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Mesenchymal stem cells

Allogeneic mesenchymal stem cell infusion for treatment of metachromatic leukodystrophy (MLD) and Hurler syndrome (MPS-IH)

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Summary:

Patients with Hurler syndrome (mucopolysaccharidosis type-IH) and metachromatic leukodystrophy (MLD) develop significant skeletal and neurologic defects that limit their survival. Transplantation of allogeneic hematopoietic stem cells results in partial correction of the clinical manifestations. We postulated that some of these defects may be corrected by infusion of allogeneic, multipotential, bone marrow-derived mesenchymal stem cells (MSC). Patients with Hurler syndrome ($n = 5$) or MLD ($n = 6$) who previously underwent successful bone marrow transplantation from an HLA-identical sibling were infused with 2–10 × 10^6/kg MSCs, isolated and expanded from a bone marrow aspirate of the original donor. There was no infusion-related toxicity. In most recipients culture-purified MSCs at 2 days, 30–60 days and 6–24 months after MSC infusion remained of host type. In two patients the bone marrow-derived MSCs contained 0.4 and 2% donor MSCs by FISH 60 days after MSC infusion. In four patients with MLD there were significant improvements in nerve conduction velocities after MSC infusion. The bone mineral density was either maintained or slightly improved in all patients. There was no clinically apparent change in patients' overall health, mental and physical development after MSC infusion. We conclude that donor allogeneic MSC infusion is safe and may be associated with reversal of disease pathophysiology in some tissues. The role of MSCs in the management of Hurler syndrome and MLD should be further evaluated.

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Patients with Hurler syndrome and metachromatic leukodystrophy (MLD) develop significant neurologic and musculoskeletal defects that limit their survival.[1–3] MLD is an autosomal recessive disease due to the deficiency of arylsulfatase A enzyme, that results in accumulation of sulfatides causing demyelination of central and peripheral nervous system. Patients develop severe gait disturbances, spasticity, tetraplegia, mental regression, and eventually absence of any voluntary function. Slowing or absence of nerve conduction correlate with peripheral nerve demyelination. Hurler syndrome is inherited in an autosomal recessive pattern. In this disorder, deficiency of α-L-iduronidase enzyme results in accumulation of heparan sulfate and dermatan sulfate in lysosomes. Progressive hepatosplenomegaly, cardiac disease, severe skeletal abnormalities, hydrocephalus and mental retardation result in early death. Transplantation of allogeneic hematopoietic stem cells (HSC) can significantly halt disease progression and improve survival of patients with MLD and Hurler syndrome.[1,3,4] This effect is believed to be due to tissue infiltration of macrophages that express normal arylsulfatase-A and α-L-iduronidase that are missing in MLD and MPS-IH, respectively,[5] and transfer of normal enzyme into defective cells by endocytosis. In Hurler syndrome, despite improvements in liver and heart, nervous system and skeletal deformities persist and often progress.[6] In MLD neurologic abnormalities persist after transplantation and commonly progress. We postulated that some of the residual defects could be corrected by use of supplemental cell therapy by infusion of donor bone marrow-derived allogeneic multipotential mesenchymal stem cells (MSC). Human MSCs were shown to differentiate into various cells of mesenchymal origin, including osteoblasts, chondrocytes, adipocytes, tendon-forming cells and myocytes *in vitro* and *in vivo*.[7–10] Furthermore Kopen and colleagues[11] have shown that murine marrow stromal cell were capable of migrating and differentiating into mature astrocytes when injected into lateral ventricle of neonatal mice. Azizi and colleges[12] have shown that human bone marrow stromal cells migrate and engraft in brain like astrocyte grafts. In addition, we have determined previously that normal donor MSCs express high amounts of α-L-iduronidase and aryl-

sulfatase-A, deficient in Hurler syndrome and MLD respectively.[13] These findings led us to hypothesize that culture expanded normal donor MSC could migrate and differentiate into tissues such as bone, cartilage, peripheral and central nervous system and repair these tissues in patients with MLD and Hurler syndrome. We previously showed that patients with Hurler syndrome or MLD treated with allogeneic bone marrow transplantation using an HLA-identical sibling donor had persistence of 'host'-type MSC in their bone marrow despite complete donor hematopoietic engraftment.[13] This finding likely reflects the relatively small numbers of MSC that are transferred in the course of an unmanipulated bone marrow transplant. *Ex vivo* culture expansion of allogeneic donor MSCs resulted in 5000- to 10 000-fold increase in the number of MSCs available for transplantation compared to unmanipulated BM transplantation. We hypothesized that high numbers of MSC infused would home to and provide normal enzyme activity to tissues and result in repair of tissues. In this manuscript we describe the safety and potential efficacy of culture-expanded allogeneic MSC infusion as treatment of patients with MLD and Hurler syndrome.

