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Replacement of Recipient Stromal/Mesenchymal Cells After Bone Marrow Transplantation Using Bone Fragments and Cultured Osteoblast-Like Cells

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ABSTRACT

We present our experience on treatment of three children with potentially fatal diseases using a unique protocol for non-myeloablative bone marrow transplantation. The protocol was designed to promote engraftment of bone marrow stromal/mesenchymal cells (SC/MSCs) based on the knowledge from preclinical models over the last three decades. Accordingly, our protocol is the first to test the use of bone fragments as an ideal vehicle to transplant such cells residing in the bone core. Because of the paucity of knowledge for optimum transplantation of SC/MSCs in humans, we used a multifaceted approach and implanted bone fragments both intraperitoneally and directly into bone on day 0 of BMT. We also infused cultured donor osteoblast-like cells intravenously post-BMT. We were able to achieve high levels of stroma cell engraftment as defined by molecular analyses of bone biopsy specimens.

KEY WORDS

Stroma • Mesenchymal cells • Osteoblasts • Bone fragments

INTRODUCTION

The adult mammalian bone marrow microenvironment comprises several different elements that together are responsible for the support of the hematopoietic stem cell, [1-3], B-cell ontogeny [4-6], and bone homeostasis [7,8]. It includes various cell types, including reticular cells, adipocytes, osteoprogenitors, endothelial cells and macrophages, which make up the stroma and are now known collectively as mesenchymal cells (reviewed in [8,9]). Early stem cells and B cells in situ or in vitro have been more closely identified to be in contact with the endosteal surface in direct proximity to the osteoblasts as an important source of chemokines for homing [10,11], growth factors for early hemopoiesis [3,12-14], and B cell development [15,16]. These stromal cells/mesenchymal cells (SC/MSCs) can be isolated from the adherent [17-21] and, more recently, from the nonadherent fraction of bone marrow [22,23] as well as bone [24,25]. After bone marrow transplantation (BMT), however, replacement of these stromal elements does not appear to occur and is the subject of considerable debate. [8, 26-38] Substitution does not take place despite the fact that the stroma may be abnormal or damaged in a variety of malignant and human genetic diseases (eg, lysosomal storage disease, Fanconi's anemia, Shwachman-Diamond syndrome, osteogenesis-imperfecta) [30,39-41]. In malignant disease, the marrow may be further damaged by high-dose chemotherapy [42-46]. The failure to replace the stroma with donor cells after BMT is thought to be responsible for poor engraftment [43,47,48], immune recovery [4-6,15], and increased bone turnover [49]. Analysis of the adherent fraction of bone marrow cells after BMT has revealed that these cells are mostly of recipient origin but may be contaminated with small populations of donor macrophages and lymphocytes [35,36,47]. Several studies have tried to engraft cultured SC/MSCs in autologous breast cancer patients and allogeneic patients with the skeletal dysplasia of

Table 1. *Donor Stromal Cell Engraftment of Patients Transplanted with Unfractionated Bone Marrow Cells, Bone Chips, and Ex Vivo Expanded Bone Marrow Mesenchymal Cells*

Patient Information*			Stem Cells†						Chimerism‡	
No.	Diagnosis	Sex	Age	Donor	TNC	CD34+	Bone chip	OBLC (day)	PBMNC	Bone biopsy (day)
1	IH	F	9 months	Dad (4/6)	1.4	0.2	Yes	Yes (+17)	0%	++ (+606)
2	HS	M	9 years	Sister (6/6)	3.5	2.7	Yes	Yes (+13)	13%→0%	26–50% (+423)
3	AD	F	12 years	Mom (6/6)	5.0	1.5	Yes	Yes (+13)	81%→97%	66% (+270)

*IH, infantile hypophosphatasia; HS, Hunter syndrome; AD, autoimmune disease with overlap syndrome and lung vasculitis.

