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Phenotypical and Functional Characterization of Bone Marrow Mesenchymal Stem Cells in Patients with Chronic Graft-versus-Host Disease

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A B S T R A C T

Chronic graft-versus-host disease (cGVHD) is a critical complication after allogeneic hematopoietic stem cell transplantation. The conditioning therapy has been involved in the impairment of bone marrow (BM) mesenchymal stem/stromal cells (MSCs). However, the potential implication of MSCs in the pathophysiology of cGVHD has not been investigated. We analyzed expanded MSCs from patients with cGVHD and compared them with those from transplantation patients without cGVHD. The MSCs from both groups were of host origin and their reserves were comparable. They showed similar morphology, immunophenotype, population doubling times, self-renewal capacity, differentiation, and migration potential. The immunomodulatory potential of the 2 groups was also identical, they were both capable of inhibiting phytohemagglutinin-activated peripheral blood mononuclear cells (PBMCs) proliferation and inducing regulatory T cells after coculturing with $CD4^+$ T cells, and the immunosuppressive factors were secreted similarly in both MSCs whether in normal culture or coculture with PBMCs. No significant differences were observed in the cellular senescence and apoptosis between 2 groups. In addition, MSCs from patients with cGVHD displayed normal phenotype and function compared with their counterparts from healthy donors, although reduced frequency in BM mononuclear cell fraction was observed in these patients. Taken together, our results suggest that MSCs do not seem to contribute to the pathogenesis of cGVHD and indicate the feasibility of autologous cell therapy in patients who are not completely responding to standard immunosuppressive therapy for cGVHD.

INTRODUCTION

Chronic graft-versus-host disease (cGVHD) is a major complication after allogeneic hematopoietic stem cell transplantation (HSCT) that causes significant morbidity and mortality, although some improvements have been made using first-line treatments [1-4]. In addition to prior acute GVHD (aGVHD), some unique risk factors are thought to correlate with cGVHD, including stem cell source [1,5] and older recipient age [6]. cGVHD is known to have characteristic clinical manifestations that resemble those observed in autoimmune diseases. However, the pathophysiology of cGVHD is complicated and still not completely understood.

Mesenchymal stem/stromal cells (MSCs) are multipotent fibroblast-like cells that can differentiate into osteoblasts, chondrocytes, adipocytes, and myoblasts [7]. In addition, such cells exhibit extensive immunomodulatory activities and they affect a broad panel of immune cells of the innate and adaptive immunity [8,9]. The mechanisms responsible for MSCs immune regulation mainly involve direct cell-cell contact and the release of soluble inducible factors, such as transforming growth factor-β1 (TGF-β1), interleukin-10 (IL-10), hepatocyte growth factor (HGF), and prostaglandin-E2 (PGE2) [8,10,11]. Given the immunomodulatory properties of MSCs and dysregulated immune responses in cGVHD, it could be hypothesized that MSCs might contribute to the pathogenesis of cGVHD.

Due to low immunogenicity, allogeneic MSCs were largely utilized in clinical settings. However, allogeneic MSCs were also found to be susceptible for lysis by cytotoxic $CD8^+$ T cells [12] and immune rejected by MHC-mismatched recipients [13]. Autologous MSCs may thus be the preferable candidate

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Table 1
Clinical Characteristics of Patients

	cGVHD (n = 25)	No cGVHD (n = 16)	*P* Value
Age, median (range), yr	26 (14-48)	28 (18-52)	NS
Sex (male/female)	18/7	12/4	NS
Diagnosis			NS
Acute myeloid leukemia	10	8	
Acute lymphoblastic leukemia	9	8	
Chronic myeloid leukemia	4	0	
Non-Hodgkin lymphoma	2	0	
Source of HSC			NS
PB	23	15	
BM	2	1	
Donor			.005
Related	19	5	
Unrelated	6	11	
HLA			.018
Matched	11	13	
Mismatched	14	3	
Acute GVHD grade II to IV	11	2	.034
Time after HSCT, median (range), mo	20 (11-67)	23 (11-93)	NS
Immunosuppressive drugs	22	7	.007
cGVHD grade			
Mild	17	0	
Moderate	7	0	
Severe	1	0	

NS indicates not significant; HSC, hematopoietic stem cells; PB, peripheral blood.

for use in patients. It has been reported that MSCs in patients with Crohn's disease and systemic sclerosis were not affected and could be considered in an autologous setting [14,15], whereas MSCs in patients with some other autoimmune diseases were impaired [16-18]. Another issue is whether MSCs derived from cGVHD patients are suitable for autologous cell therapy. However, the biological properties of MSCs in cGVHD patients have not been studied.

In this study, we investigated whether MSCs expanded from patients with or without cGVHD showed any differences concerning the reserves and proliferation, differentiation, immunomodulation, and migration potential. Furthermore, phenotypical and functional characterization of cGVHD patients derived MSCs were compared with normal counterparts isolated from healthy individuals.