Patients and methods

Patients and donors

The clinical protocol was registered with the FDA under an IND for the use of *ex vivo* expanded MSC use and approved by the Institutional Review Board for Human Subjects at the University Hospitals of Cleveland. All patients and donors (or their legal guardians) provided written informed consent. Enrollment occurred between March 1999 and July 2000. Between 1984 and 1999 all 12 patients underwent allogeneic bone marrow transplantation using HLA-identical sibling bone marrow at the University of Minnesota. At the time of this transplant harvested marrow was routinely filtered sequentially through three filters of 800 μm, 500 μm and 200 μm diameter. Hematopoietic engraftment previously was documented by either restriction fragment length polymorphism (RFLP) or PCR analysis of variable number tandem repeats (VNTR) in every patient, at the Molecular Diagnostics Laboratory of the University of Minnesota. For the current study, bone marrow specimens of 12 patients were obtained 1 to 14 years after their allogeneic bone marrow transplantation and before MSC infusion for baseline MSC growth, genotype and enzyme expression analysis and these data were reported previously.[13]

Mesenchymal stem cell cultures

Bone marrow was aspirated (10–30 ml) under local anesthesia (with or without sedation) from the posterior iliac crest, collected into sodium heparin containing tubes and transferred to the Cell and Gene Therapy Facility of the Case Western Reserve University. MSC cultures were established as described previously.[14] Briefly, bone marrow cells were washed with Dulbecco's phosphate-buffered saline (DPBS) (Gibco, Grand Island, NY, USA) and layered on to 25 ml of Percoll (1.073 g/ml) (Sigma, St Louis, MO, USA) at a concentration of 1–2 $\times$ 10^7 cells/ml and gradients were centrifuged at 900 *g* for 30 min. Mononuclear cells (MNCs) were washed with phosphate-buffered saline (PBS) and plated at a density of 1 $\times$ 10^6 cells per ml in 175 cm^2 polystyrene flasks (Falcon, Becton Dickenson, Franklin Lakes, NJ, USA) in Dulbecco's minimum essential medium (DMEM) with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA). The serum lot used was selected based on optimal MSC growth.[15] Each flask was loaded with a minimum of 30 million cells. MSCs were allowed to adhere for 72 h followed by media changes every 3–4 days. By the fifth day of culture >100 fusiform adherent cells initiating small colonies were visible, which came to confluence by day 10–14. Adherent cells were removed with 0.05% trypsin-EDTA (Gibco) and replated (passaged) at a density of 1 $\times$ 10^6 per 175 cm^2. Cell cultures were tested for sterility weekly (University Hospitals Microbiology, Cleveland, OH, USA), endotoxin by limulus amebocyte lysate test (Associates of Cape Cod, Falmouth, MA, USA), and mycoplasma by DNA-fluorochrome stain (Bionique, Saranac Lake, NY, USA) prior to infusion into patients.

Flow cytometry

In order to determine the quality of MSC cultures, flow cytometry was performed on all cultures for surface expression of SH-2, SH-3, SH-4 MSC markers and lack of contaminating CD14- and CD-45 positive cells. Cells were detached with 0.05% trypsin-EDTA (Gibco), washed with DPBS + 2% bovine albumin, fixed in 1% paraformaldehyde, blocked in 10% serum, incubated separately with primary SH-2, SH-3 and SH-4 antibodies followed by PE-conjugated anti-mouse IgG(H+L) antibody (Caltag, Burlingame, CA, USA) or with FITC-labeled CD45 and PE-labeled CD14 with appropriate isotype controls (Becton Dickinson, San Jose, CA, USA). Flow cytometry was performed on a FACScan (Becton Dickinson, Parsippany, NJ, USA) equipped with an argon laser and data were analyzed with CellQuest software (Becton Dickinson). By the third passage of MSCs, hematopoietic cells could not be detected in cultures either visually or by flow cytometry after CD45 and CD14 staining. Greater than 95% of the cells expressed MSC markers SH2, SH3 and SH4 as determined by flow cytometry.

