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Bone Marrow–Derived Mesenchymal Stromal Cells from Patients with Sickle Cell Disease Display Intact Functionality

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A B S T R A C T

Hematopoietic cell transplantation (HCT) is the only cure for sickle cell disease (SCD), but engraftment remains challenging in patients lacking matched donors. Infusion of mesenchymal stromal cells (MSCs) at the time of HCT may promote hematopoiesis and ameliorate graft-versus-host disease. Experimental murine models suggest MSC major histocompatibility complex compatibility with recipient impacts their in vivo function, suggesting autologous MSCs could be superior to third-party MSCs for promoting HCT engraftment. Here we tested whether bone marrow (BM)-derived MSCs from SCD subjects have comparable functionality compared with MSCs from healthy volunteers. SCD MSC doubling time and surface marker phenotype did not differ significantly from non-SCD. Third-party and autologous (SCD) T cell proliferation was suppressed in a dose-dependent manner by all MSCs. SCD MSCs comparably expressed indoleamine-2,3-dioxygenase, which based on transwell and blocking experiments appeared to be the dominant immunomodulatory pathway. The expression of key genes involved in hematopoietic stem cell (HSC)–MSC interactions was minimally altered between SCD and non-SCD MSCs. Expression was, however, altered by IFN-γ stimulation, particularly CXCL14, CXCL26, CX3CL1, CKITL, and JAG1, indicating the potential to augment MSC expression by cytokine stimulation. These data demonstrate the feasibility of expanding BM-derived MSCs from SCD patients that phenotypically and functionally do not differ per International Society of Cell Therapy essential criteria from non-SCD MSCs, supporting initial evaluation (primarily for safety) of autologous MSCs to enhance haploidentical HSC engraftment in SCD.

INTRODUCTION

Sickle cell disease (SCD) is caused by a β-globin mutation, which leads to recurrent vaso-occlusion and ischemia from poorly deformable RBCs. SCD patients have significant morbidity and early mortality despite complex and expensive medical treatment [1], with millions worldwide living with SCD [2], resulting in a significant burden of disease. Hematopoietic cell transplantation (HCT) is currently the only treatment with curative intent, with excellent survival after matched sibling donor transplant [3], but nearly 70% of patients lack an available HLA-matched donor [4,5]. Early results of HCT for SCD from haploidentical first-degree relatives are promising and suggest that this could substantially expand the donor pool [6]. Although haploidentical HCT has been well tolerated by SCD patients, the efficacy of this approach has been limited by high rates of graft rejection. Strategies to diminish this immune-mediated rejection have traditionally relied on augmentation of pretransplant conditioning, which leads to more global immunosuppression and risk for infection.

As extensively reviewed [7,8], mesenchymal stromal cells (MSCs) are rare, multipotent progenitors present in bone marrow (BM) that promote hematopoiesis and have immunoregulatory properties. These properties make MSCs attractive as cellular therapy to modulate the immune system post-HCT. Additionally, MSCs are believed to be less globally immunosuppressive than alternative strategies for augmenting engraftment, which may account for them being well tolerated [9]. When given peri-HCT, MSCs enhance preclinical hematopoietic stem cell (HSC) engraftment [10], and clinical trials have demonstrated promising results [11,12].

Negative outcomes of graft-versus-host disease (GVHD) trials have led to re-examination of what factors may contribute to MSC function in vivo, particularly donor source.

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Despite the previous belief that MSCs are immunoprivileged, MSCs upregulate MHC class I and express MHC class II under inflammatory conditions, and MHC-mismatched murine MSCs undergo specific immune-mediated rejection [13]. Further, elegant work by Nauta et al. [10] demonstrated that MSC donor source significantly impacts their effect on preclinical HSC engraftment, wherein recipient-derived MSCs enhance engraftment, donor-derived enhance rejection, and third-party sources have no effect. We therefore hypothesize that the use of autologous MSCs would provide a mechanistically defined remedy to the limitations of prior attempts at promoting engraftment and additionally may prevent GVHD. The objective of this study was to verify the ability to ex vivo expand functional MSCs from the BM of patients with SCD (as compared with MSCs from healthy volunteers) based on the mechanistic hypothesis that autologous MSCs could promote haploidentical HSC engraftment through the inhibition of residual recipient T cells and direct support of hematopoiesis.

