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The Effect of Granulocyte Colony–Stimulating Factor on Immune-Modulatory Cytokines in the Bone Marrow Microenvironment and Mesenchymal Stem Cells of Healthy Donors

Ikbal Ok Bozkaya [1,*], Fatih Azik [1], Betul Tavil [2], Yasin Koksal [3], Meltem Ozguner [3], Bahattin Tunc [1], Duygu Uckan Cetinkaya [2,4]

[1] *Department of Pediatric Hematology, Ankara Children's Hematology Oncology Education and Research Hospital, Ankara, Turkey*
[2] *Department of Pediatric Hematology, Hacettepe University, Ankara, Turkey*
[3] *Stem Cell Research Laboratory, Department of Pediatric Hematology Oncology, Ankara Children's Hematology Oncology Education and Research Hospital, Ankara, Turkey*
[4] *Center for Stem Cell Research and Development, Hacettepe University, Ankara, Turkey*

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A B S T R A C T

Granulocyte colony stimulating factor (G-CSF) is sometimes administered to donors before bone marrow (BM) harvest. G-CSF–primed (G-BM) and unprimed BM (U-BM)–derived mesenchymal stem cells (MSC) were obtained from 16 healthy donors and were expanded in vitro. Their proliferative characteristics, morphology, and differentiation capacity were examined. Supernatants of the second passage of MSCs were evaluated for transforming growth factor β1, hepatocyte growth factor, and prostaglandin E2 (PGE2) levels and compared with controls. The analyses of cytokines in the G-BM– and U-BM–derived MSCs supernatants revealed that PGE2 levels were significantly lower in the G-CSF–primed samples. These cytokines were also measured in BM plasma. The level of hepatocyte growth factor in G-BM plasma was significantly increased. The current study is the first to show the effects of G-CSF on the BM microenvironment of healthy human donors. The preliminary data suggest that G-BM– and U-BM–derived MSCs have similar morphologic/phenotypic properties and differentiation capacity but differ in their secretory capacity. Significant changes in cytokine levels of BM plasma in G-CSF–primed donors were also demonstrated. These findings suggest that BM MSCs and changes in the BM microenvironment may contribute to the effects of G-CSF on inflammation and immunomodulation.

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INTRODUCTION

Granulocyte colony–stimulating factor (G-CSF) is the basic hematopoietic growth factor that modulates hematopoiesis and immune system. It is widely used in clinical practice to overcome radiotherapy and chemotherapy-induced myelosuppression, to increase the proliferation and differentiation of hematopoietic cells, and to activate neutrophil functions [1]. In addition, G-CSF is administered to donors to mobilize stem cells from the bone marrow (BM) into peripheral blood before collection of peripheral blood (PB) stem cells. G-CSF is sometimes administered to donors before BM harvest [2-4]. However, if the established number of stem cells needed for the recipient will require a large volume marrow collection (>16 mL/kg) from the donor and if the donor is not eligible for PB collection, G-CSF may be used to increase the stem/progenitor cell content of the product. G-CSF has been shown to affect the immune system by modifying T cell reactivity and antigen-presenting cell function. Some studies have shown that G-CSF stimulates the inflammatory response while suppressing the adaptive immune system. However, there is a paucity of information in the literature regarding the effects of G-CSF–primed stem cells on recipient immune system. Data suggests that a G CSF–induced T helper 1 to T helper 2 shift may play a role in modification of the alloimmune reactions in the recipient [5].

Mesenchymal stem cells (MSCs) were first characterized more than 30 years ago and are described as fibroblast-like

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* Correspondence and reprint requests: Ikbal Ok Bozkaya, Mehmet Akif Ersoy Mahallesi, 266. Cadde 7B NO: 4806210, Yenimahalle/Ankara, Turkey.
E-mail address: ikbalok@yahoo.com (I. Ok Bozkaya).