MSC infusion

Confluent MSC in 175 cm^2 flasks were washed with Tyrode's salt solution (Sigma), incubated with medium 199 (M199) (Gibco) for 60 min, and detached with 0.05% trypsin-EDTA (Gibco). Cells from 10 flasks were detached at a time and MSCs were resuspended in 40 ml of M199 + 1% human serum albumin (HSA; American Red Cross, Washington DC, USA). MSCs harvested from each 10-flask set were stored for up to 4 h at 4°C and combined at the end of the harvest. A total of 2–10 $\times$ 10^6 MSC/kg were resuspended in M199 + 1% HSA and centrifuged at 460 *g* for 10 min at 20°C. Cell pellets were resuspended in fresh M199 + 1% HSA media and centrifuged at 460 *g* for 10

min at 20°C for three additional times. Total harvest time was 2–4 h based on MSC yield per flask and the target dose. Harvested MSC were cryopreserved in Cryocyte (Baxter, Deerfield, IL, USA) freezing bags using a rate controlled freezer at a final concentration of 10% DMSO (Research Industries, Salt Lake City, UT, USA) and 5% HSA. On the day of infusion cryopreserved units were thawed at the bedside in a 37°C water bath and transferred into 60 ml syringes within 5 min and infused intravenously into patients over 10–15 min. Patients were premedicated with 325–650 mg acetaminophen and 12.5–25 mg of diphenhydramine orally. Blood pressure, pulse, respiratory rate, temperature and oxygen saturation were monitored at the time of infusion and every 15 min thereafter for 3 h followed by every 2 h for 6 h.

Genetic polymorphism

To determine host *vs* donor origin of MSC we utilized PCR amplification of frequently polymorphic simple sequence repeats (SSR) in genomic DNA. The length polymorphism associated with SSRs has been previously utilized in humans to determine the cellular origin.[16] Primer pairs (Human MapPairs) specific for highly polymorphic loci (heterogeneity score >0.8) were obtained from Research Genetics (Huntsville, AL, USA). In some reactions the forward primer was end-labeled by using ^{32}P-γ-ATP and T4-polynucleotide kinase. One hundred to 500 ng of genomic DNA obtained from both donor and recipient culture expanded MSCs and their bone marrow nucleated cells, were subjected to 32 cycles of PCR amplification with at least six different pairs of labeled forward and cold reverse primers. PCR products were separated on a 1.5% agarose or 5% polyacrylamide denaturing gel along with appropriate size markers. The size and the pattern of the amplicons were determined by autoradiography. Informative primer sets were those which amplified a specific size DNA fragment in donor cells but not in patients' MSCs. Band intensities were measured by transluminescence and signals above background were identified as positive. By using mixtures of known quantities of donor- and recipient-type DNA, we could reliably detect 1% donor type DNA.

Fluorescence in situ hybridization

Fluorescence *in situ* hybridization using probes for the X and Y chromosomes was performed on MSC obtained from patients transplanted with gender mismatched donors as described elsewhere.[17] Methanol:acetic acid fixed MSC nuclei were dropped on to slides, denatured and dehydrated. Nuclei were probed with Spectrum Orange CEP X and Spectrum Green CEP Y probes (Vysis, Downers Grove, IL, USA) and counterstained with a 4,6-diamino-2-phenylindole (DAPI) solution. Nuclei were scored as male when they exhibited both X and Y signal, and scored as female when they exhibited no Y signal and two X signals. A total of 600 nuclei was counted for each specimen.

ELISPOT assay

Ninety-six well ELISPOT plates (Polyfiltronics, Rockland, MA, USA) were coated with capture antibody for interferon-γ (2G1; Endogen, Woburn, MA, USA) as described elsewhere.[18] Three hundred thousand responder peripheral blood lymphocytes (PBL) were added to each well in 100 μl of medium. The PBLs were activated *in vitro* with PHA (10 μg/ml final concentration; Sigma) or donor MSC. After 24 h the plates were washed and biotinylated detection antibody (B133.5, Endogen, 4 mg/ml) was added to the wells overnight at 4°C. Strepavidin-HPR (Dako, Carpenteria, CA, USA) was then added for 2 h at room temprature. The spots were developed using 3-amino-9-ethylcarbazole (Pierce, Rockford, IL, USA). The resulting spots were counted on a computer-assisted ELISPOT image analyzer Immunospot (Cellular Technology, Cleveland OH, USA).

Clinical follow-up

All patients were monitored closely during and within 48 h of MSC infusions. Oxygen saturation, temperature, blood pressure, pulse and respiratory rate were recorded prior to infusion and every 15 min for 1 h and hourly for 6 h and every 8 h for 48 h after infusion. Patients also had chest films before and 1 day after MSC infusion. Patients had follow-up visits every 6 to 12 months after MSC infusion. MLD patients underwent serial nerve conduction velocity (NCV) measurements at the Fairview University Medical Center EMG laboratory in Minneapolis, MN. The median, ulnar, peroneal and tibial nerves were stimulated by electrodes and surface recording of the largest amplitude of the compound muscle action potential was determined by surface electrodes. Technical error was $\pm$1–1.5 m/s for nerves with conduction velocities in the 15 m/s range that is the typical range in MLD. Bone mineral density was measured at the lumbar spine (L1-L4) by a DEXA scanner (Hologic QDR-4500A; Hologic, Waltham, MA, USA).