†Bone marrow stem cells, donor source and HLA matching in parenthesis; TNC, total numbers of marrow: cells infused $\times 10^8$/kg containing CD34+ cells count $\times$ 10^6/kg on day 0; OBLC, infusion of ex vivo expanded donor OBLCs and posttransplant day of infusion in parentheses.

‡Percent donor chimerism in peripheral blood mononuclear cells (PBMNCs) and bone biopsy on posttransplant days in parentheses.

lysosomal storage diseases but to no avail [30,50]. Preclinical studies by Ikehara and Good have shown that stromal cells from bone fragments placed intraperitoneally can migrate to the stromal and other tissues, enhance engraftment, and induce tolerance across major histocompatibility complex (MHC) barriers [51-53]. The same group could accomplish replacement of the stroma by directly injecting bone marrow into the bone marrow cavity [54]. It was also determined that the osteoblasts could also facilitate engraftment and immune recovery if cotransplanted with stem cells [55].

It was our aim to replace the stromal elements of bone marrow in patients with potentially fatal diseases to improve engraftment and facilitate immune recovery and eventually arrest the underlying disease process. This postulate was based on our hypothesis that to replace the stroma, bone fragments represent the ideal vehicle [21] and contain more SC/MSC cells [24,25], but must be placed in an appropriate microenvironment to receive the appropriate signals for proliferation and differentiation.

Three patients with potentially fatal disease were transplanted in this pilot study. The patient is with infantile hypophosphatasia presented with failure to thrive and progressive rickets at age 6 months. The second child was a 9-year-old with Hunter syndrome with some involvement of the central nervous system (CNS). Transplantation in Hunter syndrome is controversial [56], despite full lymphohematopoietic engraftment, the donors derived enzyme is not transported to the sites of substrate accumulation. The purpose was to determine whether the engraftment of bone marrow/peripheral blood as well as stromal cells could stop the further accumulation of glycosaminoglycans in tissues and repair the already damaged tissues, an experiment subsequently tried in 2 other storage diseases [57]. The third patient had an autoimmune disease classified as overlap syndrome with vasculitis, involving the skin, lung, and CNS. She was refractory to all immunosuppressive therapy and was a candidate for an allogeneic transplant.

We used a different approach to standard BMT by placing bone fragments intraperitoneally (IP) and directly into bone along with bone marrow given systemically. Cultured osteoblast-like cells (OBLCs) were given by the intravenous (IV) route. Biopsy specimens of the bone rather than bone marrow aspirates were used to demonstrate evidence of donor stroma engraftment to avoid macrophage contamination.

PATIENTS, MATERIALS AND METHODS

Preparative Regimens for Transplantation

The BMT protocol and informed consents were approved by All Children's Hospital Institutional Review Board. Patient 1, with a mismatched related donor (father), was conditioned with fludarabine (30 mg/M^2 $\times$ 5 days), low-dose busulfan (8 mg/kg), and anti-thymocyte globulin (ATG; 30 mg/kg $\times$ 3 days), and then infused systemically with T-cell–depleted (e-rosetted) bone marrow cells (Table 1). Two patients with HLA-matched related donors (patients 2 and 3) underwent a preparatory treatment of fludarabine (30 mg/M^2 $\times$ 5 days), cyclophosphamide (50 mg/kg $\times$ 2 days), and TBI (200 cGy) before IV infusion of unfractionated bone marrow cells from fully matched related donors on day 0. All patients received posttransplant immunosuppression for graft-versus-host disease (GVHD) prophylaxis with mycophenolate (MMF), 15 mg/kg p.o. bid for 25 days, and cyclosporine (CSA), 6.25 mg/kg p.o. bid, until good evidence of engraftment or graft rejection was determined.

