

ORIGINAL ARTICLE

Mesenchymal stem cell as salvage treatment for refractory chronic GVHD

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Refractory chronic GVHD (cGVHD) is an important complication after allogeneic hematopoietic SCT and is prognostic of poor outcome. MSCs are involved in tissue repair and modulating immune responses *in vitro* and *in vivo*. From April 2005 to October 2008, 19 patients with refractory cGVHD were treated with MSCs derived from the BM of volunteers. The median dose of MSCs was 0.6×10^6 cells per kg body weight. Fourteen of 19 patients (73.7%) responded well to MSCs, achieving a CR ($n=4$) or a PR ($n=10$). The immunosuppressive agent could be tapered to less than 50% of the starting dose in 5 of 14 surviving patients, and five patients could discontinue immunosuppressive agents. The median duration between MSC administration and immunosuppressive therapy discontinuation was 324 days (range, 200–550 days). No patients experienced adverse events during or immediately after MSC infusion. The 2-year survival rate was 77.7% in this study. Clinical improvement was accompanied by the increasing ratio of CD5+CD19+/CD5-CD19+ B cells and CD8+CD28-/CD8+CD28+ T cells. In conclusion, transfusion of MSCs expanded *in vitro*, irrespective of the donor, might be a safe and effective salvage therapy for patients with steroid-resistant, cGVHD.

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Keywords: mesenchymal stem cell; refractory; chronic GVHD; transplantation

Introduction

Chronic GVHD (cGVHD) is one of the main limitations to successful allogeneic hematopoietic SCT, and has a substantial impact not only on survival but also on the

quality of life of otherwise cancer-free patients. Half of the patients undergoing a HLA-identical allograft who survive beyond 100 days may require long-term immunosuppressive treatment for extensive cGVHD, often for more than 2 years.^{1–3} More than one-third of patients with cGVHD do not respond to first-line therapy, which often involves combinations of corticosteroids and a calcineurin inhibitor.⁴ There is no standard second-line or salvage therapy for these patients and they have a poor outcome.^{5–11} MSCs are multipotent non-hematopoietic stem cells that can differentiate into various lineages and have been used to repair injured tissues.^{12–17} Recently, MSCs have also shown unique immunomodulatory properties *in vitro*, including inhibition of T-cell proliferation after stimulation by allo-Ag and mitogens, and prevention of the activity of cytotoxic T cells.^{18,19} MSCs have been used for the prophylaxis of acute GVHD²⁰ and for the treatment of patients with steroid-refractory acute GVHD,^{21–24} but just one study has reported the temporary effect of MSCs in a patient with refractory cGVHD.²²

In this study, we investigated the effects of MSCs derived from HLA-identical sibling donors or HLA-disparate third-party donors as a salvage therapy for 19 patients with refractory cGVHD.

Patients and methods

Patient characteristics and definitions

Between April 2005 and October 2008, 19 patients were treated with *in vitro* expanded BM-derived MSCs as a compassionate treatment for refractory cGVHD (Table 1). Patients included in this study require the presence of one of the evidences: (1) the manifestations of cGVHD show no improvement during treatment with the standard immunosuppressive therapy, including corticosteroids and calcineurin inhibitors or combination with other immunosuppressive agents after 1 month; (2) the manifestations of cGVHD show evidence of progression after at least 2 weeks of standard immunosuppressive therapy or during steroid or other immunosuppressive agents taper. The study was approved by the Ethics Committee of Guangdong General Hospital. All patients and the MSC donors provided written informed consent.

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Table 1 Clinical characteristics of patients with refractory cGVHD and the organ response