Results

Normal donor MSC cultures

Between March 1999 and July 2000 we enrolled 12 patients and donors into this clinical protocol. *Ex vivo* MSC cultures were established from 12 normal donors who were HLA identical to respective patients and previously donated bone marrow for hematopoietic transplantation (Table 1). Cultures were initiated using a median ($\pm$s.d.) of $82.4 \pm 84.6 \times 10^6$ bone marrow-derived mononuclear cells. There was a strong correlation between the starting mononuclear cell number and the number of MSCs found

Table 1 Study population

	Donor characteristics	*Patient characteristics*
n	12	12
Age median (range)	13 (4–31)	15 (5–25)
Start BM ml mean $\pm$ s.d.	17.7 $\pm$ 5.4	—
Start MNC $\times 10^6$	82 $\pm$ 84	—
Weight (kg)	—	38 (13–82)

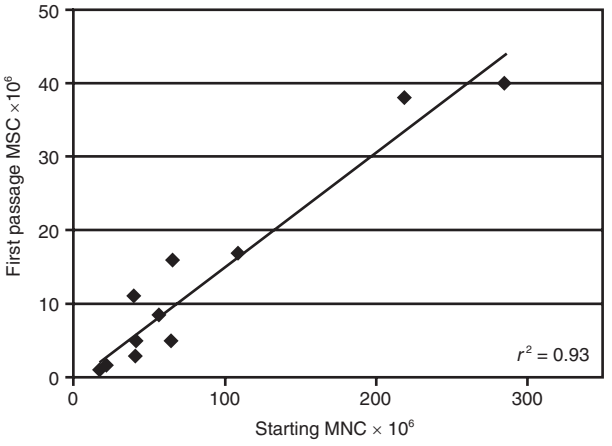

Figure 1 Correlation of bone marrow aspirate mononuclear cell number to MSC number obtained at first passage. Data from 12 normal donors.

at the first passage ($r^2 = 0.93$) (Figure 1). Number of MSC at first passage varied from 0.7 to 40×10^6 (mean 12.2). MSCs subsequently exhibited exponential growth at a similar rate among donors (Figure 2). The variability in cell number among donors at the second and third passages was related directly to the variability in starting cell number. Normal donor MSCs were evaluated for enzyme expression. We found high level of α-L-iduronidase (122 ± 69 nmol/mg protein/hour, $n = 14$) and arylsulfatase-A (49 ± 21, $n = 9$) expression in third passage normal donor MSCs that were significantly higher than enzyme activity found in normal donor leukocytes (77 ± 25 and 39 ± 15, respectively).

Infusion of allogeneic MSC

Eleven of 12 patients enrolled were given allogeneic MSC infusion between May 1999 and 31 August 2000. One patient withdrew from the trial for personal reasons before MSCs could be infused. The trial was designed to allow MSC dose escalation from 2×10^6/kg to 10×10^6/kg in cohorts of three patients. Four patients received 2×10^6 donor MSC per kg, five patients received 3.4 to 4.6×10^6 MSC per kg and three patients received 10×10^6 MSC per kg. Target donor MSC numbers were reached in eight of 11 patients (Table 2). A decision was made to limit the time and the passage number by terminating the cultures if the target dose could not be achieved based on the starting mononuclear cell numbers. One patient in the 4×10^6 MSCs/kg cohort and two patients in the 10×10^6 MSC/kg cohort received a lower number of MSCs than planned. There were no significant immediate or delayed toxicities related to MSC infusion and GVHD was not observed within a median follow-up of 2 years (15–31 months). Four patients developed self-limited grade 1 fever within 24 h of MSC infusion. One patient developed grade 2 phlebitis at the site of venous access. We used the ELISPOT assay to determine if recipient blood had alloreactive memory T cells that would activate and secrete interferon γ upon encountering donor MSCs. This is a highly sensitive assay (limit of detection five reactive lymphocytes out of 300 000) that is capable of characterizing the frequencies of alloantigen-specific T cells in short-term culture, thus providing a reflection of their function *in vivo*.[18] None of the recipients tested had detectable alloreactive T cells using the ELISPOT assay (Figure 3). In patient no. 12 we tested recipient lymphocytes obtained both before and after donor MSC infusion and again we did not detect alloreactive lymphocytes in response to donor MSC (data not shown).