Harvesting and Placement of Bone Fragments

The protocol included transplantation of donor bone fragments on day 0. Six bone fragments were obtained from each donor using a Jamshidi biopsy needle at the time of bone marrow harvest. Four bone fragments were then transferred into the adjacent operating room in medium 199 (Gibco, Rockville, MD). Two bone fragments from the donor were then placed by the surgeon IP under light anesthesia (Dirrivan; Gensia Sicor, Irvine, CA) through an incision near the umbilicus. The patient was then placed in the prone position for the removal and insertion of 2 bone fragments. After additional local anesthesia, the biopsy

needle was inserted into the patient's iliac crest, and a piece of the recipient's bone was removed with an extraction cannula, with the biopsy needle left in place in the hole. An extraction cannula with the donor specimen was then reinserted into the needle cannula, followed by insertion of a cannula to force the donor bone fragment into the defect.

The remaining 2 donor bone core biopsy specimens were expanded ex vivo for OBLCs. In the patient with hypophosphatasia (patient 1), the bone harvest and the implantation procedure were delayed for 17 days because of concern over possible iatrogenic effects of ATG on osteoblast cells known to be Thy-1+. [58] In addition, this patient had soft bones indistinguishable from other soft tissues, thus lacking firm landmarks to allow the placing of donor bone fragments directly into the iliac crest.

Cultures of Osteoblast-Like Cells

OBLCs were grown in culture using DMEM/F12 medium (Celgro; Fisher, Pittsburgh, PA) containing 10% fetal calf serum (Hyclone, Logan, UT) as originally described by Robey and Termine [59] with some modification introduced in our laboratories [55]. Briefly, under sterile conditions, small pieces of bone were placed between 2 glass slides and crushed into small particles by gentle pressure and then transferred into a tube with medium and vortexed several times to wash out attached marrow cells. Collagenase (1 mg/mL) (Sigma-Aldrich, St. Louis, MO) was added, and the tubes were incubated for 2 hours at 37°C with continuous shaking. The contents including the bone pieces were transferred to plastic culture flasks with additional medium and incubated for 10 days at 37°C and 5% CO_2. The adherent cells were then incubated with L-ascorbate (final concentration 10 mmol) and B-glycerol phosphate (50 μg/mL)(Sigma-Aldrich) for another 48 hours, harvested by brief trypsinization, washed, counted, suspended in medium 199 and infused IV into the patients on days +13 or + 17 after the bone marrow infusion. Because osteoblasts lack definitive markers, cells were characterized previously by morphology as well as by staining for alkaline phosphatase (staining kit 86-R; Sigma-Aldrich) and calcium deposition with the von Kossa technique (silver-gelatin) [60]. In our hands, the cells were a homogenous population of mottled basophilic cells with eccentric nucleoli and a web-like cytoplasm when stained with Wright–Giemsa. Although we did not exclude contamination by other mesenchymal cells, these cultures did not have a morphology characteristic of fibroblasts.

Analysis of Engraftment

Engraftment analysis for donor hematopoietic stem cells (HSCs) was studied in peripheral blood and bone marrow cells using fluorescence in situ hybridization (FISH) for X and Y chromosomes (patients 1 and 2, who had sex-mismatched donors) or polymerase chain reaction (PCR) for short tandem repeat (STR) polymorphisms (patient 3). Engraftment of donor SC/MSC was isolated from bone core biopsy tissue harvested from the posterior iliac crest using PCR for male-specific sex-determining region (SRY) sequences (patient 1) or for dinucleotide and trinucleotide STR polymorphisms (patients 2 and 3). For Patients 1 and 2, the SC/MSC chimerism was studied in freshly harvested bone tissue; the bone core fragment was washed to purge bone marrow and peripheral blood cells, ground using a mortar and pestle, and pelleted by gentle centrifugation. DNA extracted from cells in the supernatant was used for PCR. For patient 3, the chimerism was measured using ex vivo expanded cultured cells from a bone core biopsy specimen. The bone fragment was washed, ground, and cultured in RPMI medium with 20% fetal calf serum, 1% penicillin, 1% streptomycin, 1% fungizone, and 1% L-glutamine for 11 days. All nonadherent cells were removed during the culture period. Morphologically, there was an outgrowth of fibroblast-like adherent cells in the culture (> 99%). No macrophages were seen or tested for. The DNA was extracted and analyzed for STR polymorphism.