UPN	Sex	Age (years)	Disease	Organ scoring before MSC and organ response																							
				Skin			Eyes			Oral			Liver			GI			Muscle			Plt			Lung		
				A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
102309	F	35	CML	3 ^a	mPR	PR	—	—	—	—	—	—	3	mPR	CR	—	—	—	2	NR	PR	—	—	—	—	—	—
065877	F	39	ALL	2	PR	CR	1	NR	CR	3	CR	CR	—	—	—	2	CR	CR	—	—	—	—	—	—	—	—	—
125883	M	28	AML	3	PR	GPR	2 ^b	NR	PR	3	PR	CR	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
128497	M	33	AML	2	mPR	CR	2 ^b	NR	PR	2	PR	CR	2	NR	CR	—	—	—	—	—	—	—	—	—	—	—	—
112707	M	22	AML	3	NR	mPR	3 ^b	PR	PR	3	mPR	PR	2	PR	CR	3	PR	PR	—	—	—	—	—	—	—	—	PD
144925	M	26	CML	2	mPR	CR	2	PR	CR	2	PR	CR	3	PR	CR	2	PR	CR	—	—	—	—	—	—	—	—	—
152529	M	35	CML	3 ^a	mPR	mPR	1 ^b	NR	NR	2	CR	CR	—	—	3	GPR	CR	2	NR	PR	—	—	—	—	—	—	—
151639	M	36	AML	3	NR	PR	2 ^b	NR	NR	3	PR	PR	—	PR	CR	—	—	—	—	—	—	—	—	—	—	—	—
157937	F	28	CML	3	PR	PR	2 ^b	NR	PR	3	PR	CR	2	NR	CR	—	—	—	—	—	—	—	—	—	—	—	PD
202507	F	18	AML	2	PR	PR	1 ^b	NR	NR	2	PR	PR	2	CR	CR	2	PR	PR	—	—	—	—	—	—	—	—	—
167290	M	19	ALL	3	PR	PR	2 ^b	NR	PR	3	PR	PR	1	CR	CR	3	GPR	GPR	—	—	—	—	—	—	—	—	—
173264	M	28	CML	1	CR	CR	1 ^b	NR	NR	—	—	—	3	GPR	CR	—	—	—	—	—	—	—	—	—	—	—	PD
206111	F	28	CML	2	PR	PR	1 ^b	NR	NR	1	CR	CR	2	NR	NR	—	—	—	—	—	—	—	—	—	3	NR	NR
065877	M	33	AML	3 ^a	NR	NR	3 ^b	NR	NR	2	PR	PR	—	—	—	3	NR	NR	—	—	—	—	—	—	—	—	—
211188	M	36	CML	1	NR	PR	1 ^b	NR	NR	2	CR	CR	—	—	—	1	CR	CR	2	NR	NR	—	—	—	—	—	—
120560	M	20	MDS	2	mPR	mPR	—	—	—	—	—	—	—	—	3	PR	PR	—	—	—	2	NR	PR	—	—	—	—
213198	M	35	ALL	2	PR	PR	1	NR	CR	2	PR	PR	2	CR	CR	—	—	—	—	—	—	—	—	—	—	—	—
124732	M	28	CML	—	—	—	1 ^b	NR	NR	2	CR	CR	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
196003	M	31	ALL	2	PR	PR	1 ^b	NR	NR	2	PR	PR	—	—	—	2	PR	PR	—	—	—	3	NR	PR	—	—	—

Abbreviations: A = scoring before MSC treatment; B = initial response; C = final response; cGVHD = chronic GVHD; F = female; GI = gastrointestinal; GPR = good partial response; M = male; MDS = myelodysplastic syndrome; mPR = minor partial response; NR = no response; PD = progressive disease or emerge new involvement; UPN = unique patient number.

^aWith scleroderma.

^bKeratoconjunctivitis sicca.

Transplantation procedure

All patients received sibling HLA-identical PBSCs after treatment with fludarabine combined with modified BU/CY-conditioning regimes (FABC conditioning regimen), which consisted of cytarabine 2.0 g/m² on day -9, BU 3.2 mg/kg per day for i.v. on days -8 to day -6, followed by CY 60 mg/kg per day on days -5 and day -4, combined fludarabine 30 mg/m² per day for three consecutive days, on days -6 to day -4, and Me-CCNU (1-(2-chloroethyl)-3-(4-ethylnitrophenyl cyclohexyl)-1-nitrosourea) 250 mg/m² on day -3. All patients received CsA in combination with a short course of mycophenolate mofetil and four doses of MTX for GVHD prophylaxis. The dosage of CsA was 2.0 mg/kg per day i.v. from day 9 before transplantation until bowel function was normal, at which time the patient was switched to oral CsA according to the ratio of 1:2.5. The dose of mycophenolate mofetil was 0.5 g every 12 h orally from day 9 before transplantation to day 1 before transplantation. The dose of MTX was 15 mg/m² i.v. on days +1 (1 day after transplantation), then 10 mg/m² i.v. on days +3, +6 and +11 after transplantation. Patients received *Pneumocystis carinii* prophylaxis with trimethoprim/sulfamethoxazole, antiviral prophylaxis with acyclovir or equivalent, and antibacterial prophylaxis with penicillin or equivalent.

HSC donors

All donors were HLA-identical siblings. HLA typing was performed by genomic low-resolution DNA-based typing (PCR sequence-specific primer).

Diagnosis and grading of GVHD

The diagnosis, organ scoring and global assessment of cGVHD were based on the National Institutes of Health (NIH) consensus criteria for cGVHD.²⁵ The onset forms of cGVHD were determined according to the published classifications.²⁶

Treatment prior to MSC administration

All patients except one had failed to respond to at least two previous lines of immunosuppressive therapy more than 6 weeks before receiving MSCs (Table 2). The median time from cGVHD diagnoses to MSC infusion was 35.6 weeks (range, 6.4–246.1). One patient (UPN124732) suffered moderate cGVHD that resolved with calcineurin inhibitor and steroid, but recurred after discontinuing all agents 6 months later. This patient received MSC infusion only for the treatment of cGVHD recurrence.