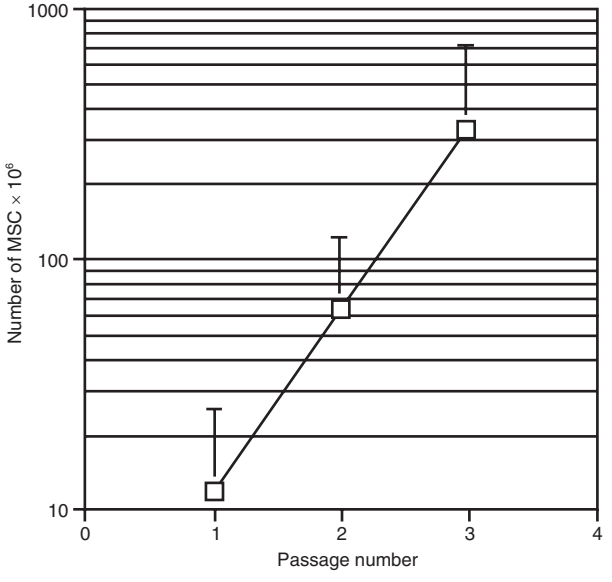

Figure 2 *In vitro* MSC expansion: number of MSC harvested during the first three passages. Mean data from 12 normal donor MSC cultures. Positive error bar represents one standard deviation.

Allogeneic MSC chimerism

Allogeneic MSC chimerism was evaluated in culture expanded (24–28 days) populations of MSC derived from recipient bone marrow 1–2 days, 1 month, 2 months and 6–24 months after allogeneic MSC infusion. Highly culture-purified MSCs were genotyped either by fluorescence *in situ* hybridization using probes to X and Y chromosomes (seven patients) or by PCR amplification of polymorphic simple sequence repeats in genomic DNA (four patients). In the majority of patients we found that both MSC and osteoblasts isolated after allogeneic MSC infusion remained of host genotype. In two patients (no. 10 and no. 12) third passage MSC cultures established 60 days after allogeneic MSC infusion contained 0.4% and 2% donor MSC by FISH analysis.

Clinical outcome

Clinical outcome was evaluated by physical and neurological examinations, psychosocial assessments as well as nerve conduction velocity and bone mineral density (BMD) measurements. There was no dramatic change in patients' overall health and no readily detectable change in mental and physical development. Three of five Hurler syndrome patients and all six MLD patients underwent BMD measurements (Figure 4). Most of these measurements were performed after the MSC infusion except for patient

Table 2 MSC dose levels

Donor No.	*Donor age*	*Disease*	*Patient age*	*Sex*	*Allo BMT*	*Wt kg*	*Dose level* $\times 10^6$*/kg*	*MSC infused* $\times 10^6$	*Passage No.*
1	15	MLD	20	F	1984	35	2	84	3
2	11	MLD	5	F	1994	13	2	30	2
3	28	MLD	21	F	1994	58	2	116	3
4	19	Hurler	17	F	1985	45	2	95	3
5	20	Hurler	17	F	1985	50	4	220	3
6	4	Hurler	5	M	1996	23	4	94	4
7	31	MLD	25	F	1989	44	3.4	150	6
8	8	Hurler	6	M	1994	20	10	200	3
9	8	ALD	13	M	1992	47	—	—	4
10	10	MLD	7	F	1999	21	10	200	
11	6	Hurler	5	M	1996	18	10	176	3
12	18	MLD	19	M	1995	82	3.5	290	3
Median or mean	13		15			38 (mean)		150 (mean)	3

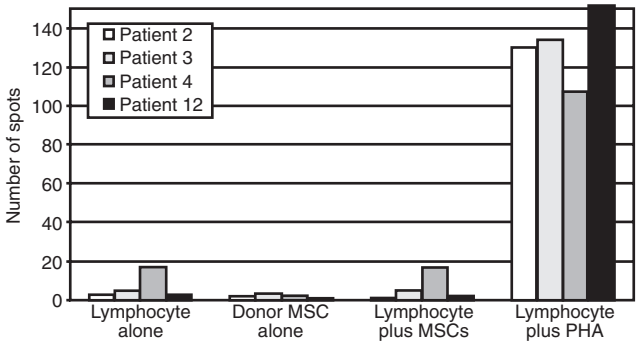

Figure 3 ELISPOT assay for lymphocyte activation. Blood lymphocytes were obtained from four patients and tested for interferon-γ secretion on ELISPOT wells in triplicate as described in Methods. Cells were plated after either no stimulation (lymphocyte alone), stimulation with donor MSC (Lymphocyte plus MSC) or stimulation with PHA. The number of spots corresponding to the number of activated lymphocytes were counted and means of three counts were recorded. In addition, donor MSC alone were tested simultaneously (Donor MSC alone).

