Cytokine 58 (2012) 218–225

Contents lists available at SciVerse ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/issn/10434666

Granulocyte colony stimulating factor expands hematopoietic stem cells within the central but not endosteal bone marrow region

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ARTICLE INFO

Article history:
Received 13 July 2011
Received in revised form 4 January 2012
Accepted 24 January 2012
Available online 16 February 2012

Keywords:
Granulocyte colony stimulating factor
Mobilization
Hematopoietic stem cells

ABSTRACT

Granulocyte colony stimulating factor (G-CSF) is clinically well established for the mobilization of hematopoietic stem cells (HSC). Extensive data on the underlying mechanism of G-CSF induced mobilization is available; however, little is known regarding the functional effect of G-CSF on HSC within the bone marrow (BM). In this study we analyzed the proportion and number of murine HSC in the endosteal and central bone marrow regions after 4 days of G-CSF administration. We demonstrate that the number of HSC, defined as $CD150^{+}CD48^{-}$LSK cells (LSKSLAM cells), increased within the central BM region in response to G-CSF, but not within the endosteal BM region. In addition the level of CD150 and CD48 expression also increased on cells isolated from both regions. We further showed that G-CSF mobilized proportionally fewer LSKSLAM compared to LSK cells, mobilized LSKSLAM had colony forming potential and the presence of these cells can be used as a measure for mobilization efficiency. Together we provide evidence that HSC in the BM respond differently to G-CSF and this is dependent on their location. These findings will be valuable in developing new agents which specifically mobilize HSC from the endosteal BM region, which we have previously demonstrated to have significantly greater hematopoietic potential compared to their phenotypically identical counterparts located in other regions of the BM.

1. Introduction

For many years HSC transplants have been used to support intensive treatment of patients with hematological and oncological diseases to overcome high dose chemotherapy and radiation induced BM destruction. Besides the reconstitution of hematopoiesis, allogeneic HSC transplants also provide the benefit of graft versus disease activity [1,2]. For transplantation, HSC are either harvested directly from the BM or collected from the peripheral blood (PB) of donors using leukapheresis following stimulation with mobilizing agents.

G-CSF was the first growth factor shown to mobilize sufficient numbers of HSC into the PB to be clinically useful [3]. Since then, extensive research on the process that enables egress of HSC into the PB after G-CSF administration has been performed. Recent data suggests that the main cause of HSC egress after G-CSF is the disruption of the $CXCL_{12}$–$CXCR_4$ interaction. G-CSF administration has been shown to result in the degradation of $CXCL_{12}$ by proteases, suppression of mature osteoblasts which are located within the endosteal BM region and consequently reduced expression of $CXCL_{12}$ [4–8]. The most convincing evidence that inhibiting this interaction causes HSC mobilization is the $CXCL_{12}$ antagonist AMD3100 (Plerixafor) which specifically disrupts this interaction and results in fast and significant mobilization of HSC [9,10]. Despite this, AMD3100 is preferably used together with G-CSF to stimulate adequate HSC mobilization for transplantation [11–13]. Although G-CSF remains the most commonly used agent for HSC mobilization it is completely ineffective in a proportion of patients [14] and commonly causes side effects such as intensive bone pain and occasionally spleen enlargement [15]. Additionally, G-CSF has a broad effect on other cell compartments like immuno-regulatory cells, endothelial cells [16] and neuronal cells [17] and the consequences of these actions are not fully understood. Accordingly, there is a strong need to develop novel mobilization agents that have a specific mode of action and solely mobilize HSC without uncontrolled side effects.

In order to achieve this, knowledge regarding the location of HSC within the BM and the regulatory mechanisms that anchor HSC within the stem cell niche is mandatory. Since the initial studies by Calvi and Zhang [18,19], we and others have provided evidence that HSC reside within the endosteal BM region, in close proximity to osteoblasts that provide regulatory stimuli through direct contact or soluble factors like osteopontin or angiopoietin [20–24].

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doi:10.1016/j.cyto.2012.01.014

In addition, we also recently demonstrated that HSC located in the endosteal region have significant higher hematopoietic potential than cells with the same CD150CD48LSK phenotype located in the central BM region [25]. This leads to the hypothesis that mobilizing agents should preferentially act in the endosteal BM region, enabling the mobilization of biologically superior HSC. Potentially this would result in fewer donor cells being required and/or improved engraftment after transplantation and therefore benefit to transplant recipients. To date there is very little data on the effects of G-CSF administration on HSC within the BM cavity. Morrison et al. showed that G-CSF administration in combination with Cyclophosphamide results in a proliferation of HSC and progenitor cells (HSPC) prior to mobilization [26]. However, there is no data about the effect of G-CSF administration alone on HSPC within different BM regions.

Therefore, we investigated the effect of G-CSF administration on HSC residing within the endosteal and central BM regions. As previous studies routinely use HSPC (for example lineage negative, Sca-1$^+$, c-kit$^+$ cells; LSK) or colony forming cells as a marker of mobilization efficiency, we added the SLAM markers (CD150 and CD48) [27] to investigate the effect of G-CSF administration on HSC within the BM and PB.