FISH and PCR Analyses

The FISH and PCR analyses were conducted by the clinical laboratories of All Children's Hospital using standardized methods with commercially available probes (Vysis, Inc, Downers Grove, IL). The primers for SRY were SRY (XES2): 5′-CTG TAG CGG TCC CGT TGC TGC GGT G-3′ and SRY (XES7): 5′-GAC AAT GCA ATC ATA TGC TTC TGC-3′. The primers for STRs were GABRB3: (forward) 5′-CTC TTG TTC CTG TTG CTT TCA ATA CAC-3′ and (reverse) 5′-CAC TGT GCT AGT AGA TTC AGC TC-3′ [61]; D9S15: (forward) 5′-TAA AGA TTG GGA GTC AAG TA-3′ and (reverse) 5′-TTC ACT TGA TGG TGG TAA TC-3′ [62]; MDPK: (forward) 5′-GCT CGA AGG GTC CTT GTA GC-3′ and (reverse) 5′-GTG GAG GAT GGA ACA CGG AC-3′ [63]; and IT15: (forward) 5′-ATG AAG GCC TTC GAG GCC TCC CTC AAG TCC TTC-3′ and (reverse) 5′-GGC GGT GGC GCC TGT TGC TGC TGC TGC TGC-3′ [64]. Fragment sizes were run on 4.5% polyacrylamide gels on an ABI 373A automated analyzer (Applied Biosystems, FosterCity, CA) and analyzed using Genescan and Genotyper software packages (Applied Biosystems).

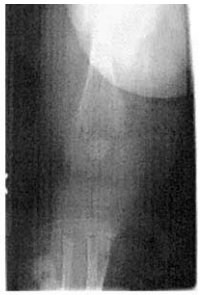

Figure 1. Patient 1: patient at 6 months showing the extensive cupping and flaring with associated erosive changes in the metaphysis of the right femur.

RESULTS

The 3 patients tolerated the BMT protocol without any complications of GVHD or infections. They all have shown clinical improvement in their conditions. The engraftment of donor SC/MSC was convincing in these 3 patients, even though 2 patients lacked any evidence of peripheral or bone marrow engraftment of donor HSC.

Patient 1

A 9-month-old with worsening infantile hypophosphatasia (Figure 1) received bone marrow from her father (4/6 match) on day 0. She had early posttransplant cytogenetics by FISH on days 17 and 55, which revealed only host (XX) cells [65]. But 3 months post-BMT she showed some unexplained improvement in her clinical condition, marked by correction of her hypercalcemia and bone pain with an increase in her appetite and weight. Serial x-rays and DEXA scans of her bones showed gradual but steady improvement of her osteopenia and severe rickets. A PCR analysis of bone biopsy specimen obtained on day +606 revealed a band at the expected base pair marker of 600 for SRY under increased stringency (at 55°C annealing temperature), suggestive of donor SC/MSC engraftment despite negative PCR on peripheral blood or bone marrow cells (even at 60°C annealing temperature) for donor HSC engraftment. Now at 4.6 years post-BMT, the patient is maintaining her height (5%) and weight (7%) and exhibits almost normal mineralization and complete resolution of the radiolucencies. She still has mild scoliosis and bowing of the long bones that is slowly correcting with the help of braces (Figure 2).

Patient 2

A 9-year-old with mucopolysaccharidosis IIB (Hunter syndrome) received bone marrow from his HLA-matched sister. The first cytogenetic study of his peripheral blood revealed 13% donor cells as measured by FISH, which over 1 year decreased to 2% and finally to 0. This patient was being followed post-BMT at the University of Colorado (Dr. Ralph Quinones) and found to have improvement with increased joint range of motion. He is now able to open his fingers and toes, which he was unable to do before the transplant. In addition, the extent of his hepatosplenomegaly is diminishing. There has been no further deterioration in his condition despite a lack of evidence for any circulating iduronate-2-sulfatase enzyme activity. The STR analysis of his bone core biopsy specimen, on posttransplant day +423 using the MDPK and HD-IT15 microsatellite polymorphisms revealed a chimeric status in his bone specimen at 50% and 25% donor markers, respectively, whereas donor chimerism of peripheral blood cells was negative for these markers when measured simultaneously.

