Leukemia Research 38 (2014) 594–600

Contents lists available at ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

Decreased frequency, but normal functional integrity of mesenchymal stromal cells derived from untreated and Imatinib-treated chronic myeloid leukemia patients

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ARTICLE INFO

Article history:
Received 21 November 2013
Received in revised form 18 February 2014
Accepted 20 February 2014
Available online 2 March 2014

Keywords:
Imatinib
Mesenchymal stromal cells
Bone remodeling
Chronic myeloid leukemia
Fibrosis

ABSTRACT

In vitro, Imatinib inhibits the proliferation and stimulates the osteogenic and adipogenic differentiation of mesenchymal stromal cells (MSC). However, it is unknown whether Imatinib affects the biology of MSC in vivo. We asked whether MSC from long-term Imatinib-treated CML patients were affected by the in vivo treatment. MSC from untreated and Imatinib-treated patients displayed normal functional properties (i.e. proliferation, immunophenotype, differentiation and hematopoietic supportive capacity) – but a decreased frequency. In vitro, Imatinib lost its effect when discontinued; which suggest that it has a reversible effect on MSC. Therefore it might lose its effect on MSC after discontinuation in vivo.

1. Introduction

Imatinib, a drug designed to inhibit the Bcr-Abl oncoprotein – the hallmark of chronic myeloid leukemia (CML) – has become the first-line treatment for newly diagnosed CML in chronic phase [1,2]. Initial clinical trials described manageable immediate side effects and infrequent toxicity in Imatinib-treated patients [3]; however, cumulative experience reported long-term side effects on bone remodeling, including increased parathyroid hormone levels, cortical bone mineralization, and trabecular bone volume [4–8]. Most CML patients require continuous Imatinib administration to avoid relapse [9]; thus, it is necessary to characterize the long term side effects induced by such a drug.

In vitro, Imatinib inhibits the proliferation of mesenchymal stromal cells (MSC) and osteoblasts – two key regulators of bone remodeling – [6,10,11], decreases their hematopoietic support [10], and promotes MSC differentiation toward osteoblasts [6,11,12] and adipocytes [10,13]. To date, however, it is not known whether Imatinib affects the biology of MSC in vivo. Accordingly, we asked whether MSC from long-term Imatinib-treated CML patients were affected by the in vivo treatment. We hypothesized that the presence of Imatinib is required in order to have an effect on MSC and that it will affect their functional properties only temporarily; thus, MSC from long-term Imatinib-treated CML patients will display normal proliferation, differentiation and hematopoietic support capacity.

We compared the number of MSC in bone marrow aspirates from long-term Imatinib-treated CML patients, untreated CML patients, and normal bone marrow, as a control. We then analyzed their in vitro proliferation, differentiation potential (toward osteoblasts and adipocytes) and hematopoietic support capacity. To the best of our knowledge, this is the first report on the functional integrity of MSC from Imatinib-treated CML patients.

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http://dx.doi.org/10.1016/j.leukres.2014.02.011
0145-2126/

2. Materials and methods

2.1. Cell samples

Bone marrow (BM) cells, collected according to institutional guidelines, were obtained from 30 patients, after signing a consent form, with CML in chronic phase as a part of their routine clinical tests. All of them fulfilled the standard diagnostic criteria for CML, including the presence of the Ph+ chromosome translocation in direct marrow preparations. Fifteen patients were studied at the time of diagnosis (CML patients). Fifteen patients (CML-IM patients) were studied after being treated with Imatinib Mesylate (IMATINIB; Gleevec®, Novartis Pharmaceuticals) at 400 mg/day for at least six months (mean of two years), two patients were treated for five and six years, respectively. Normal bone marrow (nBM) was collected from 5 hematologically normal adult bone marrow transplant donors, at the Bernardo Sepulveda Hospital, "Siglo XXI" National Medical Center, IMSS; and 10 samples were obtained from hip replacement surgery patients, at the General Regional No. 2 Hospital, IMSS. Mobilized Peripheral Blood was obtained from 5 used leukapheresis bags after allogenic bone marrow transplants. All patients were hematologically normal. The Ethics Committee of the National Medical Center, IMSS, has approved all the procedures. Patient confidentiality was maintained by codifying the samples.