METHODS

MSC Expansion

After institutional review board approval and informed consent, BM was aspirated from the posterior iliac crest of up to 9 healthy adult volunteers (Emory University) and up to 11 pediatric patients with SCD (Aflac Blood and Cancer Disorders Center BMT Program, before matched related HCT). MSC culture and isolation occurred as previously described [14]. In brief, BM aspirates were diluted 1:2 with PBS and layered onto a Ficoll density gradient. The cells were centrifuged at 400 *g* for 20 minutes, and thereafter the mononuclear cells were plated in complete human MSC medium (α-MEM, 10% human platelet lysate, 100 U/mL penicillin/streptomycin) at 100,000 to 300,000 cell/cm^2. Nonadherent hematopoietic cells were removed by changing the medium after 3 days of culture, and MSCs were allowed to expand for 7 to 12 days. Thereafter, the cells were passaged weekly by treatment with trypsin/EDTA and reseeded in fresh MSC medium at 1000 cells/cm^2. MSCs were counted at passage 0 (P0) and P1 using a Countess automated cell counter (Invitrogen, Grand Island, NY).

In Vitro Assays

MSCs underwent flow cytometric analysis for cell surface antigen expression as previously described [14]. In brief, MSCs were cultured for 5 to 7 days in human platelet lysate media, harvested, and resuspended at a concentration of 1×10^6 cells/mL and then analyzed by flow cytometry for the expression of CD45, CD34, CD44, CD73, CD90, CD105, CD19, HLA-I, and HLA-DR (BD BioSciences, San Jose, CA). All samples were run on a Canto II flow cytometer (BD Biosciences, San Jose, CA) with the appropriate isotype controls. Data are presented as histogram overlay.

RNA from MSCs ± IFN-γ stimulation was extracted and reverse transcribed, and reverse transcriptase PCR assay was performed for indoleamine-2,3-dioxygenase (IDO) and β-actin, with primers designed using the NCIB/Primer Blast designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/; flanking primers IDO_F: 5′TGTAATGCCTACTGAAGAAAC, IDO_R: 5′CTTAAATTATTTTTTGGCTGAATTCAA). Data were then analyzed using the relative quantification method, as previously described [15]. MSCs (± IFN-γ stimulation) and peripheral blood mononuclear cells (PBMCs; from either SCD participants [autologous] or healthy volunteers [third party] after informed consent on an institutional review board–approved protocol) were co-cultured as previously described, with proliferation assessed by Ki67 assay according to manufacturer instruction (BD Biosciences) [14]. Co-culture experiments were repeated (1) with the addition of 1-methyl-DL-tryptophan (1 mM; Sigma Aldrich, St. Louis, MO) to block IDO and (2) in a transwell system to assess noncontact-dependent T cell suppression (Costar .4 μM transwell cell culture inserts; Corning, Corning, NY).

Quantitative reverse transcriptase PCR was performed on MSCs ± IFN-γ stimulation using a Fluidigm 48 × 48 nanofluidic array (Fluidigm, San Francisco, CA) [16] targeting 47 hematopoiesis genes (plus IDO). Primers were designed to amplify ~20 bp cDNA targets (with product size between 150 and 200 bp) and were synthesized by Integrated DNA Technologies (Coralville, IA). The targeted genes were preamplified in a single 14-cycle PCR reaction after combining cDNA with pooled primers and TaqMan Pre-Amp Mastermix, as described in the manufacturer's protocol (Fluidigm BioMark, San Francisco, CA). Quantitative amplification of the individual genes (in all samples, with duplication) was subsequently detected using the EvaGreen detection assay on a Biomark I machine and following standard Fluidigm protocols with 30 PCR cycles. Primary data are available online at http://cig.gatech.edu/people/Greg%20Gibson.

