Mesenchymal Stem Cells as a Salvage Treatment for Severe Refractory Graft-vs-Host Disease in Children After Bone Marrow Transplantation

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ABSTRACT

Application of mesenchymal stem cells (MSC) enables a novel approach to the therapy of graft- vs-host disease (GVHD) after hematopoietic stem cell transplantation. Herein we present our preliminary experience with the use of allogeneic bone marrow–derived MSC in 9 pediatric patients after hematopoietic transplantation complicated by severe acute or chronic GVHD (aGVHD, cGVHD) resistant to steroids and second-line immunosuppressants. The MSC therapy was applied concurrently with immunosuppressive treatment in 5 patients as a single infusion, in four patients as 2-6 infusions. The median dose of cells per infusion was 1.9×10^6/kg of recipient body weight (range, 0.1–6.5×10^6/kg). The median quantity of cells applied to patients was 1.2×10^6/kg (range, 0.2–30.9×10^6/kg). We did not observe any adverse symptoms of MSC therapy. Overall, partial, or complete remission (PR and CR, respectively) was obtained in 56% of patients after the first MSC infusions, and 44% after completing therapy. In those with skin involvement 50% achieved permanent CR, 38% in those with gastrointestinal manifestations, and 33% in those with liver GVHD. Three patients with overlap syndrome had amelioration, but none had permanent remission. Long-term improvement after consecutive MSC doses was observed in 3 patients. In the 4- to 8-year follow-up, 3 patients are alive and 2 have attained permanent remission. Six patients died during follow-up: 4 with aGVHD and 2 with infectous complications. Co-treatment of streoid-resistant GVHD with MSC and conventional immunosuppression can improve the outcome, although therapy regimens remain to be established.

GRAFT-VS-HOST disease (GVHD) represents one of the most severe complications of hematopoietic stem cell transplantation (HSCT) and remains as the leading cause of procedure-related morbidity and mortality. Up to 50% of patients with acute GVHD (aGVHD) do not respond well to the conventional first-line glicocorticosteroid treatment [1]. Although various second-line treatment options have been introduced, ranging from high-dose steroids and immunosuppressants to mono- and polyclonal antibodies, phototherapies, or cellular treatments, prognosis for steroid-refractory patients remains poor with mortality reaching 80% [2]. The major cause for long-term morbidity and mortality in the later period after HSCT is chronic GVHD (cGVHD), with an incidence of 40–70% in the general population and of 20–50% in children [3]. In a cohort of 105 pediatric patients with cGVHD assessed in a recent Japanese report, only 45% of achieved remission in

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Table 1. Patients' Characteristics

Patient	Age (Years)	Gender	Diagnosis	Stem-cell Source HLA Matching	Conditioning Regimen	GVHD Prophylaxis	$CD34^+ \times 10^6$/kg
1	11	M	SAA	UD BMT 10/10	FluCyTBI	ATG, CSA, MTX	1.9
2	5	M	CGD	UD BMT 8/10	BuCy	ATG, CSA, MTX	2.0
3	0.5	F	Osteopetrosis	UD BMT 9/10	TreoFlu Thiotepa	ATG, MMF	6.6
4	11.5	M	Congenital neutropenia	UD BMT 10/10	BuCy	ATG, CSA, MTX	2.22
5	12.5	M	ALL, second CR	FD BMT 10/10	TBI VP16	CSA, MTX	1.91
6	2	M	Infant ALL, second CR	Sib-BMT 10/10	TreoFlu Thiotepa	CSA	3.0
7	16	M	ALL, second CR	FD BMT 10/10	TBI VP16	ATG, CSA, MTX	3.4
8	14	F	CVID	Sib-BMT II* 6/6	TreoFlu Thiotepa	CSA, MTX	1.8
9	18	M	ALL, second CR	UD BMT 10/10	TBI VP16	ATG, CSA, MTX	1.47

Abbreviations: ALL, acute lymphoblastic leucaemia; ATG, antithymocyte globulin; BMT, bone marrow transplantation; Bu, busulphan; CGD, chronic granulomatous disease; CR, complete remission; CSA, cyclosporine A; CVID, common variable immunodeficiency; Cy, cyclophosphamide; FD, family donor; Flu, fludarabine; MMF, mofetil mycophenolate; MTX, methotrexate; SAA, severe aplastic anemia; Sib, sibling; TBI, total body irradiation; Thiotepa, tepadine; Treo, treosulfan; UD, unrelated donor; VP16, etoposide.
*Patient 8 was re-transplanted with the same donor due to the first graft loss.

the 8-year observation period, with an overall mortality of 38% [4].