2.2. Frequency of MSC and hematopoietic progenitors

Frequency of MSC was determined by the fibroblast colony-forming unit (CFU-F) assay from bone marrow aspirates as previously described [14]. Hematopoietic progenitors capable of forming colonies in vitro (colony-forming cells; CFC) were assayed in methylcellulose-based semisolid cultures (MethoCult™, Stem Cell Technologies Inc., Vancouver, Canada) as described previously [14]. Total numbers of colonies (erythorid and myeloid) were scored as colony forming units (CFU-C).

2.3. Isolation and culture of MSC

We seeded 1–2 × 10^6 cells/cm^2 mononuclear cells (MNC), from bone marrow aspirates, in Lg-DMEM (Gibco) supplemented with 10% FBS (Hyclone). After 1 day of culture non-adherent cells were removed and medium change was performed weekly. Cultures were trypsinized after they reach 80% confluence (0.05% trypsin, 0.53 mM EDTA; Gibco) and subcultured at a density of 7.5 × 10^4 cells/cm^2 into T75 flasks (Corning). Cells from the second and third passage were used. All tissue culture conditions were performed in a humidified atmosphere of 5% CO_2 in air at 37 °C.

2.4. Morphology and Immunophenotype of MSC

We analyzed the morphology and immunophenotype of MSC as previously described in detail [15]. The following monoclonal antibodies were used, anti-CD13-PE, anti-CD14-PE, anti-CD29-FITC, anti-CD31-FITC, anti-CD44-PE, anti-CD45-FITC, from Caltag Laboratories, Burlingame, CA; anti-CD49b-PE, anti-CD54-PE, anti-CD58-PE, anti-CD73-PE, anti-CD105-PE, anti-CD166-PE, anti-CD140b, anti-HLA-DR-PE (Beckton Dickinson/PharMingen, San Jose, CA, USA) and anti-CD90-FITC (Immunotech, Marseille, France). Labeled cells were analyzed on a FACS Calibur (Beckton Dickinson) by collecting a minimum of 10,000 events.

2.5. Bcr-abl analysis of MSC

Presence of *bcr-abl*+ cells was determined by detecting the *bcr-abl* gene rearrangement by RT-PCR and the ribosomal 18S subunit, as previously described [16]. For these studies, a *bcr-abl*-positive cell line (K562) and normal mononuclear peripheral blood, obtained from healthy volunteers, were used as positive and negative controls, respectively.

2.6. Cell proliferation

2500 MSC from nBM, CML or CML-IM-derived (at the first passage) were cultured on 24 well plates (Corning) on Lg-DMEM with or without PDGF-BB (10 ng/ml) for 12 days. Additionally, nBM MSC were cultured for 12 days in the presence of Lg-DMEM with or without PDGF-BB (10 ng/ml) media and Imatinib (2.5 mM) during 3, 6 or 12 days and then transferred into Lg-DMEM without the drug until day 12. Cultures without Imatinib were also performed as control. Cell counting was performed using a hemocytometer.

2.7. Differentiation potential

To induce adipogenic differentiation, 1 × 10^5 MSC were incubated in 35 mm petri dishes (Corning) containing 2 ml of Lg-DMEM for 24 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. According to manufacturer's instructions, media was replaced with adipogenesis differentiation kit media (STEMPRO®, Gibco) and cultured for 14 days with two medium changes per week. Cells were trypsinized and 7500 cells were then cytospined and fixed with 1% of formaldehyde (Sigma–Aldrich, Taufkirchen, Germany) for 1 min onto glass slides. The slides were stained with oil-red O (Sigma) and counterstained with hematoxylin (Sigma) and mounted (Supermount, Biogenex) for their evaluation. A minimum of 400 single cells were scored, positive cells were score for the presence of bright red vacuoles.