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cells adhering to plastic when they were cultured from BM in vitro. It has been shown that MSCs may be isolated from most mesoderm-derived tissues and have differentiation capacity to mainly connective tissue and different mesoderm tissues [6]. To date, several studies have been performed on the biological properties and function of these cells. MSCs are believed to play a role in supporting other types of cells/stem cells by establishing cellular interactions, providing secretory factors for growth and differentiation and other biological functions, and by contributing to angiogenesis and immune modulation [7]. MSCs have been shown to affect the functions of immune cells mainly by secreting immune-modulatory factors, such as transforming growth factor beta 1 (TGF-β1), hepatocyte growth factor (HGF), prostaglandin E2 (PGE2), indoleamine 2,3 dioxygenase (IDO), and HLA G-5 through cell-cell interactions [8]. In recent years, these cells have aroused increasing attention in regenerative medicine and in the treatment of autoimmune and inflammatory conditions, including steroid-resistant graft-versus-host disease (GVHD) [9].

To our knowledge, the effect of G-CSF on the immunomodulatory functions of MSCs and on the secretion capacity of immune-modulatory cytokines has not been studied. In the present study, it is hypothesized that G-CSF–primed BM (G-BM)–derived MSCs may modulate secretion of immune-modulatory cytokines in the BM microenvironment.

MATERIALS AND METHODS

This study enrolled 16 HLA-identical healthy related donors for children who underwent allogeneic hematopoietic stem cell transplantation (HSCT) at Ankara Children's Hematology Oncology Education and Research Hospital. Eight donors received G-CSF (lenograstim, Granocyte) when the predicted marrow harvest volume was assumed to be more than 16 mL/kg of donor's weight. Eight age-matched donors unexposed to G-CSF (U-BM) were included as the control group.

Informed consent was obtained from all patients, donors, and their legal guardians for HSCT. This study was approved by the local ethical committee.

Harvesting BM

Among 16 donors, 8 received G-CSF at a dosage of 10 μg/kg/day (lenograstim, Granocyte) as a single injection for 3 consecutive days. The other 8 donors had unstimulated BM.

The BM was obtained from the posterior iliac crest of healthy donors under general anesthesia. G-BM and U-BM harvest were performed with a target volume to meet the optimal cell numbers for engraftment and not to exceed 16 mL/kg of donor's body weight. For this study, 2 to 3 mL of BM was separated and frozen.

Collection of Plasma from BM

BM samples were centrifuged at 2000 rpm for 10 minutes. The plasma at the upper part of the tube was collected and stored at −80°C.

Isolation of Human BM–Derived MSCs

Mononucleated cells separation procedure

BM samples were diluted with Dulbecco's PBS (Biochrom, Germany) after the plasma collection and layered on Biocoll (1.077 g/mL) separating solution (Biochrom, Germany) (1:1) and centrifuged at 2200 rpm for 20 minutes.

Mononucleated cell freezing procedure

The buffy coat containing the mononucleated cells (MNCs) was washed with PBS and frozen in DMEM low glucose (DMEM-LG) (Biochrom, Germany) containing 20% FBS (Biochrom, Germany) and 10% DMSO. Cryovials were kept in liquid nitrogen tanks at −196°C.

MNC Thawing procedure

MNC samples in the cryovials were thawed in a 37°C water bath before the seeding procedure. The cells were disaggregated by gentle pipetting several times and centrifuged with PBS at 2000 rpm for 5 minutes to remove freezing solution. The supernatant was discarded, and the pellet was resuspended in 10 mL DMEM-LG, 10% FBS, and 1% penicillin-streptomycin (Biochrom, Germany) and at least 20×10^6 cell were seeded in 75 cm^2 plastic flasks. Flasks were kept at 37°C in a humidified atmosphere containing 5% CO_2 (Galaxy 170R incubator, Eppendorf Company, Hamburg, Germany).

Culture of Human BM–Derived MSCs

After 72 hours, nonadherent cells were removed. The culture medium was changed every 3 days. When 70% to 80% adherent cells were confluent, they were trypsinized (.05% trypsin) at 37°C for 5 minutes (Biochrom). Characterization of MSCs, collection of supernatant for ELISA assay, and MSC differentiation assay were set up within passage 2 of MSC culture.