Thus, novel therapeutic approaches in steroid-resistant GVHD are necessary. Due to their immunomodulatory properties and low immunogenicity, mesenchymal stem cells (MSC) represent a promising therapeutic tool for GVHD. First isolated from bone marrow as a rare subset of non-hematopoietic marrow stromal cells, MSC have been since been derived from different adult tissues, as well as from the newborn cord blood and cord parenchyma [5]. MSC have been shown to mediate immunoregulatory activites by interactions with many cell types. It was reported that MSC inhibit T-cell activation, induce expansion of regulatory T cells, inhibit cytotoxic T-cell reactions and natural killer (NK)-cell activities, reduce pro-inflammatory and increase anti-inflammatory cytokine secretion, and inhibit lymphocyte proliferation in response to mitogens [6]. MSC influence B-cell, monocyte, and dendritic-cell proliferation and function [6,7]. When applied in severe forms of GVHD, MSC have been suggested to inhibit the T-cell cycle and ameliorate T-cell cytotoxicity [8].

The safety of MSC infusions was demonstrated by Sundin et al [9], who showed the absence of immunization in HSCT recipients, neither to HLA-matched MSC, nor third-party (mismatched) MSC, thus showing unresponsiveness to minor histocompatibility antigens as well. After MSC treatment the recipients remained unresponsive to the donor MSC, but maintained alloreactivity to the donor peripheral blood lymphocytes, indicating no development of immunologic memory restricted to MSC antigens.

MATERIALS AND METHODS

Patients

Between July 2008 and March 2014, 9 patients who had undergone allogeneic bone marrow transplantation, aged 0.5–18 years and diagnosed with steroid-resistant aGVHD grade III or IV (6 patients) or steroid-resistant severe cGVHD (3 patients), received MSC treatment for compassionate use after failing to respond to at least 2 lines of immunosupressants after ineffective first-line steroid treatment. Median age at transplant was 11.5 years. After ablative conditioning, all patients received a bone marrow transplant (2 from sibling donors, 2 from other family members, and 5 from unrelated donors) for treatment of acute lymphoblastic leukemia in 4 cases and for nonmalignant diseases in 5 cases. Patients' characteristics are detailed in Table 1. Six children developed severe forms of aGVHD over the mean time period of 26 (range, 14–38) days posttransplant. One patient had several flareups of the disease, finally developing extensive cGVHD overlap syndrome after 5 months. Two consecutive patients presented with ovelap sydrome features within the first 3 months posttransplant. One patient was diagnosed primarily with cGVHD.

In all patients, the diagnosis of GVHD was established upon clinical evidence, which was confirmed by histopatologic findings in 5 of them. Clinical diagnosis and grading of acute and chronic GVHD were based on criteria defined by the Keyston Consensus Conference and National Institutes of Health (NIH) consensus, respectively [10,11]. All patients were administered MSC therapy concurrently with salvage immunosuppressive treatment. MSC were isolated from bone marrow sourced from hematopoietic stem cell