No. 7 who had three measurements conducted prior to MSC infusion. Following allogeneic MSC infusion BMD was either maintained or slightly improved in all patients. Patient No. 7 who had declining BMD prior to MSC infusion had improvement in BMD after the MSC infusion. Patients 2, 7, 8 and 10 had BMD that was lower than age-adjusted average BMD. Six patients with MLD had electrophysiological assessment (Figure 5) and in four there was clear evidence of improvement of nerve conduction velocity (NCV). In these patients NCV was 6 to 12 m/s faster after MSC infusion that is significantly above the potential technical error ($\pm$1–1.5 m/s). None of the patients had a similar degree of improvement in their NCV spontaneously. Patient No. 3 had a similar degree of improvement in her NCV after the allogeneic bone marrow transplantation. In this patient NCV declined again over the next 3 years and improved once more with MSC infusion. In all patients change in NCV was evident within the first year post-MSC infusion. In addition to changes in the NCV, there were changes in distal motor and F-wave latency, motor and sensory amplitudes parallel to what was observed in NCV (data not shown). NCV has not been reported to improve spontaneously in MLD patients who have not received either BMT or MSC infusion (Peters and Krivit, personal communication).

Discussion

Allogeneic bone marrow transplantation provides long-term engraftment with donor monocyte–macrophage system that is effective in correction of pathophysiology and clinical manifestations of selected lysosomal and peroxisomal storage disorders.[19] Clinical correction of the disease phenotype is variable based on the organ systems involved, patient age at the time of transplant and the nature of the metabolic defect. Patients with MLD and Hurler syndrome have persistent progressive neurologic and skeletal defects despite achieving 100% donor hematopoietic chimerism. Bone marrow-derived mesenchymal stem cells have the potential to differentiate into cells of bone, cartilage, tendon, muscle and other adventitial tissues[20] and offer potential for corrective cellular therapy. Although bone marrow contains both hematopoietic and non-hematopoietic progenitors, including MSCs, allogeneic BMT does not result in replacement of non-hematopoietic tissues and bone marrow-derived MSC remain of host origin.[13] This may be due to very low numbers of non-hematopoietic progenitors present in the bone marrow. Therefore, we expanded the number of MSCs obtained from a bone marrow aspirate using an *ex vivo* culture system. We infused 2–10 $\times$ 10^6/kg allogeneic donor MSC into Hurler syndrome and MLD patients in an attempt to both replace mesenchymal tissues with normal MSC and to provide a source of normal enzyme in tissues to which they distribute. This is the first series of patients who were infused with allogeneic donor bone marrow-derived MSC for treatment of their Hurler syndrome or MLD.

We found that relatively small amounts of starting bone marrow aspirates were adequate to grow up to 10 $\times$ 10^6/kg human MSC for small children (>40 kg). The number of MSC that could be culture-expanded correlated directly with the number of mononuclear cells obtained from the initial 20 ml bone marrow aspirate. All procedures were

