation (data not shown). This suggests that the high frequency of myeloid and erythroid colonies in MIOP PB was not due to the presence of a stimulatory cytokine in the serum, although these tests were not repeated with serum from other patients.

CFU-F were generated from all normal BM samples. Their frequency showed the expected linear relationship with number of cells plated: 27 ± 3 colonies were formed when cultured at 10^5 cells per milliliter. Of the MIOP samples, 2 (patients 7 and 8) showed dispersed fibroblasts, but none grew CFU-F colonies, thus implying that these cells lack mesenchymal stem cells.

Autologous Harvest Potential and Reinfusions

The estimated and actual PB harvest requirements to provide a potential backup of stem cells (2×10^6 $CD34^+$/kg body weight) before BM transplantation were calculated (Table 2). For the 8 patients with typical MIOP (including patient 5 after G-CSF stimulation), this ranged from only 8.3 to 83.7 mL, equating to a CFU-GM content of 0.5 to 126×10^4/kg, respectively. In patient 1, the calculated volume was 482 mL, although only 300 mL was taken at exchange transfusion.

Autologous PB backups were returned to 2 children (patients 1 and 4). Both had undergone high-dose haploidentical parental SCT after conditioning therapy with busulphan, cyclophosphamide, and antithymocyte globulin, and both experienced primary graft rejection confirmed by HLA typing of residual white blood cells. The $CD34^+$ cell content in returned MNCs was 1.26 and 2.35×10^6/kg for patients 1 and 4, respectively.

In patient 1, the transplantation had been complicated by acute respiratory obstruction requiring urgent tracheostomy (on a background of long-standing upper respiratory tract obstruction exacerbated by mucositis), adenovirus infection, and early cytomegalovirus reactivation. After transient donor neutrophil recovery, the lymphocyte count increased, and neutrophils decreased to 0; the lymphocyte population was shown by microsatellite polymerase chain reaction to be exclusively of recipient origin, and this suggested graft rejection. Because of the patient's critical clinical situation, autologous PB was reinfused on day 15 after transplantation (the lymphocyte count at this stage was 0.7, and neutrophils were 0×10^9/L). Neutrophils were sustained $>0.5 \times 10^9$/L on 3 consecutive days by 17 days after reinfusion. Recovery was slower in patient 4, who received autologous reinfusion on day 19 after SCT (the white blood cell count was 0 at infusion). Although neutrophils of 0.3×10^9/L were achieved 13 days later, a sustained neutrophil count of $>0.5 \times 10^9$/L was not reached until 27 days after reinfusion. Platelet independence followed 12 days later. These children were subsequently discharged 57 and 55 days after their initial allogeneic transplantations.

Posttransplantation Observations

Posttransplantation $CD34^+$ assays were performed in only 2 children. Patient 2 underwent transplantation for a form of MIOP that showed greatly increased numbers of osteoclasts on biopsy from a 1 antigen–mismatched sibling donor. The PB $CD34^+$ cell count was 91.4×10^7/L before transplantation. A sustained neutrophil count of $>0.5 \times 10^9$ was achieved by day +26, and $CD34^+$ cell counts decreased to $<1 \times 10^3$/mL on day +14 and 2.3×10^3/mL on day +28. Patient 6 was also studied at 4 and 6 months after transplantation, and the $CD34^+$ cell count was 4×10^3/mL and 1×10^3/mL, respectively.

DISCUSSION

It has long been recognized that children with MIOP have a leukoerythroblastic blood picture and that myeloid colonies can be freely grown from peripheral blood mononuclear cells; indeed, this growth can be so exuberant as to impair interpretation of mixed lymphocyte reactions performed for graft-versus-host disease prediction unless recipient MNCs are first isolated [16]. This is assumed to be due to displacement of hemopoiesis to the extramedullary compartment and is reminiscent of primary myelofibrosis. In the latter, PB has been shown to contain 25- to 75-fold more CFU-GM precursors than normal PB and 13-fold more BFU-E [17-19].

We have been concerned that patients undergoing alternative donor SCT for MIOP are required to undergo transplantation without previously storing autologous cells, even though graft failure is common in this patient population [5]. This is particularly true where T-cell depletion has been used, especially with Campath antibodies and after haploidentical transplantations. Although further infusions from the original, or a second, allogeneic donor can be used in this circumstance, autologous rescue may be preferred by some families or may be dictated by clinical circumstance. Examples of the latter include situations in which severe infection occurs (eg, patient 1) or addi-

tional stem cells cannot be sourced (eg, cord blood transplants).

