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REGULAR ARTICLE

G-CSF increases mesenchymal precursor cell numbers in the bone marrow via an indirect mechanism involving osteoclast-mediated bone resorption

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Abstract During the course of studies to investigate whether MPC circulate in response to G-CSF, the agent most frequently used to induce mobilization of hematopoietic progenitors, we observed that while G-CSF failed to increase the number of MPC in circulation (assayed *in vitro* as fibroblast colony-forming cells, CFU-F), G-CSF administration nevertheless resulted in a time-dependent increase in the absolute number of CFU-F within the BM, peaking at Day 7. Treatment of BM cells from G-CSF-treated mice with hydroxyurea did not alter CFU-F numbers, suggesting that the increase in their numbers in response to G-CSF administration is not due to proliferation of existing CFU-F. Given previous studies demonstrating that G-CSF potently induces bone turnover in mice, we hypothesized that the increase in CFU-F may be triggered by the bone resorption that occurs following G-CSF administration. In accord with this hypothesis, administration of an inhibitor of osteoclast differentiation, osteoprotegerin (OPG), prevented the increase of CFU-F numbers induced by G-CSF. In conclusion, these data indicate that the cytokine treatment routinely used to mobilize hematopoietic stem cells could provide a readily applicable method to induce *in vivo* expansion of MPC for clinical applications.

Introduction

The bone marrow (BM) contains a rare population of multipotent mesenchymal precursor cells (MPCs) with the capacity to differentiate into all of the cellular elements of the marrow stroma, including osteoblasts, chondrocytes, and adipocytes as demonstrated by *in vitro* and *in vivo* experiments (Friedenstein et al., 1987; Grigoriadis et al., 1988). The multilineage differentiation potential of MPCs, their unique immunosuppressive properties (Tyndall et al., 2007; Uccelli et al., 2008; Joo et al., 2010) coupled with the relative ease of their isolation and *ex vivo* propagation, has engendered considerable interest in their application in a broad range of cellular and gene therapies (reviewed in Gronthos and Simmons, 1996; Fouillard et al., 2007; Bianco et al., 2008; Porada et al., 2006; Feng et al., 2008; Giordano et al., 2007; Caplan and Dennis, 2006; Berry et al., 2006; Yan et al., 2009). A

Abbreviations: BM, bone marrow; MPCs, mesenchymal precursor cells; G-CSF, granulocyte colony-stimulating factor; CFU-F, fibroblast colony-forming cells; OPG, osteoprotegerin.

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notable impediment to the use of MSC in these proposed clinical applications is the low incidence of MPC in the bone marrow and the consequent need to aspirate significant volumes of bone marrow to obtain sufficient starting numbers to avoid the requirement for *ex vivo* expansion of MPC and the attendant risk of senescence or transformation through excessive proliferation.

Hematopoietic stem/progenitor cells (HSC/HPCs) are also present at low incidence in bone marrow but their rarity is partially overcome by the use of various agents which result in their release into the peripheral circulation, a process termed stem cell mobilization (for review, see Nervi et al. (2006)). Among a variety of agents shown to elicit mobilization of primitive hematopoietic cells, granulocyte colony-stimulating factor (G-CSF) is the most frequently used clinically for stem cell mobilization.

A number of previous reports have described the generation of colonies of fibroblastic cells following *in vitro* culture of normal peripheral blood from a range of adult mammalian species including humans, mice, rats, rabbits, guinea pigs, and dogs (Kuznetsov et al., 2001; Fernandez et al., 1997; Zvaifler et al., 2000; Huss et al., 2000; Rochefort et al., 2006). In addition, cells with the characteristics of MPC have also been described in the circulation at early stages of human development (Campagnoli et al., 2001) and in umbilical cord blood (Gutierrez-Rodriguez et al., 2000; Erices et al., 2000; Markov et al., 2007). While not all of the above studies provide quantitative analysis of CFU-F numbers, the consensus is that the incidence of MPC in the circulation under steady-state conditions is extremely low.

In the present study, we sought to investigate whether MPC can be mobilized in mice in response to administration of G-CSF. Although we failed to demonstrate increases in the numbers of circulating CFU-F, administration of G-CSF was found to elicit a transient increase in the absolute number of CFU-F within the bone marrow compartment compared to saline-treated controls. From a series of experiments designed to investigate the underlying mechanism of this phenomenon, the transient increase in CFU-F numbers in the bone marrow was not the result of a direct mitogenic effect of G-CSF on CFU-F or to proliferation of the marrow resident pool of MPC. Rather, the data are consistent with a model in which the increase in CFU-F in the marrow is due to recruitment of MPCs from a reservoir in the bone tissue as a consequence of an increase in bone turnover initiated by G-CSF. In conclusion, these data demonstrate that G-CSF, a cytokine routinely used to mobilize hematopoietic stem cells, could also provide a readily applicable method to induce *in vivo* expansion of MPC for clinical applications.