MSC preparation and administration

BM-derived MSCs were aspirated (20 mL) under local anesthesia from HLA-matched donors or HLA-disparate third-party adult donors. This study was approved by the institutional review board of the Guangdong General Hospital, and all donors provided written informed consent. Human MSCs were isolated and cultured as previously described with minor modifications.^{27,28} Briefly, 20 mL BM aspirates were diluted 1:1 with human MSC growth medium (consisting of low glucose Dulbecco's modified Eagle's medium (L-DMEM; Hyclone, Logan, UT, USA) and 10% fetal bovine serum (FBS; Hyclone))

Table 2 Immunosuppression and global response in patients with refractory chronic GVHD

UPN	Immunosuppressive agents before MSC infusion		No. of MSCs	Primary response	Final response	Immunosuppressive agents at the last follow-up		Outcome (no. of days of follow-up) and disease status at the last follow-up
	PSL, mg/kg	Other agent				PSL (mg/kg)	Other agents	
102309	0.5	FK506	3	NR	PR	—	—	Alive (1823) in PR
065877	0.5	CsA/Penicillanmine/Thal	1	PR	CR	—	—	Alive (1375) in CR
125883	0.5	CsA/MMF	3	NR	PR	—	CsA	Alive (1367) in PR
128497	0.5	CsA/MMF/Thal	2	NR	CR	—	—	Alive (1210) in CR
112707	0.5	FK506/MMF	4	NR	MR	—	FK506	Died (1083) in MR
144925	0.5	CsA	2	PR	CR	—	—	Alive (1013) in CR
152529	0.3	FK506/MTX/Azathioprine	5	NR	PR	—	FK506/MTX	Alive (922) in PR
151639	0.5	CsA/MTX	1	NR	PR	—	CsA/MTX	Alive (1073) in PR
157937	0.6	CsA/MTX	1	PR	MR	0.3	—	Alive (1040) in MR
202507	0.5	CsA/MTX	1	NR	PR	—	CsA	Died (331) in PR
167290	0.4	CsA	2	NR	PR	—	FK506/MTX	Died (763) in PR
173264	0.3	CsA/MMF	1	PR	MR	0.5	—	Alive (1719) in MR
206111	1	CsA	2	NR	NR	—	CsA	Died (234) in NR
065877	0.3	MMF	2	NR	NR	0.05	MMF	Alive (2791) in NR
211188	0.5	FK506/Azathioprine	1	PR	PR	—	FK506	Alive (546) in PR
120560	1	FK506/Budesonide	2	PR	PR	0.1	FK506	Alive (711) in PR
213198	0.8	CsA	1	PR	PR	—	CsA	Died (261) in PR
124732	—	—	1	PR	CR	—	—	Alive (1343) in CR
196003	1.6	CsA/MTX/Penicillanmine	3	NR	PR	0.3	CsA/Penicillanmine	Alive (511) in PR

Abbreviations: FK506 = tacrolimus; MMF = mycophenolate mofetil; MR = mixed response; NR = no response; PSL = prednisone; Thal = thalidomide; UPN = unique patient number.

and layered over Ficoll-Paque solution (1.077 g/mL; Amersham Biosciences, Uppsala, Sweden). After centrifugation at 800 g for 20 min, mononuclear cells were collected from the interface, washed and resuspended in human MSC growth medium at a density of 5000 cells/cm². After 3 days, the non-adherent cells were removed by replacing the spent medium with fresh medium. Adherent cells were further cultured with media changes every 3 days. When they were 70–80% confluent, cells were detached by trypsin-EDTA and passaged at a ratio of 1:3. Multiple individual donor preparations were performed and second or third passage MSCs were used for individual experiments. All these samples have been tested for their ability to differentiate into osteoblasts, adipocytes and chondrocytes. Flow cytometry was performed using a FACSort and analyzed with CellQuest software (Becton Dickinson, San Jose, CA, USA). The culture-expanded cells expressed CD29, CD44, CD73, CD90, CD105 and CD166, but not CD11a, CD34 or CD45. They could be induced to differentiate into cells of osteogenic, adipogenic and chondrogenic lineages under proper induction medium (Figure 1). The MSCs were screened negative for pathogens and contaminants (for example, bacteria, fungi, virus, mycoplasma and endotoxin) before infusion.