We reasoned that high precursor frequencies may be reflected by high spontaneous PBSC counts and that these would facilitate easy collection of autologous harvests capable of accelerating return to transfusion independence if reinfused after rejection. This study has shown that the vast majority of patients with severe MIOP have extremely high numbers of circulating PBSCs (typically in the range of 1%-5%) and that these have progenitor potential comparable to that of normal donor BM. However, they behave like PBSC or cord blood samples in their inability to generate CFU-F, and this implies a lack of significant mesenchymal stem cells. One proposed mechanism of this increase in circulating undifferentiated cells may be differences in the presence of cytokines stimulatory to these cells in patients with severe OP. However, when serum from a patient with OP and a high proportion of circulating $CD34^+$ cells was added to an in vitro colony-forming assay, no change was observed in the number of colonies (data not shown). The density of PBSC is such that very small exchange transfusions (calculated range, 8.3-83.7 mL) may be required to obtain a desirable number of cells for reinfusion (in this study set, the goal was 2×10^6 $CD34^+$ cells per kilogram). Arguably, it may be wise to take multiples of this figure if it can be done easily, to maximize the reconstitution potential of the harvest. We therefore took volumes up to 300 mL in children ranging in weight from 3.8 to 12.1 kg, all without clinical incident and in a procedure lasting 15 to 45 minutes. Exchange transfusion was preferred over repeated blood draws to avoid multiple cryopreservation procedures, although invariably the concentration of $CD34^+$ cells will decrease during a single exchange procedure.

The return of autologous PBSCs in 2 patients allowed transfusion independence and successful discharge. Patient 1 is alive and well 4 years later, although she continues to have low platelet counts in the range of 19 to 55 $\times$ 10^9/L (secondary to the development of antiplatelet antibodies that caused her to be platelet refractory during transplantation). Patient 4 subsequently underwent a second elective transplantation, but this was complicated by fatal pulmonary arterial hypertension, which is now recognized as a common complication of SCT for MIOP [20,21]. It cannot be known to what extent the autologous reinfusions speeded count recovery, although we were encouraged that both patients recovered uneventfully from their rejection and infection episodes. Of particular note, patient 1 had undergone haploidentical transplantation complicated by respiratory obstruction requiring emergency tracheostomy; cytomegalovirus; and adenovirus infections. Considering the ease of performing these collections, we advocate that this should become standard practice for any child undergoing alternative donor transplantation for MIOP.

There are 2 potential added values to the collection of PB in OP children. The first provocative observation is that the 2 children with the lowest $CD34^+$ cell counts had atypical clinical forms of OP; if confirmed in larger studies, this may allow better identification of such patients. We also will follow up future patients to see how $CD34^+$ cell counts vary with time in untransplanted disease. The second has come with the development of techniques for growing osteoclasts from PB or BM in vitro on animal bone slices in the presence of macrophage colony-stimulating factor and receptor activator nuclear factor-κB ligand [10]. Osteoclasts can be identified unequivocally by coexpression of calcitonin and $\alpha_v\beta_3$ vitronectin receptors, cathepsin K, and tartrate-resistant acid phosphatase, thus allowing assessment of their ability to be formed, seal on to the bone surface, and form resorption pits. With this technique, it has been possible to show that children with the less common hypo-osteoclastic variants of OP will not form osteoclasts in vitro; this shows that their mechanism of disease is different and is not due to a lack of intrinsic macrophage colony-stimulating factor or receptor activator nuclear factor-κB ligand production [9].

Currently, the molecular mechanisms and genetics of this disease are still unresolved for approximately one third of affected families [22,23]. For these, antenatal diagnosis is still restricted to ultrasound or plain radiologic studies late in pregnancy—investigations with a recognized failure rate [24]. The many animal models of OP [25] and the very variable phenotype (eg, classic, neuronopathic, and intermediate) suggest that a range of genes may be involved. Mechanistic in vitro analysis of their osteoclast function may eventually allow grouping of families with similar pathology and, hence, more refined and productive searches for the causative genes.

In conclusion, we propose that the collection of PBSCs by drawing sufficient blood or by limited exchange transfusion should be considered for all children about to undergo SCT for MIOP from alternative donors. The procedure is quick and well tolerated, and the autologous harvest obtained could provide an option not otherwise available should life-threatening graft failure or infection occur. We also suggest that measurement of PB $CD34^+$ cells, in conjunction with bone biopsies and in vitro culture of osteoclasts, may allow valuable insights into the pathophysiology of the unclassified forms of human OP.

REFERENCES

1. Fasth A, Porras O. Human malignant osteopetrosis: pathophysiology, management and the role of bone marrow transplantation. *Pediatr Transplant*. 1999;3(suppl 1):102-107.