Table 2. GVHD Features and Patients' Response to MSC Treatment

Patient (MSC Dose Number)	GVHD Diagnosis After Tx	Before MSC Infusion				Day of MSC Infusion	MSC Donor, MSC Load (10^6/kg)	14 Days After MSC infusion				28 Days After MSC Infusion				Overall Response	Follow-up
		GVHD Grading	Affected Sites	Stage	GVHD Treatment			GVHD Grading	Affected Sites	Stage	GVHD Treatment	GVHD Grading	Affected Sites	Stage	GVHD Treatment		
1 (1)	26	aGVHD grade IV	Skin Liver GI	3 1 4	MP MMF CSA ETA	95	0.4	aGVHD grade IV	Skin Liver GI	2 0 4	MP CSA ETA	aGVHD grade III	Skin Liver GI	0 0 2	MP CSA ETA	PR	GI aGVHD reflaring
1 (2)		aGVHD grade III	Skin Liver GI	0 0 3	MP CSA	130	0.1	aGVHD grade III	Skin Liver GI	0 0 3	MP CSA	aGVHD grade III	Skin Liver GI	0 0 3	CSA	NR/PD	Development of ileus
1 (3)		aGVHD grade IV	Skin Liver GI	0 0 4	CSA	162	1.8	aGVHD grade IV	Skin Liver GI	0 0 4	Terminal state	–	–	–	–	PD	Death of aGVHD, day 175
2 (1)	19	aGVHD grade IV	Skin Liver GI	3 2 4	MP BUD MMF	36	0.3	aGVHD grade III	Skin Liver GI	2 1/2 3	MP ↓ BUD MMF ETA	–	–	–	–	PR	Next MSC infusion in 12 days
2 (2)		aGVHD grade III	Skin Liver GI	2 1/2 3	MP ↓ BUD MMF ETA	48	0.4	aGVHD grade II	Skin Liver GI	1/2 1 1/2	MP ↓ BUD MMF ETA	aGVHD grade II	Skin Liver GI	1 1 1	MP ↓ BUD MMF	CR	CR, 8-year-follow-up
3	32	aGVHD grade IV	Skin Liver GI	0 4 0	TAC MP ETA	99	3.0	aGVHD grade IV	Skin Liver GI	0 4 0	TAC MP	–	–	–	–	NR/PD	Death of aGVHD, day 113
4	24	aGVHD grade IV	Skin Liver GI	3 4 4	MP ETA	69	1.1	aGVHD grade IV	Skin Liver GI	3 4 4	MP ETA	–	–	–	–	NR/PD	Death of aGVHD, day 74
5	38	aGVHD grade IV	Skin Liver GI	3 4 3	MMF MP ETA	57	0.2	aGVHD grade IV	Skin Liver GI	4 4 3	MMF MP ETA	–	–	–	–	NR/PD	Death of aGVHD, day 72
6 (1)	14	aGVHD grade IV	Skin Liver GI	3 0 4	MP TAC ETA BUD	32	5	aGVHD grade III/IV	Skin Liver GI	2 0 3/4	MP↓ TAC ETA BUD	–	–	–	–	PR	Improvement: skin lesions, GI bleeding relief; next MSC infusion in 17 days
6 (2)		aGVHD grade IV	Skin Liver GI	2 0 4	MP↓ TAC ETA BUD	49	5.9	aGVHD grade III	Skin Liver GI	2 0 3	TAC ETA BUD	–	–	–	–	PR	Continuous relief of diarrhea, bleeding remnants; the third MSC infusion in 26 days
6 (3)		aGVHD grade III	Skin Liver GI	2 0 3	MMF TAC ETA BUD	75	6.5	aGVHD grade III improvement	Skin Liver GI	2 0 3	TAC ETA BUD	–	–	–	–	PR	The fourth MSC infusion in 15 days

6 (4)		aGVHD grade IV	Skin Liver GI	2 0 4	TAC MP BUD	90	3	aGVHD grade III	Skin Liver GI	2 0 3/4	TAC MP BUD	–	–	–	–	PR	The fifth MSC infusion in 25 days
6 (5)		aGVH persistent grade IV	Skin Liver GI	2 0 3	TAC MP↓ BUD	115	5.3	aGVH persistent grade II	Skin Liver GI	1 0 1	MMF MP↓↓ BUD	aGVH persistent grade II	Skin Liver GI*	1 0 nd	MMF MP↓↓ BUD	PR	Rectoscopy* complicated by mesenteric vessel rupture, massive bleeding, surgical ligature
6 (6)		cGVHD overlap s. severe	Skin GI	2 3	MMF MP↓↓	154	5.2	cGVHD overlap moderate	Skin GI	1 2	MP↓↓	cGVHD overlap s. moderate	Skin GI	1 2	MP↓↓	PR	Severe cGVHD –musculoskeletal manifestation, sclerodermia; 4-year follow-up
7 (1)	38	cGVHD overlap s. severe	Skin KCS Mouth GI PLT Lymph	1 2 3 3 3 ↓↓	MP CSA	120	0.9	cGVHD overlap severe	Skin KCS Mouth GI PLT Lymph	1 1 1 2 3 ↓↓	MP↓ CSA	cGVHD overlap s. moderate	Skin KCS Mouth GI PLT Lymph	1 1 0 1 3 ↓↓	ETA MP ↓	PR	Amelioration of aGVHD; no result in overlap symptoms
7 (2)		cGVHD overlap s. severe	Skin GI PLT Lymph	1 4 3 ↓↓	ETA MP BUD	167	2.7	cGVHD overlap severe	Skin GI PLT Lymph	1 3 3 ↓↓	MP↓ BUD	cGVHD overlap s. severe	Skin GI PLT Lymph	1 1 3 ↓	TAC MP↓	PR	Amelioration of aGVHD, poor result in overlap symptoms
7 (3)		cGVHD overlap s. severe	Skin Liver GI PLT Lymph	0 1 3 3 ↓↓	TAC BUD	219	2,2	cGVHD overlap moderate	Skin Liver GI PLT Lympha	0 0 2 3 ↓	TAC BUD	cGVHD overlap syndrome-moderate	Skin Liver GI PLT Lympha	0 0 2 3 ↓	TAC↓ BUD	PR	Marked improvement of clinical status
7 (4)		cGVHD overlap s. severe	Skin Liver GI PLT Lymph	0 0 4 3 ↓	TAC SIR	324	2.0	cGVHD overlap severe	Skin Liver GI PLT Lymph	0 0 3 3 ↓↓	SIR TAC BUD	cGVHD overlap s. moderate	Skin Liver GI PLT Lymph	0 0 2 3 ↓↓	TAC SIR	PR	Lung mycosis; adenoviral infection; death of mucotic/ adenoviral pneumonia day 371
8	16	cGVHD overlap s. severe	Skin Liver GI Lungs PLT	2 1/2 2 2 2	TAC MP BUD	104	1.1	cGVHD overlap severe	Skin Liver GI Lungs PLT	2 2 1 2 2	TAC MP↓ BUD	cGVHD overlap s. severe	Skin Liver GI Lungs PLT	1 2 1 2 1	TAC MP ↓ BUD	PR	Mild mucosal cGVHD; 4.5-year follow-up