To induce osteogenic differentiation 7.5 × 10^4 MSC were incubated in 35 mm petri dishes (Corning) containing 1.5 ml of low glucose Dulbecco's modified Eagle medium (Lg-DMEM; Gibco) supplemented with 15% Osteogenic Stimulatory Supplements (Stem Cell Technologies), 10^{-8} M dexamethasone, 0.2 mM ascorbic acid for three weeks. During the last week, 10 mM β-glycerol phosphate was added to the culture. Cells were trypsinized (0.05% trypsin, 0.53 mM EDTA; Gibco BRL) and detached with the aid of a cell scraper (Corning). Cells were washed and resuspended in PBS (Gibco) containing 2% FBS (Hyclone) and 1 mM EDTA (Sigma). 1 × 10^5 cells/50 μl were fixed (4% paraformaldehyde) for 10 min and washed. Cells were permeabilized (0.1% saponin (Sigma) in balance salt solution (HBSS, Gibco) and stained with osteocalcin-PE antibody (R&D Systems) and fixed with a buffer lysis (Becton-Dickinson) according to manufactures instructions. Cells were analyzed using FACS Calibur (Becton-Dickinson). 10,000 events were recorder and an istoype control was used (R&D Systems).

Chondrogenic differentiation was evaluated by Alcian blue dye as previously described [15].

Table 1
Mesenchymal stromal cell and hematopoietic progenitor cell content from nBM, CML and CML IM bone marrow aspirates.

	nBM	CML	CML IM
CFU-F	11.7 ± 10.6 n = 15	2.1 ± 3.4* n = 15	1.0 ± 2.8* n = 15
CFU-C	195.8 ± 89.2n = 11	126.1 ± 94.5n = 8	157 ± 120.7n = 15

Number of colonies/1 × 10^5 MNC, mean ± standard deviation, *$p \leq 0.05$ Student's t test.

2.8. Hematopoietic supportive capacity of MSC

CD133+ lineage-cells were isolated from mPB MNC by immunomagnetic separation according to manufacturer's instructions (Diamond CD133 MicroBead Kit, Miltenyi). Previous to the co-cultures, we incubated overnight confluent MSC with 0.2 μg/ml Mytomicin C [17], and washed three times with PBS, in order to inhibit the feeder layer growth. Ten thousand mPB cells were layered on a confluent layer of MSC derived from either normal donors or CML, CML IM patients, on a 6-well plate (Corning, Inc., Costar, NY, USA), in the presence of myelocult medium (Stem Cell Technologies), for 5 weeks. At weekly intervals we performed a half medium change and evaluated the numbers of non-adherent nucleated cells (trypan blue, Gibco) and hematopoietic progenitors (Methocult H4434, Stem Cell Technologies), at day 21 and 35 we trypsinized the cultures and evaluated the number of nucleated cells, hematopoietic progenitors and their immunophenotype by flow cytometry (CD133-PE Miltenyi, CD34-APC, Caltag) of non-adherent and adherent cells.

2.9. Statistical analysis

Data were analyzed using Student's t-test with GraphPad PRISM statistics software and Excel.

3. Results

3.1. Frequency of MSC

The frequency of MSC was three times lower in CML and CML IM patients compared to nBM (Table 1). We found no differences in colony size between all three groups. Larger colonies account for half of all colonies, and medium and small colonies for the rest. In contrast, we found that CML and CML-IM MNC have a normal content of hematopoietic progenitor cells (CFU-C) (Table 1).

3.2. Development of MSC layers and cellular morphology

MSC layers were established from all bone marrow samples (nBM, CML and CML-IM), reaching confluence by day 14 (Fig. 1A); with a normal cell growth kinetics (Fig. 1C). Layers appeared as a well-defined population, consisting of three distinguishable cell types (Fig. 1B) fibroblast-like spindle-shaped cells (a) – comprising more than 90% of the cells in culture, – large cells (b) and rare cells with neuritic morphology (c).