2. Key LL Jr, Ries WL, Rodriguiz RM, et al. Recombinant human interferon gamma therapy for osteopetrosis. *J Pediatr.* 1992; 121:119-124.
3. Gerritsen EJ, Vossen JM, van Loo IH, et al. Autosomal recessive osteopetrosis: variability of findings at diagnosis and during the natural course. *Pediatrics.* 1994;93:247-253.
4. Key LL Jr, Rodriguiz RM, Willi SM, et al. Long-term treatment of osteopetrosis with recombinant human interferon gamma. *N Engl J Med.* 1995;332:1594-1599.
5. Driessen GJ, Gerritsen EJ, Fischer A, et al. Long-term outcome of haematopoietic stem cell transplantation in autosomal recessive osteopetrosis: an EBMT report. *Bone Marrow Transplant.* 2003;32:657-663.
6. Gerritsen EJ, Vossen JM, Fasth A, et al. Bone marrow transplantation for autosomal recessive osteopetrosis. A report from the Working Party on Inborn Errors of the European Bone Marrow Transplantation Group. *J Pediatr.* 1994;125:896-902.
7. Cournot G, Petrovic M, Trubert CL, et al. Cultured circulating mononuclear cells from osteopetrotic infants express the osteoclast-associated vitronectin receptor and form multinucleated cells in response to 1,25-dihydroxyvitamin D3. *J Bone Miner Res.* 1993;8:61-70.
8. Marcus JR, Fibach E, Aker M. Circulating myeloid and erythroid progenitor cells in malignant osteopetrosis. *Acta Haematol.* 1982; 67:185-189.
9. Flanagan AM, Massey HM, Wilson C, et al. Macrophage colony-stimulating factor and receptor activator NF-kappaB ligand fail to rescue osteoclast-poor human malignant infantile osteopetrosis in vitro. *Bone.* 2002;30:85-90.
10. Flanagan AM, Sarma U, Steward CG, et al. Study of the nonresorptive phenotype of osteoclast-like cells from patients with malignant osteopetrosis: a new approach to investigating pathogenesis. *J Bone Miner Res.* 2000;15:352-360.
11. Shapiro F. Osteopetrosis. Current clinical considerations. *Clin Orthop*. 1993;34-44.
12. Srinivasan M, Abinun M, Cant AJ, et al. Malignant infantile osteopetrosis presenting with neonatal hypocalcaemia. *Arch Dis Child Fetal Neonatal Ed.* 2000;83:F21-F23.
13. Steward CG. Neurological aspects of osteopetrosis. *Neuropathol Appl Neurobiol.* 2003;29:87-97.
14. McNiece IK, Langley KE, Zsebo KM. Recombinant human stem cell factor synergises with GM-CSF, G-CSF, IL-3 and epo to stimulate human progenitor cells of the myeloid and erythroid lineages. *Exp Hematol.* 1991;19:226-231.
15. Clarke E, Potter MN, Oakhill A, et al. A laboratory comparison of T cell depletion by CD34+ cell immunoaffinity selection and in vitro Campath-1M treatment: clinical implications for bone marrow transplantation and donor leukocyte therapy. *Bone Marrow Transplant.* 1997;20:599-605.
16. Sieff CA, Chessells JM, Levinsky RJ, et al. Allogeneic bone-marrow transplantation in infantile malignant osteopetrosis. *Lancet.* 1983;1:437-441.
17. Chikkappa G, Carsten AL, Chanana AD, et al. Increased granulocytic, erythrocytic, and megakaryocytic progenitors in myelofibrosis with myeloid metaplasia. *Am J Hematol.* 1978;4:121-131.
18. Andreasson B, Swolin B, Kutti J. Patients with idiopathic myelofibrosis show increased CD34+ cell concentrations in peripheral blood compared to patients with polycythaemia vera and essential thrombocythaemia. *Eur J Haematol.* 2002;68:189-193.
19. Hibbin JA, Njoku OS, Matutes E, et al. Myeloid progenitor cells in the circulation of patients with myelofibrosis and other myeloproliferative disorders. *Br J Haematol.* 1984;57:495-503.
20. Steward CG, Pellier I, Mahajan A, et al. Severe pulmonary hypertension: a frequent complication of stem cell transplantation for malignant infantile osteopetrosis. *Br J Haematol.* 2004; 124:63-71.
21. Kasow KA, Bonfim C, Asch J, et al. Malignant infantile osteopetrosis and primary pulmonary hypertension: a new combination? *Pediatr Blood Cancer.* 2004;42:190-194.
22. Sobacchi C, Frattini A, Orchard P, et al. The mutational spectrum of human malignant autosomal recessive osteopetrosis. *Hum Mol Genet.* 2001;10:1767-1773.
23. Frattini A, Pangrazio A, Susani L, et al. Chloride channel ClCN7 mutations are responsible for severe recessive, dominant, and intermediate osteopetrosis. *J Bone Miner Res.* 2003; 18:1740-1747.
24. Sen C, Madazli R, Aksoy F, et al. Antenatal diagnosis of lethal osteopetrosis. *Ultrasound Obstet Gynecol.* 1995;5:278-280.
25. Popoff SN, Schneider GB. Animal models of osteopetrosis: the impact of recent molecular developments on novel strategies for therapeutic intervention. *Mol Med Today.* 1996;2:349-358.