Table 2. (continued)

Patient (MSC Dose Number)	GVHD Diagnosis After Tx	Before MSC Infusion					MSC Donor, MSC Load (10^6/kg)	14 Days After MSC infusion				28 Days After MSC Infusion				Follow-up
		GVHD Grading	Affected Sites	Stage	GVHD Treatment	Day of MSC Infusion		GVHD Grading	Affected Sites	Stage	GVHD Treatment	GVHD Grading	Affected Sites	Stage	GVHD Treatment	Overall Response	
9	402	cGVHD severe	Skin	1	TAC	441	1.2	cGVHD severe	Skin	1	MP	–	–	–	–	NR	Death of lung mycosis at day 479
			KCS	2	ETA				KCS	2	BUD						
			Mouth	2	P				Mouth	2							
			GI	2	BUD				GI	3							
			Joints	3					Joints	3							
			PLT	3					PLT	3							

Abbreviations: aGVHD, acute graft-vs-host disease; BUD, budesonide oral; cGVHD, chronic graft-vs-host disease; CR, complete remission; CSA, cyclosporine A; ETA, etanercept; GI, gastrontestinal tract; KCS, keratoconjunctivitis sicca; MSC, mesenchymal stem cells; P, prednisolone; PLT, platelets; PR, partial remission; Lymph, lymphocytes; MP, methylprednisolone; MMF, mofetil mycophenolate; TAC, tacrolimus.

family donors of 3 patients (6 MSC infusions performed) or from healthy volunteer third-party HLA-mismatched donors, who were family members of 3 patients (7 MSC infusions) or unrelated in 4 children (7 MSC infusions). Two children were given consecutive MSC infusions from more than 1 donor. Patients were evaluated for the response to MSC treatment on day 14 after each MSC infusion and reevaluated on day 28 (evaluation concerned the global effect of MSC and concomitant immunosuppressive medication). Response criteria were as follows: complete response (CR) referred to the resolution of all signs of GVHD; partial response (PR) was defined as improvement in staging of at least 1 involved organ and at least steady state on other symptoms; no response (NR) included cases of persisting symptoms without any visible amelioration; and progressive disease (PD) was defined as progressed stage of any affected sites or deterioration of clinical state. The detailed data on the GVHD characteristics and therapy are summarized in Table 2.

All patients were treated in the Transplantation Unit at University Children's Hospital in Kraków. Use of the MSC clinical protocol was approved by the ethics committee at Jagiellonian University. Conditions and risks associated with the procedure were fully explained to the patients or their legal guardians and written informed consent was obtained in each case. The same conditions were fulfilled with regard to the volunteer donors of bone marrow for MSC isolation.

MSC Isolation and Expansion

The procedures of mesenchymal stromal cell isolation and expansion have been detailed by our team in an earlier report [12]. Two MSC isolation strategies were applied, as described in what follows.

Isolation of MSC from bone marrow mononuclear cells. Bone marrow mononuclear cells obtained by density gradient centrifugation were plated into vented 25-cm^2 tissue culture flasks (Sarstedt) with Dulbecco's modified Eagle medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Stem Cell Technologies) and antibiotics (PAA Laboratories). Medium plating density was 17×10^6. Flasks were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and, after 7 days, half of the medium was replaced with a fresh medium. The cells were then cultured with a half medium change each week until the fibroblastlike cells at the base of the flask reached confluence. On reaching confluence, the adherent cells were detached using 0.25% trypsin and reseeded at 1×10^5 cells per 25-cm^2 flask.

Isolation of bone marrow–derived CD271 MSC. A CD271-positive population enriched in MSC was isolated by positive immunoselection. Mononuclear cells were incubated with allophycocyanin (APC)-conjugated ME20.41.H4 monoclonal antibody (MAb) labeling the LNGF receptor (or p75 NTR) (Miltenyi Biotec) for 10 minutes at 4°C, rinsed, incubated with anti-APC immunomagnetic beads for 15 minutes at 4°C, and placed on a miniMACS column. After isolation, the cells were plated into tissue culture flasks with non-hematopoietic expansion medium (NHEM, Miltenyi Biotec) or human basal medium (MesenCult, Stem Cell Technologies) and processed as described previously.