The planned MSC dose scheme was 1.0×10^6 /kg body weight, but there were some difficulties in growing sufficient MSCs on time for patient infusion as planned. As a result, the median MSC dose given was 0.6×10^6 /kg body weight (range 0.23 – 1.42×10^6 /kg per body weight). MSCs were harvested fresh from culture and administered to the patients by i.v. infusions over 30 min. The median number of MSC administrations was 2 (range, 1–5). The median duration between the first and second MSC application was 188 days (range, 13–944 days).

Evaluation of response and monitoring

Evaluation of response to cGVHD treatment is fairly difficult owing to the lack of uniform assessment criteria and the various disease manifestations of this disease. In this pilot study, we used the NIH consensus criteria for organ scoring and global assessment of cGVHD²⁵ and measures of assessing response.²⁹ Patients were evaluated at 3-month intervals after the first MSC administration, including scoring and the following domains were scored: skin, oral mucosa, liver, gastrointestinal system, eyes, muscle, joint, and lung. These domains were chosen to make the evaluation as objective as possible. The patient investigation form used to obtain information about symptoms and the physician investigation form was collected at each follow-up visit. A detailed symptom list and physical examination findings were recorded, and these two forms were used to confirm responses. In the case of a discrepancy, objective data, such as biological sign and laboratory results, were used to clarify ambiguity. Each patient's response was assessed at 3 months and at his or her final assessment. The organ response and the overall response were used to determine the therapeutic effects of MSC for refractory cGVHD.

Assessment of organ response

According to the criteria of organ scoring proposed by NIH for cGVHD,²⁹ each organ was assigned scores of 0 (none) to 3 (worst). The response definitions were in agreement with current studies:^{5,7,11,16} CR was defined as the resolution of all clinical manifestations of the involved organ, except the irreversible injury (such as severe scleroderma and keratoconjunctivitis sicca). Damage caused to certain organs by cGVHD is not reversible or