Patient 3

A 12-year-old girl with very severe autoimmune disease (overlap syndrome/vasculitis) was given cells from her HLA-matched (6/6) mother. She had serial cytogenetics studies that initially revealed 81%–86% by day +176. On day + 270, the fibroblast cultured from a bone biopsy specimen revealed 66% donor cells as demonstrated by the presence of the donor polymorphism, FA:D9S15 (Figure 3), whereas the bone marrow specimen was found to be 89% donor. Her last peripheral blood analysis on day +640 was all donor cells without any clinical GVHD during the posttransplant course. She slowly was weaned off her CSA by 9 months and off daily steroids by 24 months. Currently, at 39 months posttransplant, she is off all medicines and in clinical remission.

DISCUSSION

Herein we present a modified protocol of nonmyeloablative BMT unique for achieving engraftment of SC/MSCs. Our protocol is distinctive by incorporating implantation of donor bone fragments both IP and directly into bone as well as ex vivo expanded

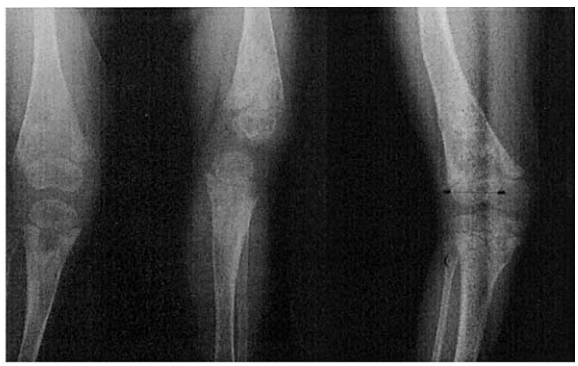

Figure 2. Patient 1: right knee posttransplant at 14, 43, and 49 months showing correction of the rachitic changes with some residual radiolucencies.