After isolation the cells were counted and assessed for viability. Their purity was determined by flow cytometry with CD3, CD45, CD73, CD90, and CD105 labeling (Becton Dickinson).

RESULTS

The clinical course of therapy and the detailed results of MSC infusions are summarized in Table 2. Overall, 20 MSC were applied in 9 patients. Five patients were administered a single infusion of MSC. Of these, 4 were children, with

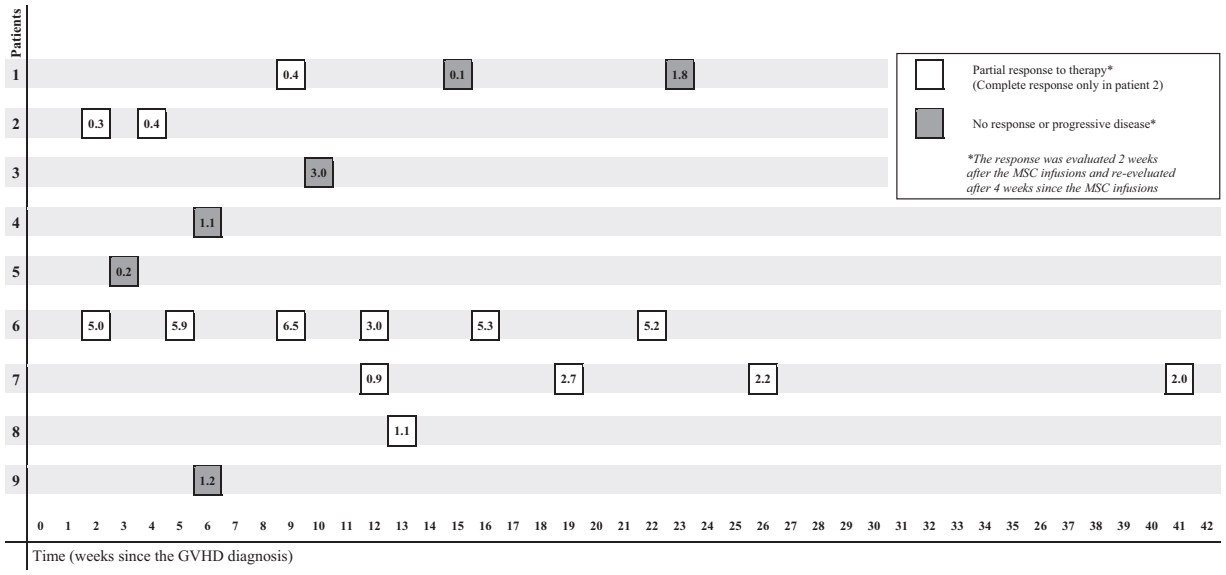

Fig 1. Response to the MSC therapy in relation to the MSC doses and the time of infusions since GVHD diagnosis (values of cell count x 10^6/kg are shown in boxes).

MSC being administered as a final attempt in advanced-stage disease with multiorgan failure syndrome. Four patients were given multiple, 2–6 repeated MSC infusions. Consecutive MSC portions were applied 12–105 (median, 32) days after the previous infusions if poor response to treatment or GVHD reflaring occurred. The median dose of cells per infusion was 1.9×10^6/kg of recipient's body weight (range, $0.1–6.5 \times 10^6$/kg). The median summarized quantity of cells applied to each patient was 1.2×10^6/kg (range, $0.2–30.9 \times 10^6$/kg). Median time from stem cell transplantation and the first episode of MSC treatment of aGVHD (or cGVHD featured early-onset ovelap syndrome) was 82 (range, 32–120) days and the patient showing primary cGVHD was given the MSC infusion on day 441 after stem cell transplant. Table 2 shows that a positive response to the therapy, if any, could be seen after 14 days in all cases. After 28 days, the effect of therapy was maintained or even showed improvement when compared with 14 days. We observed no acute side effects during or after infusions, and none of the patients revealed any perceptible late side effects in the follow-up period of 4–8 years.