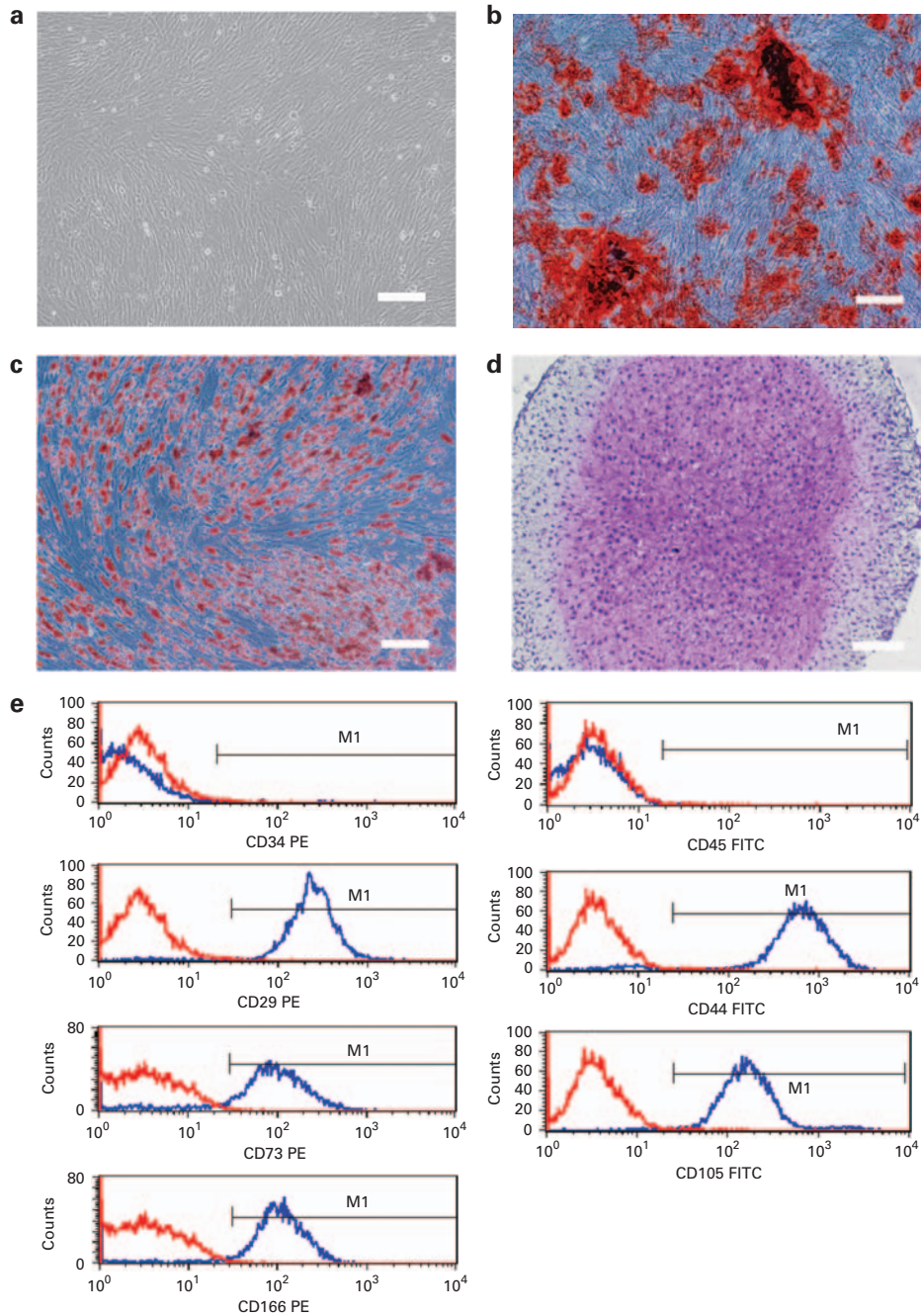


Figure 1 Characterization and differentiation of human MSCs. (a) Phase-contrast microscopy of human MSCs at passage 3. (b) Alizarin red S staining of osteogenic differentiated human MSCs. (c) Oil red O staining of adipogenic differentiated human MSCs. (d) Toluidine blue staining of chondrogenic differentiated human MSCs. (e) Flow-cytometric analysis of cell surface Ags of human MSCs. Bar = 200 μ m.

curable,^{29,30} such as chronic dry eyes, esophageal stricture, bronchiolitis obliterans, or advanced skin sclerosis or contractures. To objectively assess the therapeutic response of a novel salvaged treatment for refractory cGVHD, we excluded ‘irreversible’ injuries of scleroderm and keratoconjunctivitis sicca from the CR to avoid bias of responses assessment. Good PR (GPR) was defined as reduction in clinical manifestations and laboratory data of more than 75% or improvement by more than one point on a

four-point scale without achieving CR; PR 10 was defined as improvement by at least one point in the involved organ, or reductions in clinical manifestations and laboratory data of more than 50% but less than 75%. Minor PR (mPR) was defined as improvement of less than one point in one organ or clinical symptom score and reductions in laboratory data of more than 25% but less than 50%. Progressive disease (PD) was defined as worsening in any domain of at least 25% or deterioration of more than one

point while on treatment or steroid taper. No response (NR) was defined as no improvement or deterioration in the affected organ. Each organ in cGVHD after MSC infusion was estimated as showing meaningful response (CR, GPR and PR), progression or NR. Patients with an initial improvement but subsequent worsening were considered as showing disease recurrence.

Assessment of overall response

Patients were evaluated according to the NIH consensus criteria of cGVHD,²⁹ and the grade was reassessed (none, mild, moderate or severe) at the first assessment (between 2 weeks to 3 months after MSC infusion) and final follow-up visits. Overall response was defined as follows: CR was defined as the resolution of all clinical manifestations of cGVHD in all the involved organs, except the irreversible injury, or the scoring of all organs being zero. PR was defined as the global assessment improvement by at least one point or at least a 50% improvement of clinical manifestations but without CR, and with no worsening in any domain. No response (NR) was defined as no improvement or deterioration of all affected organs. Patients who experience early deaths due to GVHD prior to assessment of response were considered NR as well. Mixed response (MR) was defined as improvement in at least one organ with deterioration in another organ or emergence of newly involved manifestation. Patients with an improvement but later worsening were considered to have PD as well. The response rate including CR and PR, and no response was defined as NR and PD. The OS rate was calculated from the date of hematopoietic SCT to the date of death or the last follow-up.

Flow-cytometric analysis

Flow cytometry was performed on a FACScan (Becton Dickinson) (BDIS) equipped with a 15 mW air-cooled argon laser tuned at 488 nm. Data acquisition and analysis were performed by the CellQuest (Becton Dickinson) (BDIS) research software.

Statistical analysis

The Kaplan–Meier method was used to estimate OS and significance of differences with the log-rank test (Mantel–Cox). The proportion of lymphocyte subsets was compared using a two-sided Wilcoxon signed ranks test. *P*-values <0.05 were considered statistically significant. Analysis was performed with the SPSS software package (SPSS 13.0, Inc., Chicago, IL, USA).