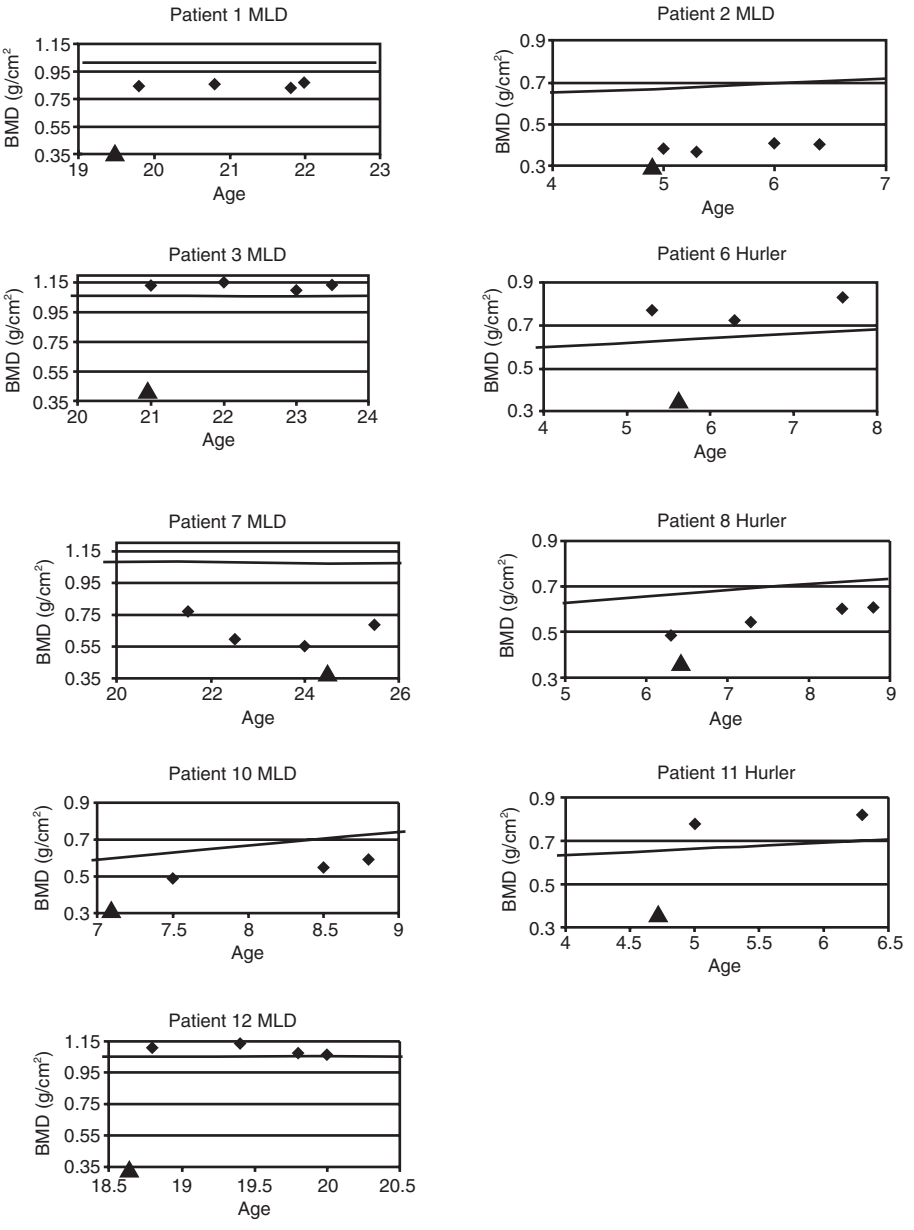

Figure 4 Bone mineral density (BMD) measurements (g/cm^2) over time. Arrows indicate time of donor allogeneic MSC infusion. Thick gray line represent age adjusted average BMD in normal individuals.

done as outpatient with local anesthesia or sedation for children under 12 years of age. Infusion of up to 10×10^6 allogeneic MSC/kg was well tolerated in all patients without pulmonary symptoms, changes in chest radiograms or changes in oxygen saturation. Despite prolonged *ex vivo* culture with fetal calf serum-containing media we have not seen any hypersensitivity reactions. Since MSC cultures are virtually devoid of lymphocytes and our patients were already engrafted with donor-type hematopoiesis, we did not observe recurrent or new onset GVHD. In addition, as expected, we did not detect any anti-donor MSC reactive T lymphocytes even after donor MSC infusion, strongly indicating lack of such reactive lymphocytes in recipients.

In this clinical trial we could not ascertain the distribution and homing pattern of infused MSC. Our analysis of MSC distribution was limited to recipient bone marrow, aspirated at various time points after MSC infusion. To avoid contamination by allogeneic hematopoietic cells, we only analyzed highly purified (third passage) MSC for their genotype. Despite these stringent conditions, MSC cultures of two patients showed evidence of donor MSC growth. Similar low degrees of donor stromal cell chimerism had been reported previously in animal models using stromal cell infusion[21,22] and in one clinical trial.[23–25] Overall, our MSC genotype results indicate that culture-expanded donor MSCs do not repopulate recipient bone marrow and do not re-grow in adherent cultures of recipient bone marrow. Reasons for this observation may include: (1) poor survival and marrow homing of culture-expanded donor MSCs; (2) lack of preparative therapy to provide a competitive advan-