Four patients showed long-lasting benefit from the MSC therapy, among them 2 children with aGVHD (patients 2 and 6) and 2 with aGVHD followed by early-onset overlap syndrome (patients 7 and 8). Patient 2 was given 2 infusions of MSC for grade IV aGVHD affecting the skin, gastointestinal tract (GI), and liver. The boy recovered completely and is currently well after an 8-year observation period. Patient 6 was given 5 courses of MSC for extremely severe intestinal aGVHD. He improved continously after each dose, reaching recovery of acute abdominal disease; however, he developed overlap syndrome affecting the musculoskeletal system, which was followed by a sixth MSC course and amelioration of symptoms. At the end of the observation period at 4 years after transplant he continues to be treated for musculoskeletal and sclerodermic manifestations of cGVHD. Patient 7, affected with aGVHD with severe GI manifestations accompanied by early-onset overlap syndrome, was given 4 doses of MSC, which resulted in partial improvement, especially of intestinal symptoms, after each dose. This encouraged the team to continue the therapy, but the boy died of lung mycosis aggravated by adenoviral infection on day 371 after bone marrow transplant. Patient 8 presented with early-onset overlap syndrome after a second HSCT from the same donor, which had been performed due to graft failure. She improved partially after a single dose of MSC, and was able to taper immunosuppressants; however, she continued with mild oral mucosal manifestations of GVHD after the 4.5-year observation period.

A lack of response or GVHD progression affected 5 patients in the study group: 4 patients with aGVHD (patients 1, 3, 4, and 5) and 1 with an extensive cGVHD (patient 9). In all of these cases, MSC treatment was administered for long-term, severe disease with an extreme decrease in clinical performance and meeting the criteria of multiorgan failure. The time of MSC therapy was determined by delayed availability of MSC production in patients 1 and 4, or by a very late decision of the parents of patient 3. Furthermore, in patient 1 (in the second infusion) and patient 5, only very low numbers of MSC were attainable. In patient 1, transient amelioration was observed after the first MSC infusion, which was nevertheless followed by rapid GVHD reflare and progressive deterioration with no response to treatment, which included 2 additional MSC infusions. All these patients died of aGVHD in a median period 93.5 days after stem cell transplantation (range, 72–175

Table 3A. Organ-specific and Global Response After the First MSC Infusion

	PR/CR after 14 days	PR/CR after 28 days	PR/CR All
Organ response, n (%)			
Skin (n = 8)	3 (38%)	2 (25%)	5 (63%)
Liver (n = 6)	2 (33%)	0	2 (33%)
GI (n = 8)	4 (50%)	1 (13%)	5 (63%)
Overlap features (n = 2)	1 (50%)	1 (50%)	2 (100%)
Global response, n (%)			
All affected sites (n = 9)	4 (44%)	1 (11%)	5 (56%)

Abbreviations: CR, complete remission; GI, gastrointestinal; MSC, mesenchymal stem cell; PR, partial remission.

days). Patient 9, who was given MSC therapy for extensive cGVHD, died of lung mycosis after a brief follow-up period.

The cell count applied in each MSC infusion and the result of treatment are shown in Figure 1. Application of lower doses of MSC, starting at 0.3–0.4 $\times$ 10^6/kg, could have been associated with amelioration of aGVHD in patients 1 and 2, as well as higher doses, up to 6.5 $\times$ 10^6/kg. When applied in the critical condition, MSC therapy was ineffective regardless of the cell count administered.

Referring to organ-specific response to MSC treatment (Tables 3A and 3B), in skin involvement the first infusion as well as consecutive doses were effective in 63% of affected patients, among whom 50% (4 children) achieved long-term complete remission of skin symptoms. In the GI manifestation of the disease, 63% of patients responded to the first MSC therapy, 50% to subsequent infusions, but, at the end of the observation period, 38% (3 children) showed permanent resolution of intestinal disease. In children with liver GVHD, 33% (2 children) responded to the first and subsequent MSC doses, achieving permanent recovery.

In summary, 14 of 20 MSC infusions were followed by either complete or partial response in 5 patients (Fig 1). After the first MSC infusions, we observed amelioration of GVHD symptoms in 5 patients (56%), but consecutive MSC therapies resulted in elimination of GVHD symptoms in 4 of them (44%), as shown in Tables 3A and 3B. These 4 children achieved permanent remission of acute GVHD symptoms, but only 1 (a boy with aGVHD with no signs of overlap features) showed a complete disease resolution. Three patients, with overlap syndrome/chronic GVHD at the beginning of MSC treatment, showed visible amelioration after the first MSC infusion, but none reached complete long-term recovery of chronic GVHD symptoms after global MSC therapy. At the end of the observation, 2 children still needed maintenance treatment for cGVHD. In 1 patient, progression of chronic disease was noted on further observation.