Statistical Analysis

Data are reported as mean ± standard deviation. Calculations were carried out using GraphPad Prism software (La Jolla, CA). Comparisons between groups were made by 2-sample *t*-tests. Statistical analysis of Fluidigm data was performed using SAS JMP Genomics (Cary, NC) as previously described [17].

RESULTS

We obtained 11 BM samples from patients with SCD (HbSS genotype; before undergoing matched related HCT) ranging in age from 2.6 to 19.9 years (8.3 ± 4.7) and weighing 12.5 to 50.8 kg (27.1 ± 11.5). Eight patients received hydroxyurea treatment pre-HCT, which was discontinued approximately 2 weeks before transplant admission. Nine samples were obtained fresh (6 to 10 mL) and 2 frozen (2 mL, post-Ficoll), with a starting mononuclear cell count of $49.7 \pm 26.5 \times 10^6$ in fresh samples. SCD MSCs were expanded from BM harvest to P1 for a total of 14.4 ± 2.5 days.

MSC phenotype was compared between non-SCD and SCD MSCs, with MSCs displaying MSC phenotype ($CD73^+CD90^+CD105^+CD45^-$) consistent with International Society for Cellular Therapy (ISCT) definition [18] (Figure 1A) and with our phase I evaluation of low passage (majority P1) autologous MSCs for Crohn's disease [19,20]. Notably, there was minimal to no $CD45^+$ contamination, consistent with MSCs being the dominant cell at P1. Doubling time (P0 to P1) was calculated and did not significantly differ between SCD and non-SCD samples (Figure 1B). MSCs were then co-cultured with α-CD3/CD28-stimulated PBMCs to evaluate their immunosuppressive function. MSCs suppressed third-party T cell proliferation in a dose-dependent manner, with more potent suppression by SCD MSCs (Figures 1C,D). Given our mechanistic hypothesis, co-culture experiments were repeated using autologous T cells (eg, MSCs and PBMCs from the same SCD donor). As shown in Figure 1E, SCD PBMC proliferation was responsive to autologous MSCs, with comparable suppression by either SCD or non-SCD MSCs.

As IFN-γ–stimulated MSCs have augmented immunomodulatory function on upregulation of IDO and MSC responsiveness to IFN-γ is a surrogate measure of potency [21], we next compared IDO gene expression in MSCs ± IFN-γ. Unstimulated MSCs had a negligible expression of IDO, whereas IFN-γ stimulation resulted in significant upregulation of IDO by both non-SCD and SCD MSCs (Figure 2A). There was no substantial difference in IDO expression between non-SCD and SCD MSCs (± IFN-γ). To evaluate the contribution of IDO and other contact-independent factors to the suppression of T cell proliferation by SCD MSCs, MSC/PBMC co-culture experiments were repeated in a transwell system. As shown in Figure 2B, MSCs (both non-SCD and SCD) suppressed the proliferation of third-party T cells (%$CD3^+Ki67^+$ cells), consistent with 1 or more contact-independent pathways being mechanistically dominant. Compared with non-SCD MSCs and as seen in initial experiments, SCD MSCs more potently suppressed T cell proliferation ($P = .0186$). Finally, to confirm IDO as the principal pathway for MSC suppression of T cell proliferation, MSC/PBMC co-culture experiments were repeated with a pharmacologic blocker of IDO, 1-methyl-DL-tryptophan. Consistent with blockade of IDO, the addition of 1-methyl-DL-tryptophan fully abrogated the suppression of T cell proliferation by both MSC groups (non-SCD and SCD; Figure 2C,D).