Results

Clinical characteristics

Nineteen patients with refractory cGVHD received MSC transfusions based on the primary immunosuppressive therapy. All patients except one were heavily pretreated with immunosuppressive treatment more than 6 weeks before the first MSC transfusion, including CsA or FK506 (*n* = 17), steroids (*n* = 18), MMF (*n* = 5), MTX (*n* = 6), penicillamine (*n* = 3), azathioprine (*n* = 2), thalidomide

(*n* = 2) and budesonide (*n* = 1). The characteristics of the patients with cGVHD are listed in Table 1. Sixteen patients were progressing during the immunosuppressive agents taper, two patients showed no response after 2 months of initiation and one relapsed when discontinued immunosuppressive therapy for 6 months. Eleven patients had *de novo*, five had a progressive and three had the quiescent form of cGVHD. cGVHD was diagnosed at a median of 160 days (range, 73–352) after transplantation. According to NIH classification, 14 patients (73.7%) were classified as severe grade and 5 patients (26.3%) as moderate grade. Of 18 patients (94.7%) with skin involvement, three had severe sclerotic features with a skin scoring of 3 in all areas tested, and with decreased range of motion in at least one joint or ulceration at the same time. The remainder had lichen planus-like features, poikiloderma and maculopapular changes involving the majority of their body surface. Fourteen of the 17 cases of cGVHD of eyes were keratoconjunctivitis sicca (Schirmer test <5 mm/5 min). Among them, two patients had severe impairment with loss of vision and were unable to work because of ocular symptoms. Oral cGVHD was severe in six of sixteen patients (84.2%). The major symptoms and signs were pain with ulcer or mucositis, lichen, hyperkeratotic plaques, xerostomia, pseudomembranes and mucocele. Eleven patients with liver cGVHD (57.9%) had elevations of serum alanine transaminase, alkaline phosphatase and bilirubin. In patients with gastrointestinal (GI) involvement (*n* = 10, 53%), eight had nausea, vomiting and abdominal pain. One had diarrhea and one had strictures of the esophagus. Other cGVHDs were those of the muscle and joint (*n* = 3), thrombocytopenia (*n* = 2) and lung (*n* = 1). Histological information was available for five of the nineteen patients with cGVHD. No infusion-related toxicity was observed during or immediately after the administration of MSCs.

Primary response to therapy

According to different patients, the primary assessment was carried out from 2 weeks to 3 months after the first MSC transfusion. A total of 8 patients (42.1%) had response after the first dose of MSC infusion. Eleven patients showed no response. The median time from the first MSC infusion to meaningful improvement was 29 days (range, 1–100 days). Skin was the organ that was the most involved (*n* = 18) and had 50% CR/PR rate. Two of three patients with severe sclerodermatosis had minor PRs after the first MSC infusion. Responses were also seen in patients with refractory cGVHD of oral mucosa (*n* = 15, 93.8%), GI tract (*n* = 9, 90%), liver (*n* = 7, 63.6%) and eye (*n* = 2, 11.1%). No meaningful response was seen in the patients with muscle or joint, lung or platelet involvement at the first follow-up.

Overall response to therapy

After the first MSC administration, 11 patients received at least two doses because of NR (*n* = 9) or consolidation (*n* = 2). The median period from the first to the second infusion was 188 days (range, 13–944). The data are summarized in Table 2. After the median follow-up time of

697 days (range, 81–1294), a total of 14 patients (73.7%) had an overall response (CR, $n=4$; PR, $n=10$). The median duration from the first MSC transfusion to the best response was 233 days (range, 81–761 days). The cumulative response rate of skin was 78% ($n=14$, CR=4, GPR=1, PR=9). Out of three patients with scleroderma, one had a PR, one had a minor PR and the remaining one had no response to the MSC infusion. The cumulative response rate of eyes was 44.4% ($n=8$) and three patients with conjunctivitis achieved CR. Keratoconjunctivitis sicca developed in 14 patients, and only 5 patients (35.7%) showed amelioration of clinical symptoms but without improvement in Schirmer's test. Cumulative response was also seen in oral mucosa ($n=16$, 100%), liver ($n=10$, 90.9%) and GI tract ($n=9$, 90%). Of three patients with moderate joint stiffness or myositis, two of them had a delayed PR more than 7 months later. Two patients with thrombocytopenia showed delayed responses at days 223 and 366 after multiple MSC infusions. The patient (UPN206111) with lung involvement had no response to MSC treatment and died of invasive fungal infection at 81 days after MSC infusion. The other three patients achieved mixed response because of developing lung cGVHD after MSC infusion, although their initial involved lesions were improved.