DISCUSSION

In this study we have addressed the issue of the treatment of the most severe forms of refractory aGVHD (clinical grade III or IV) and the extensive refractory cGVHD in children, with MSC administered in compassionate-use circumstances after at least 2 ineffective lines of immunosuppression and lack of response to standard steroid treatment. As the MSC were given at this same time as immunosuppressive salvage therapy containing tacrolimus or etanercept in most cases, we analyzed a combined result of pharmacologic therapy and MSC infusions. However, all our patients, aside from those in a terminal condition at the time of MSC application, benefited from the MSC support. Regarding the prognosis of resistant progressive grade III or IV GVHD, such a result could not have been anticipated as an effect of immunosuppression only, when patients had failed to respond to at least 2 previous second-line therapeutic attempts [2]. Nevertheless, in our group, MSC treatment was followed by amelioration of GVHD symptoms in 56% of children after the initial infusion and 44% after completing MSC therapy. Patients achieved long-lasting remission after repeated MSC infusions with stepwise release in GVHD symptoms after consecutive doses. At the end of the observation period (4–8 years after the last MSC infusion), 3 patients are still alive, including 1 with permanent complete remission, 1 with mild cGVHD features, and 1 with extensive cGVHD.

Table 3B. Organ-specific and Global Response After Complete MSC Therapy

	PR	CR	PR/CR	CR in Further Observations (Permanent CR)
Organ response, n (%)				
Skin (n = 8)	4 (50%)	1 (13%)	5 (63%)	4 (50%)
Liver (n = 6)	1 (17%)	1 (17%)	2 (33%)	2 (33%)
GI (n = 8)	4 (50%)	0	4 (50%)	3 (38%)
Overlap features (n = 3)	3 (100%)	0	3 (100%)	0
Global response, n (%)				
All affected sites (n = 9)	4 (44%)	0	4 (44%)	1 (11%)

Abbreviations: CR, complete remission; GI, gastrointestinal; MSC, mesenchymal stem cell; PR, partial remission.

In multiple studies, when MSC were applied in an earlier stage of resistant aGVHD, the complete remission rates were higher. Le Blanc et al [13], in their study of a group of steroid-resistant grade II-IV patients, described 55% CR and 16% PR. Lucchini et al [14] reported on MSC treatment in the cohort of grade I-IV GVHD children and achieved 23.8% CR and 47.6% PR. Our findings may be compared with those of Lucchini et al if we do not consider the poor results when MSC were applied in critically ill (with multiorgan failure) patients.

In our work we observed that GVHD remission after single-dose MSC was transient and usually there was a need to apply several infusions for stable recovery. Our results refer to previous studies, beginning from the first case report on the MSC treatment of refractory GVHD, presenting the achievement of permanent disease remission after two MSC infusions in a 9-year-old boy [15]. In our study, 3 patients benefited from multiple doses of MSC (2-6 infusions), although the consecutive doses were given over longer intervals, after GVHD had reflared. Better results were seen when treating the most severe stages of GVHD, if the MSC doses were applied in multiple doses scheduled as twice weekly, once weekly, or every 2 weeks. Prasad et al [16] repeated MSC infusions twice a week for 4 weeks and continued the therapy once a week in poor responders, achieving 58% CR and 17% PR in groups with grade III or IV aGVHD. Zhao et al [17] proposed a schedule of MSC therapy at a median of 1×10^6 cells/kg given once weekly until aGVHD showed a complete response or administration of MSCs for a total of 8 doses. They observed a response rate of 75% after a median of 4 doses. Even better results with use of 4 once-weekly MSC infusions were seen in recent study by Bader and colleagues [18], who obtained an overall response of 83% estimated after 28 days of therapy, in which 61% of patients achieved CR and 25% of them PR. Erbey et al [19] treated grade III and IV aGVHD with MSC infusions every 2 weeks, showing CR in 54.5% of patients and PR in 21.2% of patients, with similar efficacy to that of a twice-per-week schedule.

Regarding organ-specific effect, we observed the best response to the therapy in the setting of skin and GI involvement. In the skin manifestation of aGVHD, 63% of patients showed remission after the first MSC infusion and 50% of them achieved stable release of skin symptoms on long-lasting follow-up. In GI involvement, 63% of patients similarly experienced amelioration after the first MSC dose and 38% showed long-lasting remission. Poorer effectiveness of the therapy was seen in the liver GVHD manifestation, with permanent remission of the disease reached in only 2 patients. Similar observations were seen in a Turkish study [19], which demonstrated GVHD remission in 75%, 65.5%, and 35.7% of patients with skin, GI, and liver involvement, respectively. Even better results, but still pointing at skin and GI GVHD localization as the most curable by MSC therapy, were shown by Prasad and colleagues [16]. They reported 100%, 75%, and 25% CR in skin, GI, and liver involvement, respectively.