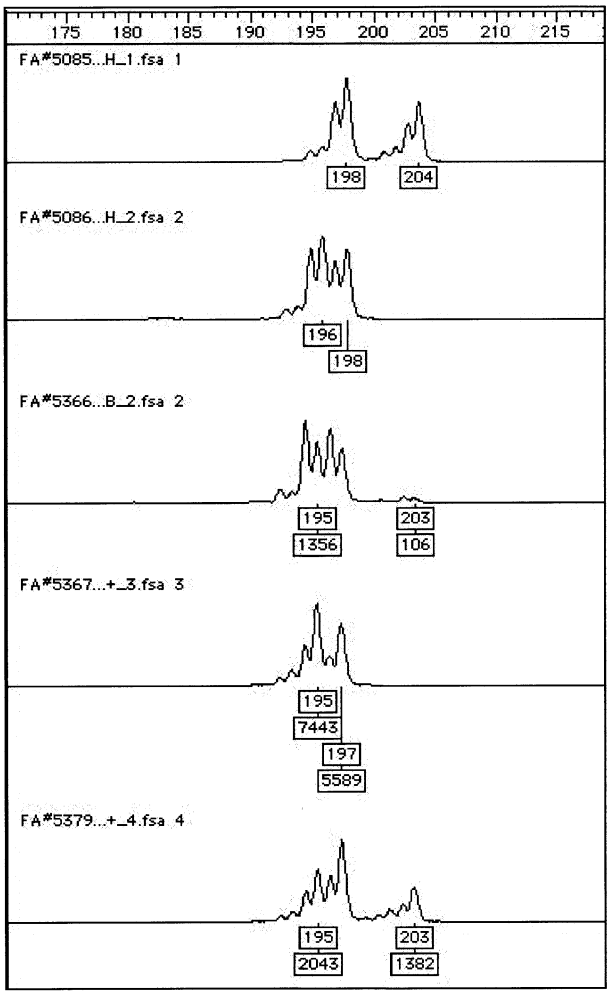

Figure 3. Documentation of successful bone marrow engraftment using DNA polymorphisms. The presence of transplant recipient (#5085) and donor (#5086) genotypes was monitored by PCR amplification using the dinucleotide repeat polymorphism at the FA (D9S15) locus. Flags indicate amplification fragment sizes in base pairs (upper) and peak height (lower). The bone core biopsy specimen (#5366) indicated a donor/recipient chimerism, with a low level (7%) of recipient marker. Bone core biopsy cells were separated into nonadherent cells (#5367) which were entirely donor type, and explanted adherent cells (#5379), which was a chimeric mixture of 40% recipient/ 60% donor marker.

donor bone marrow stromal cells. The protocol developed was an adaptation of preclinical studies over the last 3 decades [19,21,51-53]. It was clearly shown in the laboratories of Drs Good and Ikehara, as well as others, that implantation of donor bone intraperitoneally can promote engraftment of donor stromal cells as well as donor hematopoietic stem cells [51,52,66,68]. The first 2 patients without any evidence of peripheral or bone marrow engraftment had proof of donor stromal cells by PCR on DNA extracted from crushed bone. In patient 3, who is a stable mixed chimera (97% donor), 66% donor cells were detected in fibroblasts cultured from a bone biopsy.

Controversy remains on the ability of unmanipulated bone marrow cells or expanded mesenchymal cells to replace the stroma after infusion despite an abnormal or damaged stroma [8,26,28-30,32,34-38,57,69,70]. One of the first reports of the replacement of stroma with host cells after allogeneic BMT was in 1982 [29]. However, subsequent studies by others have not demonstrated engraftment of donor cells [30,32,35,36,57,71,72]. Simmons et al. were the first to look comprehensively at the stroma and found that the FISH positive donor derived cells were also positive for nonspecific esterase (NSE+), [35] a marker for macrophages. This may have accounted for the false-positive results in some of the previous and current studies [28,29,38,57]. Agematsu et al. performed in situ hybridization studies using Southern-blot analysis and PCR with MCT 118 probes on cultured fibroblasts and found all the cells to be of recipient origin [32]. Lee et al. were concerned over the high incidence of bone turnover, or osteoporosis after transplant [49]. They isolated and induced bone marrow cells to osteoblastic lineage in 7 patients and compared them to cultured cells from their donors. No recipient had evidence of donor osteoblasts as determined by PCR analysis using YN222 mini-satellite probes. Koc et al. analyzed 13 patients with lysosomal and peroxisomal storage disease 1–14 years after BMT and found no evidence of donor cells in the stromal compartment despite having skeletal dysplasia and damaged nervous tissue [30]. Subsequently, they tried to correct both defects by giving expanded (5,000–-10,000-fold) mesenchymal cells at doses of approximately 2–10 $\times$ 10^6/kg, plus bone marrow in a prospective study [57]. In 2 of the 11 patients, they found less than 2% donor cells. There was no improvement of their skeletal dysplasia despite evidence that the expanded cells retained the capacity to form osteoprogenitors. However, this technique may have enhanced engraftment and prevented serious GvHD [30,57,70].

In Seattle 15 years later Awaya et al. continued their earlier studies from 1987 to see if patients 1–27 years post-BMT had now replaced the stroma after full engraftment. Again, they found only small numbers of donor cells, <8.5% (range, 0–8.5%), from 3 patients in long-term bone marrow cultures that were NSE+ [36]. Using a different technique in one patient 8 years after conventional BMT, Awaya et al. established long-term cultures from a bone biopsy specimen [36]. Despite 100% donor engraftment in the bone marrow, there were less than 0.5% donor cells, and <0.1% were NSE+ cells detected in the culture. Earlier reports by other investigators had demonstrated <2.0% donor cells in the stroma isolated from bone marrow after BMT [28,29,38,57].