Survival and immunosuppressive therapy

In this study, five patients (26.3%) died after the first MSC infusion. Their median survival duration was 183 days (range, 61–805 days). Reasons for death were invasive fungal infection ($n=2$), primary malignant disease relapse ($n=2$) and bronchiolitis obliterans ($n=1$), and the latter was related to cGVHD. As yet, no surviving patient has experienced relapse of the malignant disease at the median follow-up duration of 669 days (range, 226–1294 days). The 2-year probability of survival rate was the same in patients with refractory cGVHD administered MSCs (77.7%) and in the 14 cases without refractory cGVHD (68.8%) ($P=0.547$). All of the patients of these two groups had the same age, sex, primary diseases, treatment and the grade of initial cGVHD (data not shown). But the median times between cGVHD diagnoses to discontinuation of immunosuppressive agents were 611 (337–1112) days and 427 (295–764) days in the refractory group and non-refractory group, respectively. In the non-refractory group, 58.8% of the patients were able to discontinue immunosuppressive agents at the median time of 427 days and with a stable CR duration of more than 375 days.

At the final follow-up visit, ten out of fourteen surviving patients (CR=4, PR=7, MR=2, NR=1) were able to taper and/or discontinue their immunosuppressive therapy after MSC transfusion (Table 2). Four patients with CR and one with PR (UNP102309) who was suspected to have post-transplantation lymphoproliferative disorder discontinued immunosuppressive agents within 324 days (range, 200–550 days) after the first MSC infusion, and maintained continuous CR or stable disease for 675 days (range, 317–1198 days). Five patients with PR began to taper (decreasing) immunotherapy at a mean time of 233 days (range, 136–345 days) after the first MSC infusion and

maintained improvement or were stable for a mean of 464 days (range, 90–842 days). The dose of prednisone was successfully discontinued or reduced to no more than 0.1 mg/kg in those responding patients during the whole observation period. One NR and one PR patient maintained their basic therapies without needing further escalation of immunosuppressive agents after the first MSC infusion, and two MR patients were retreated with steroid 0.4 mg/kg daily. Patient UPN124732 only accepted MSC infusion and achieved a CR without further immunosuppressive therapy.

Unfortunately, three patients developed lung involvement during the tapering period or after withdrawal of immunosuppressive therapy at 6 months after MSC infusion, although improvements in their oral mucosa, skin, eye or liver function were reported. These patients were regarded as MR. Two patients with PR who progressed to lung involvement were retreated with prednisone 0.3–0.5 mg/kg, and maintained stable disease. The NR patient died of bronchiolitis obliterans on day 816 after the first MSC infusion, although tacrolimus was combined with four times MSC.

No response was seen in two patients; one patient died from invasive fungus disease on day 81 after the first MSC infusion; and one alive patient (UPN 065877) had severe skin, esophagus and eye involvement, had received immunosuppressive agents for more than 6 years and did not respond to MSC treatment that was given twice.

Lymphocyte subset analysis

Flow cytometry was performed to detect lymphocyte subsets pre- and post-MSC infusion. There were no significant changes in the proportion of T cells, B cells, NK cells and activation in NR patient post-MSC treatment. In the responded group, the lymphocyte ($35.45 \pm 11.45\%$ vs $25.32 \pm 11.15\%$, $P<0.0001$) and CD3+CD4+ T cells ($45.08 \pm 13.71\%$ vs $39.15 \pm 11.99\%$, $P=0.001$) increased significantly 3 months after MSC transfusion, and the CD3+CD8+ T cells ($45.13 \pm 16.53\%$ vs $50.06 \pm 18.02\%$, $P=0.018$) decreased. However, the proportion of CD4+CD25+ T cells and CD8+CD25+ T cells did not change significantly post-MSC transfusion compared with pre-MSC infusion in patients with response (the P -value was 0.493 and 0.327, respectively). The CD8+CD28+ T cells decreased when the cGVHD improved ($42.42 \pm 15.33\%$ vs $38.87 \pm 17.09\%$, $P=0.023$) and CD8+CD28- T cells increased ($58.87 \pm 15.27\%$ vs $63.31 \pm 16.65\%$, $P=0.025$) post-MSC infusion. In addition, the CD5+CD19+ B cell subset increased ($29.42 \pm 15.33\%$ vs $40.99 \pm 17.20\%$, $P=0.033$) and CD5-CD19+ B cells decreased ($70.58 \pm 15.33\%$ vs $59.01\% \pm 17.20\%$, $P=0.033$) after MSC infusion in the responsive group.