In our study, MSC therapy was more effective in acute GVHD than in extensive chronic disease and overlap syndrome. Although all the patients with overlap syndrome showed some amelioration in symptoms after both the first infusion and complete MSC therapy, none had complete recovery. Some studies revealed promising data on cGVHD treatment with the use of MSC. In one of the largest reported cohorts of 19 adult patients studied on MSC in refractory cGVHD therapy, Weng et al [20] showed partial (53%) or complete remission (21%). Lucchini and colleagues [14] decribed 4 pediatric patients with overlap syndrome or cGVHD (1 of them previously treated with MSC for aGVHD), of whom 3 presented with partial or complete response after this therapy. Introna et al [21] reported on MSC treatment in 40 patients with grade II-IV acute GVHD and found that 27 achieved partial or complete remission, although 11 of the 27 patients developed cGVHD within 6 months. This finding supports our observation that the effect after MSC application is not itself long-lasting, thus it does not eliminate the risk of transformation into cGVHD or overlap syndrome. Even so, multiple infusions of MSC could potentially ameliorate its course.

Although multiple studies on intravenous MSC therapy for GVHD have been reported, controversy still exists about the optimal dose of these cells. In our work we applied a median quantity of 1.9×10^6/kg of recipient body mass, with the wide range of $0.1–6.5 \times 10^6$/kg. We did not observe any positive effect after the 2 lowest doses applied, 0.1 and 0.2×10^6/kg. If only the number of cells administered in 1 dose was higher (beginning from 0.4 and 0.3 + 0.4) the majority of patients achieved remission thereafter. In the first series of reports on MSC therapy for GVHD, the number of cells administered varied from 0.4 to 9.0×10^6/kg (median 1.4) [13], $0.4–3.0 \times 10^6$/kg [22], or $0.6–9.0 \times 10^6$/kg (median 1.15) [23]. The lowest reported effective MSC doses were applied by Weng et al [20]. They observed 74% partial or complete GVHD remissions in adult patients with cGVHD who had been administered $0.23–1.42 \times 10^6$/kg (median 0.6×10^6/kg) MSC. Currently, the most frequently proposed MSC single dose in GVHD therapy is 1×10^6/kg, with repeated infusions recomended [17,19,24]. On the other hand, efficacy and safety of considerably higher doses of MSC in an intensive regimen have been presented inter alia in a pediatric cohort of 12 patients with grade III or IV aGVHD with the use of MSC portions of 2×10^6/kg or 8×10^6/kg twice a week with a response rate (partial or complete) of 100% [16].

No direct adverse events of MSC infusions have been reported until now. Still, although we have touched upon the issue of delayed effects of the highest dose MSC therapies, we do not have the issue solved. Suggested delayed MSC effect on malignant relapse risk when MSC were applied contemporary with stem cell transplant [25] was not shown in the MSC treatment of GVHD [21,26].

Immunosupressive activities of MSC in the therapy of GVHD were investigated as a risk factor for late infectious complications and increased infection-related mortality [27], which was not confirmed in other studies [28]. According to the meta-analysis by Balan and colleagues [29], in-vitro studies and experimental trials with animal models validated and detailed multidirectional antimicrobial effects of MSC, but in patients with severe GVHD the reported impact of MSC therapy on infection-related death risk varied across the clinical studies. Mesenchymal treatment of grade II-IV GVHD may be related to the higher risk of pneumonia-associated death or fatal invasive fungal infections, but probably not with viral (cytomegalovirus or Epstein-Barr virus) reactivation. The authors described late infection-related deaths of patients who had undergone HSCT and were treated with MSC for severe GVHD. In any case, in steroid-resistant GVHD treatment, several lines of immunosuppressive therapies themselves can result in escalated risk of severe infectious complications. In our experience, 1 patient, who achieved remission of GVHD after a high cumulative dose of MSC (7.8×10^6/kg in 4 portions) and treated concomitantly with high-dose steroids, calcineurin inhibitors, etanercept, and sirolimus, died of lung mycosis aggravated by adenoviral pneumonia 47 days after the last MSC infusion and 371 days after HSCT. Another patient, a boy with cGVHD, died of lung mycosis 38 days after a single MSC dose at 479 days after HSCT.

The evidence of efficacy and safety of MSC therapy of severe aGVHD and extensive chronic GVHD has continued to build for years since the first data on this subject was presented by the group from the Karolinska Institutet in Stockholm [13,15,23]; nevertheless, the detailed immunosuppressive activity of MSC, as well as their precise targets in cytotoxic reaction interference, remains unclear. Our work supports the advantages and safety of this therapy, but only if applied ahead of GVHD-related critical organ damage. Further studies are needed to establish MSC as a reliable therapeutic tool with specific indications and scheduling.

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