We tried to circumvent the problem of macrophage contamination by analyzing only bone biopsy

specimens for donor SC/MSC engraftment. Of the first 2 patients, neither had evidence of HSC engraftment in the peripheral blood or bone marrow but patient 2 had >25% donor cells in his bone biopsy specimen. Patient 3′s bone biopsy specimen at day + 270 was cultured for 11 days and grew only fibroblast (> 99%). No macrophages were seen. The fibroblasts were at least 60% donor, as determined by 2 separate donor probes.

There has been no good explanation for the lack of stromal and nonhematopoietic tissue engraftment after the systemic infusion of bone marrow or mesenchymal cells. This question was studied by Noort et al., who examined the homing of fetal mesenchymal cells (fMSCs) as well as the effect on engraftment of stem cells derived from human cord blood in an animal model [73]. The expanded fMSCs from fetal lung showed a fibroblast-like morphology and were able to differentiate into osteoblasts or adipocytes. Although cotransplantation of the fMSCs enhanced engraftment of the human cord blood, these investigators found the fMSCs exclusively in the lung. They hypothesized that the fMSCs were trapped permanently in the lung and were thus unable to migrate to other tissues. This hypothesis was disproven by the intracardiac injection of the fMSCs, which failed to show any cells in the lung as well as other tissues. The cells expanded in culture lost their expression of CD34 as well as of CD50, CD102, CD106, and L-selectin. Other groups also found the loss of adhesion molecules after culture [15,74,75], including the functional expression of all CCR- and CXCR-family members with failure to migrate across Transwell filters [76-78]. These studies suggest that the loss of these adhesion molecules is responsible for the lack of homing and engraftment. Banfi et al. found that MSCs even after the first passages lost some of their ability to proliferate and to differentiate into multiple cell types [79]. The osteogenic potential of these expanded cells also decreased when compared with fresh fibroblasts isolated from bone marrow. Devine et al in a preliminary study in a nonprimate model after systemic infusion demonstrated early homing but nonsustainable engraftment of gene marked mesenchymal cells in recipients' bone and bone marrow [69]. Subsequently, in a follow-up study they were able to show that a very small number (0.1%–2.7%) of cells homed only to and persisted in nonhematopoietic tissues [80].

Although infusion of the ex vivo expanded cells may prove useful, the vehicle providing 3-dimensional scaffolding for the SC is likely to be crucial for introducing large numbers of SCs with preserved functional and morphological properties [21,25,67,68,81-83]. Krebsbach repeated and expanded the earlier experiments of Friedenstein and Haynesworth [84] by comparing CFU-F derived from mice and humans [21]. New bone formation was observed in the human cells only if they were absorbed onto hydroxyapatite/ceramics or first subjected to osteoinductive media before being placed in diffusion chambers. They concluded that the vehicle was important for the proper proliferation and differentiation of these cells into bone.

Reports of the cotransplantation of cultured MSCs and HSC may have enhanced engraftment [50,57,85]. Our method did not enhance engraftment of HSC after a nonmyeloablative preparative regimen in 2 of the 3 patients. This problem is commonly seen in nonmalignant diseases, where the immune system is relatively intact. The exception was patient 3, who had long-term immunosuppressive therapy for her autoimmune disease before the transplant. Also, the infusion of OBLCs was thought to be insignificant due to the small numbers of cells (ratio of 1 to 15,000 TNC) and the data from others showing that these expanded/cultured cells do not engraft when given systemically [21,57,73]. However, we cannot rule out an initial effect on HSC engraftment when the OBLCs are cotransplanted [55,69,86] or with bone fragments [51] that can be sustained only in patients with adequate immunosuppression. The importance of replacing the host SC/MSCs with this method may be more advantageous for treating bone diseases like hypophosphatasia, osteogenesis imperfecta, and the skeletal dysplasia of lysosomal disease. In bone diseases, providing even a small amount of normal SC/MSCs may be all that is needed for the osteoprogenitor cells to undergo self-renewal, proliferation, and differentiation after receiving the appropriate signals from the modified host microenvironment.

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