Discussion

cGVHD, a multi-organ disorder, is the major cause of late non-relapse mortality after allogeneic hematopoietic SCT. More than one-third of cGVHD patients do not respond to first-line therapy comprising corticosteroid and calcineurin

inhibitor combination therapy,⁴ and their prognosis is often poor.³¹ There is no standard second-line or salvage therapy for these patients. Recent reports have described the use of various therapies^{5–11} for refractory cGVHD, but the results have demonstrated limited efficacy and low long-term survival due to toxicity. MSCs are multipotent non-hematopoietic stem cells that can differentiate into various tissues,¹³ repair injured tissues^{12,14–17} and modulate allogeneic immunoreactions.^{18,19,32} Recently, MSCs have been used for the prophylaxis of acute GVHD²⁰ and the treatment of patients with steroid-refractory acute GVHD, at a dose of 1.0×10^6 /kg per patient weight.^{21–24} However, there are few data regarding the efficacy and safety of MSC for cGVHD. There is only one published report that demonstrated a temporary response of MSC in one patient with liver cGVHD.²²

This is the pilot study of MSCs combined with initiation of immunosuppressive therapies for refractory cGVHD, in heavily pretreated patients who have exceeded 6 months of treatment. A total of 14 patients (73.7%) had either CR ($n=4$) or PR ($n=10$) and 71.4% of surviving patients were able to discontinue or taper immunosuppressive agents after MSC infusion. In addition, the 2-year survival rate (77.7%) was greater than those of previous reports.^{5,31} MSCs seemed to be more promising and effective for cGVHD of the oral mucosa, GI fluid, liver and skin involvement. Depending on the responding organs, the manifestations of the oral mucosa, GI fluid and liver tended to improve within 24 h or a few weeks after the initiation of MSC treatment, whereas it might take a long time for the eyes and sclerotic lesion to achieve response. As we know, damage to certain organs by cGVHD is not reversible or curable. Interestingly, in the results of the present study, we found that two patients (UPN102309 and UPN 152529) with severe scleroderma had a PR or a minor PR. We also noticed that in patients with keratoconjunctivitis sicca (Schirmer's test <5 mm/5 min) in this study, although Schirmer's test had not improved, their clinical symptoms showed improvement by way of reduction in eyedrops and pain. Therefore, some 'irreversible' damages of chronic GVHD may be improved after MSC infusion. Owing to the long duration required to attain a response, patience should be exercised. The results of the present study indicate that MSCs might be ineffective in cases with bronchiolitis obliterans or may develop to bronchiolitis obliterans.

In more than half of the patients, a single dose of MSC was able to create a temporary or continuing response, whereas some patients needed several doses to generate a lasting response. On the contrary, despite several MSC transfusions, no response or mixed response was seen in a few patients. Clinical benefit might not require sustained engraftment of many MSCs, but could possibly result from production of growth factors or temporary immunosuppression. MSC transfusion by the venous system is distributed to different organs and tissues according to the blood flow.^{33–35} Thus, MSCs may disperse to the oral mucosa, GI fluid and liver, which have an abundant blood supply, repair the injured tissues and modulate immunoreaction. *In vivo* differentiation of transplanted MSCs into non-immunosuppressive cells in a short time appears to be

one of the factors responsible for the temporary responses seen in some patients.³⁶

To study the possible mechanism, we detected the lymphocyte subsets to assess whether MSCs might participate in the amelioration of GVHD. In our study, we did not observe any significant change in the CD4+CD25+ T-cell subset accompanied by the improvement of cGVHD, but found T lymphocyte subsets of CD8+CD28+ cells, which served as active CTLs that participated in transplantation immunity and rejection reaction, and were down-regulated, accompanied by clinical remission, whereas the proportion of CD8+CD28– cells were increased. CD8+CD28– T cells, namely T-suppressor cells, are MHC class I restricted and operate in an Ag-dependent manner.³⁷ CD8+CD28– T cells served as regulated cells to induce immune tolerance, inhibited the function of APCs and thus controlled further activation of T-helper cells and inhibited Ab production.^{38,39} We also found that B1 cells (CD5+CD19+) increased and B2 cells (CD5–CD19+) decreased. The involvement of B cells in the pathogenesis of cGVHD is well known. However, recently, knowledge regarding the function of B1 cells was renewed, and they were found to protect from autoimmunity by producing cytokines such as IL-10.⁴⁰ Balances of CD8+CD28–/CD8+CD28+ T cells and CD5+CD19+/CD5–CD19+B cells may be involved in the pathogenesis of cGVHD, but more immunological studies specifically addressing this issue are needed to be performed to confirm this conjecture.

In summary, MSCs derived from BM might be a safe and effective salvage treatment for patients with refractory chronic GVHD who do not respond to corticosteroids and other immunosuppressive therapies. MSCs in combination with systemic immunosuppressive agents may improve survival rates in patients with refractory cGVHD. The small number of cases investigated in this study might not provide more definitive conclusions, and, therefore, further large-scale randomized clinical studies are needed to compare this treatment with more conventional approaches. In addition, the number of infusions needed, the optimum dose of cells in each infusion, the mechanism of MSC treatment and the possible interactions of cells with other drugs for chronic GVHD require further investigation.

Conflict of interest

The authors declare no conflict of interest.

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