MATERIALS AND METHODS

Patients

Bone marrow (BM) samples from 25 patients with cGVHD and 16 patients without cGVHD after allogeneic HSCT were studied. Detailed patient characteristics are summarized in Table 1. The severity of cGVHD was classified according to the National Institutes of Health consensus criteria [19]. Nineteen normal BM samples were obtained from age- and sex-matched healthy individuals. Written informed consent was obtained from all subjects before sample collection, and the study was approved by the local ethics committee.

Isolation, Culture, and Expansion of MSCs

BM mononuclear cells (BMMNC) were prepared from BM aspirates by density gradient centrifugation and cultured in low-glucose Dulbecco's modified Eagle's medium (Gibco, Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum (FBS; Gibco) and penicillin/streptomycin [20]. When they reached ≥80% confluence, MSCs were trypsinized and reseeded at 8×10^3 cells/cm^2. Passage (P) 3 to 5 MSCs were used for most experiments. Further details are provided in the Supplementary Methods.

MSC Frequency in the BMMNC Fraction

The assay for colony-forming unit fibroblasts (CFU-F) was used to calculate MSC frequency within the BMMNC fraction [15,21]. Briefly, BMMNC were seeded at 1×10^5 cells/well in 6-well plates and expanded for 14 days. The clonogenic efficiency was calculated as the number of colonies per 10^6 BMMNC seeded.

Characterization of MSCs

MSC origin, immunophenotype, self-renewal, differentiation potential, senescence, apoptosis, and migration potential were studied. The detailed information is described in the Supplementary Methods.

Immunosuppressive Properties of MSCs

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteers and labeled with 5 μM carboxyfluorescein diacetate succinimidyl ester (Invitrogen). Labeled PBMCs were then stimulated with 2 ug/mL phytohemagglutinin (PHA, Sigma, St. Louis, MO) in the presence or absence of MSCs at different ratios. After 5 days, carboxyfluorescein diacetate succinimidyl ester–labeled cells were harvested and analyzed for cell division by flow cytometry.

Supernatants from cultures of MSCs or cocultures of MSCs/PBMCs were collected and cytokines were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits: TGF-β1, IL-10, HGF, (eBioscience, San Diego, CA), and PGE2 (Cayman Chemical, Ann Arbor, MI).

For inducing regulatory T cells (Tregs), MSCs were cocultured with purified CD4$^+$ T cell for 5 days, then nonadherent cells were separated from the MSCs and evaluated for the proportion of Tregs present by flow cytometry using monoclonal antibodies specific to CD4-FITC (BD Pharmingen, San Diego, CA), CD25-PE, CD127-APC (Biolegend, San Diego, CA). Further details are provided in the Supplementary Methods.

Real-time Quantitative Polymerase Chain Reaction Analysis

Total RNA was extracted from MSCs using Trizol reagent (Invitrogen) and reverse transcribed into complementary DNA (cDNA) using PrimeScript RT reagent Kit (Takara, Dalian, China). Quantitative polymerase chain reaction (qPCR) was performed in a LightCycler system (Roche, Basel, Switzerland) using SYBR Premix Ex Taq (Takara). Each sample was performed in triplicate and all results were normalized to the expression of glyceraldehydes 3-phosphate dehydrogenase (*GAPDH*). Primer sequences are provided in the Supplementary Methods.

Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM). The results were compared using one-way ANOVA with Fisher's least significant difference post hoc test and Student's *t*-test (SPSS 18.0 statistical software). Statistical significance was defined as *P* value of $< .05$.

RESULTS

Origin of MSCs in Patients after Allogeneic HSCT

BM MSCs were successfully expanded from all patients and healthy donors. To study chimerism of MSCs derived from patients after allogeneic HSCT, P4 cells from 10 patients (5 with cGVHD, 5 without cGVHD) were analyzed. Although PBMCs chimerism was donor type, MSCs in all patients remained of host origin whether the patients had cGVHD or not (Supplementary Figure 1). Time after transplantation did not have any influence on MSC origin, nor was any difference observed between patients receiving BM and peripheral blood grafts.

Characterization of BM MSCs

MSCs from healthy donors (HD-MSC) and patients with cGVHD (cGVHD-MSC) or without cGVHD (no cGVHD-MSC) displayed a similar spindle-shape morphology (Figure 1A). The immunophenotype of cells was analyzed by flow cytometry. All MSCs highly expressed CD73, CD90, and CD105 and moderately expressed HLA-ABC, they were negative for CD34, CD45, CD11b, CD19, and HLA-DR (Figure 1B). As determined by CFU-F assay, the frequency of cGVHD-MSC (n = 16) was significantly lower than HD-MSC (n = 12) ($19.93 \pm 3.73/10^6$ BMMNC and $45.92 \pm 10.21/10^6$ BMMNC, respectively; $P < .01$). The frequency of no cGVHD-MSC ($23.52 \pm 6.57/10^6$ BMMNC; n = 9) was also lower than HD-MSC ($P < .05$), probably due to the influence of preconditioning treatment before HSCT. However, there was no