We demonstrate that endosteal HSC have a different response to G-CSF than central HSC and that proportionally more HSPC are mobilized into the PB than HSC. This data can be useful to develop novel mobilization agents that have specific activity within the endosteal BM region, releasing a higher number of biological superior HSC than occurs following G-CSF administration and with fewer side effects.

2. Materials and methods

2.1. Mice

C57BL/6 (C57) mice were bred at Monash Animal Services (MAS, Monash University, Clayton, Australia). Mice were 6–8 weeks of age and sex matched for all experiments. All experiments were performed with the approval of the MAS Animal Ethics Committee.

2.2. Mobilization regimes

Mice were mobilized with G-CSF as previously described [28]. Briefly, mice received subcutaneous injections of G-CSF (Filgrastrim; Amgen, Thousand Oaks, CA, USA) at 250 μg/kg in saline in 100 μL/10 g body weight twice daily, 6–8 h apart, for four consecutive days. Control animals received an equivalent volume of saline. Marrow, PB, and spleen cells were harvested within 12 h of the last injection.

2.3. Hematopoietic cell isolation

Populations of endosteal and central murine BM cells were isolated as previously described [28,29]. Briefly, one femur, tibia and iliac bone were excised and cleaned of muscle. After removing the epi- and metaphyseal BM regions, bones were flushed with phosphate buffered saline 2% fetal calf serum (PBS2%Se) to obtain central BM cells. Flushed long bones and epi- and metaphyseal fragments were pooled and crushed using a mortar and pestle. Bone fragments were digested with Collagenase I (3 mg/ml) and Dispase II (4 mg/ml) at 37 °C in an orbital shaker at 750 rpm. After 5 min, bone fragments were washed once with PBS and once with PBS2%Se to collect the endosteal BM cells. All materials are available in a kit (Millipore #SCR051, Billerica, MA, USA).

PB was collected by retro-orbital puncture. Red blood cells were lysed using an ammonium chloride buffer for 5 min at room temperature (RT). White blood cells were washed twice with PBS2%Se and stained for flow cytometry.

2.4. Flow cytometry

Flow cytometric analysis of BM, spleen and PB cells was performed using a BD LSR II (Becton Dickinson, Franklin Lakes, NJ, USA), as previously described [28] after staining with a lineage cocktail (CD3, CD4, CD8, CD41, Ter-119, Gr-1, Mac-1, B220; all antibodies FITC conjugated, BD Pharmingen) and anti-Sca-1-PECy7, anti-CD150-PE (Biolegend, San Diego, CA, USA), anti-CD48-Pacific Blue and anti-c-kit-APC (BD Pharmingen) antibodies. For BM and PB, typically 5×10^5 and $1–2 \times 10^6$ events, respectively, were analysed with a flow rate of approximately 10,000 events per second. Candidate cells for colony forming assays were sorted on a Cytopeia Influx (BD) as previously described [28]. Antibody combinations were chosen to minimize emission spectra overlap.

2.5. Low- and high-proliferative potential colony-forming cell assays

Low- and high-proliferative potential colony-forming cells (LPP-CFC and HPP-CFC, respectively) were assayed by plating defined numbers of candidate cells in 35-mm Petri dishes in a double-layer nutrient agar culture system as previously described [30], except that mouse stem cell factor was added to colony-stimulating factor 1, interleukin (IL)-1, and IL-3 to analyze HPP-CFC.

2.6. Cell cycle analysis

Cells were fixed and permeabilized by dropping them into 70% ethanol and stored at 4 °C for 4 h. Consequently, cells were washed twice with PBS and stained with 1 mg/ml Propidium Iodide (PI, Invitrogen) and 200 μg/ml RNAse A (Quiagen, Lane Valencia, CA, USA) for 30 min at 37 °C. Cells were immediately analyzed by flow cytometry for deoxyribonucleic acid (DNA) staining using a BD LSR II flow cytometer.

2.7. Image acquisition and manipulation

Images were acquired using microscopes as previously described [28] and adjusted with Adobe Photoshop to adjust brightness and contrast.

2.8. Statistical analysis and data presentation

Flow cytometric analysis was performed using FlowJo for Mac (Treestar, Ashland, OR, USA). Where appropriate *unpaired t-test* (one tailed) was used to determine statistical significance.

3. Results

3.1. CD150$^+$CD48$^-$ LSK cells circulate in steady state PB

HSPC form colonies of mature blood cells *in vitro* after stimulation with different cytokine combinations. This functional capacity is frequently used to determine the number of HSPC within phenotypically defined cell populations [31]. Additionally, numbers of HSPC within a defined population can also be determined by flow cytometric analysis using cell surface markers specifically expressed by HSPC. In this context SLAM markers (CD150 and CD48) were recently introduced to define LT-HSC within the BM with the phenotype LIN$^-$CD150$^+$CD48$^-$ [27]. Subsequent analysis and transplantation studies have shown that addition of Sca-1 and c-kit antibodies for flow cytometric sorting of BM results in