Characterization and Differentiation Assay of MSCs

In vitro differentiation capacity of MSCs towards adipogenic and osteogenic lineages were tested. Adipogenic Stimulatory Supplements (Human) (MesenCult Adipogenic Differentiation Medium; Stemcell Technologies, Vancouver, Canada) induction was used for adipogenic, and Osteogenic Stimulatory Supplements (Human) (MesenCult Osteogenic Stimulatory Kit, Stemcell Technologies, Vancouver, Canada) was used to induce osteogenic differentiation. Oil red o and alizarin red stains were used to verify, respectively, adipogenic and osteogenic differentiation capacity of MSCs at day 21 of induced cultures. MSCs were also tested for positive staining of HLA ABC, CD90, CD73, CD44, and CD49e (BD Biosciences, Piscataway, NJ, USA) and negative antibody staining for CD34, CD3, CD4, and HLA DR (BD Biosciences, Piscataway, NJ, USA). Flow cytometry with a BD-FACS Aria (BD Biosciences) was used for surface phenotyping of MSCs.

MSC's Coculture with PB MNC

PB MNCs isolation and activation test

MNC were purified from heparinized PB by density-gradient centrifugation using Biocoll (1.077 g/mL) separating solution (as described in MNC separation procedure).

MNCs from PB samples were plated at a density of 3×10^5 cells/cm^2 on T-75 flask (Cellstar, Grenier Bio-one, Kremsmünster, Austria) with a density of 10 μL pythohemagglutinin (PHA)/1×10^6 cells in DMEM supplemented 10% FBS and 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO_2 for 72 hours for activation of lymphocytes.

BM MSC isolation

BM MSC isolation was performed as described previously. All assays were performed using MSCs at passage 2.

Coculture

Coculture were carried out in 6-well tissue plates (Cellstar, Grenier Bio-one) with PB MNCs from a healthy donor and allogeneic human MSCs from 2 different sources, G-BM 8 samples and U-BM 8 samples.

Control cultures consisted of MNC in the absence of MSCs, with or without PHA stimulation. Also, another control culture was set that consisted of U-BM MSCs for the evaluation of proliferation capacity of MSCs. For the immunological assays, different sets of cocultures were generated as below: PHA-activated MNCs + G-BM MSCs and PHA-activated MNCs +U-BM MSCs (8 different samples for each condition).

For the coculture experiments, MNCs were washed with PBS at least twice after activation with PHA. Cocultures were performed as in each well, we added 30,000 MNCs, with a ratio of 10:1 (MNC:MSC). The coculture plates were kept at 37°C in a humidified atmosphere containing 5% CO_2 for 4 days. After 4 days, the cultured cells were washed with PBS and centrifuged at 2000 rpm for 5 minutes.

The number of cells and cellular viability were determined.

Flow Cytometry Data Acquisition and Analysis

Cells were acquired on a Beckmann Coulter Navios using Kaluza version 1.2 software. For the immunological assays and to define the different stages of lymphocyte activation, we used mAb against CD3-PC7, CD4-FITC, CD25-PE, CD69-PC5, and HLA-DR-ECD (Beckmann Coulter, Brea, CA, USA).

Quantification of Immune Modulatory Factors

To quantify PGE2, HGF, and TGF-β1, MSCs supernatants were collected at passage 2 and stored at −80°C until measured by ELISA. When MSCs reached 70% to 80% confluency at passage 2, cells were trypsinized and counted before freezing and stored at −80°C. BM plasma was harvested and frozen at −80°C until measured by ELISA. PGE2 ELISA was performed using the Human PGE2 Assay ELISA (R&D Systems, Minneapolis, MN), TGF-β1 ELISA was assayed using the Human TGF-β1 ELISA (R&D Systems), and HGF ELISA was performed using the Human HGF ELISA (R&D Systems). MSC supernatants and BM plasma were analyzed on BioTEK ELx808 Absorbance Microplate Readers (BioTEK, Winooski, VT, USA). Measured ELISA test from supernatant of MSC for standard results were taken as the value for the 10×10^4 per MSCs.

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