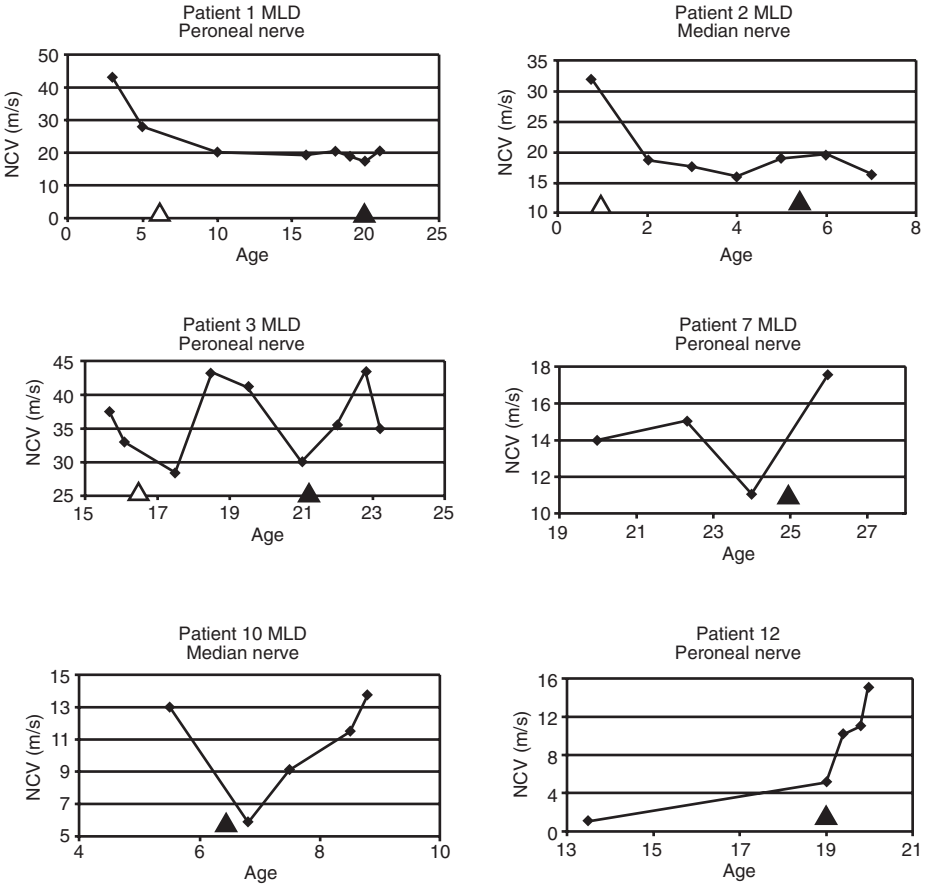

Figure 5 Nerve conduction velocity measurements over time. Open arrows indicate time of allogeneic BMT. Solid arrows indicate time of donor allogeneic MSC infusion.

tage to infused donor MSC; (3) proliferative defect of infused donor MSC compared to endogenous host MSC. Our results, however, do not preclude the possibility that MSC might have been distributed in various organs including the bone marrow and differentiated in mature mesenchymal cells.

We were intrigued to observe changes in BMD measurements and NCV after donor MSC infusion. We decided to use BMD measurements in this trial as a surrogate marker for skeletal health since it was performed regularly in children with genetic disorders who had BMT. In two patients (Nos 7 and 8) there was a clear increase in BMD 6 months following MSC infusion. Since changes in BMD are multifactorial and observed over long periods it is impossible to draw firm conclusions from our BMD data at this time. In addition BMD measurements are not useful in assessment of skeletal deformities seen in patients with Hurler syndrome. It remains to be seen whether dysostosis multiplex of Hurler syndrome can be halted with MSC infusion. Most of our patients had skeletal deformities at the time of MSC infusion and reversal of these fully developed anatomic defects is unlikely. Since the safety of donor MSC infusion is now established, it is important to consider incorporation of MSC infusion early in the course of the disease (<2 years of age) either at the time of hematopoietic transplant or shortly afterwards.

The most surprising finding was the changes in NCV in patients with MLD. There was clear and consistent improvement in NCV temporally associated with MSC infusion. We speculate that either MSC are providing normal enzyme in or around peripheral nerves or that there is Schwann cell differentiation of MSC *in vivo*. The transient nature of the improvement in one patient suggests passive enzyme transfer into peripheral nerves. The clinical significance of increased NCV of 2–12 m/s, however, is not apparent. In general there is minimal functional difference caused only by changes in conduction velocity. However, it is likely that improvement in NCV reflects reversal in the neuropathology that leads to the loss of motor and sensory amplitudes. Thus further neurophysiologic studies are warranted before and after MSC therapy both in clinical as well as pre-clinical in animal models in order to better understand the mechanism of these changes.

In conclusion, in MLD and Hurler syndrome patients, allogeneic donor MSC infusion was well tolerated and free of toxicity at up to 10×10^6 cells/kg cell dose at a median follow-up of 2 years. Improvements in some clinical parameters observed warrants further study of MSC as therapeutic tool in these disorders. Multiple infusions of donor MSC, co-infusion of donor MSC at the time of hematopoietic transplantation and targeted delivery of MSC may result in improved outcome in these and other storage disorders.

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