Results

Effect of G-CSF administration on the number of circulating CFU-F

From control and G-CSF-treated mice, we harvested 250 to 500 μl of blood per animal by various methods as described under Materials and methods: intracardiac puncture and retro-orbital puncture using capillary tubes or 26 G needles. Following hypotonic hemolysis cells were plated in 24-well plates in α-MEM 20% FCS, cultured for 10 days, fixed, and stained with toluidine blue. The range of cell number plated was from 0.1 to 1.2×10^6, with a mean of $382\pm60\times10^3$ cells for saline-injected mice and $400\pm50\times10^3$ for G-CSF-treated mice. In four independent experiments (Fig. 1A), we observed the formation of colonies of monocyte macrophages, typically between 1 and 3 colonies per blood sample) from the blood of both control and G-CSF-treated mice (Fig. 1B). These colonies are readily distinguished from the colonies derived from bone marrow which comprised stellate or spindle-shaped fibroblastic cells (Fig. 1C). In experiment No. 2, two blood samples were collected from each mouse, one from the retro-orbital sinus using a 26 G needle and the second sample by intracardiac puncture. None of the tested mice yielded CFU-F colonies from blood harvested by either route. Analysis of the data accumulated from four independent experiments demonstrated that the presence of CFU-F in the peripheral circulation was observed only rarely with 1 out of 22 mice treated with the saline vehicle and 3 out of 30 mice treated with G-CSF. In each of these assays, only 1 to 2 colonies were formed. Based on these data, we concluded that G-CSF administration does not induce a significant release of CFU-F into the blood.

G-CSF administration increases the number of CFU-F in the bone marrow

The failure to mobilize CFU-F into the peripheral circulation does not preclude the possibility of effects on CFU-F which remain resident within the bone marrow. To examine this possibility, bone marrow samples obtained at the same time as the blood samples from experiment 4 were assayed in parallel for their content of CFU-F. In contrast to the observations in blood, G-CSF administration resulted in a statistically significant increase in CFU-F numbers in the BM of G-CSF-treated mice compared to saline-treated mice with an approximate doubling in the incidence of CFU-F in the marrow at Day 7. To determine the time course of this phenomenon, mice were administered saline or G-CSF and CFU-F numbers in the marrow were assayed at Days 5, 7, 9, and 11. Spleens were harvested and weighed as a surrogate measure of the mobilization of hematopoietic stem and progenitor cells as previously described (Molineux et al., 1990). As shown in Fig. 2A, at 5 to 11 days of treatment, the weight of the spleens from G-CSF-treated mice was significantly increased compared to saline-treated mice by 1.8- to 2.3-fold ($P<0.001$) and accompanying this was a reduction in the cellular content of the bone marrow of mice treated with G-CSF compared to the control group that is statistically significant at Day 7 (Fig. 2B). In regard to CFU-F numbers, we detected an increase in CFU-F number in the marrow of G-CSF-treated mice compared to saline-treated mice that is maximum and statistically significant at Day 7 (234 ± 22.9 and 98 ± 8.2 CFU-F per femur, respectively) and declines to pretreatment steady-state levels thereafter (Fig. 2C). Thus G-CSF appears to elicit a transient increase in CFU-F numbers in the bone marrow but these increases are not reflected in enhanced numbers of these stromal progenitors in the peripheral blood.

G-CSF does not directly stimulate the proliferation of MPC in vivo or in vitro

A number of potential mechanisms may be responsible for the increase in CFU-F numbers in the bone marrow in

A

	Saline	G-CSF 5 days	G-CSF 7 days	G-CSF 9 days	G-CSF 11 days
Exp. 1 *	0/3		1/3		
	1/3				1/3
Exp. 2 #, &	0/3	0/3			
	0/3		0/3		
	0/3				1/3
Exp. 3 #	0/4	0/3	0/3	0/3	0/3
Exp. 4 #	0/3		0/3		
Total	1/22	0/6	1/12	0/3	2/9
	1/22	3/30			

Blood collection method: *: retro-orbital sinus with capillary tube, #: retro-orbital sinus with 26G needle, &: intra-cardiac.

B C

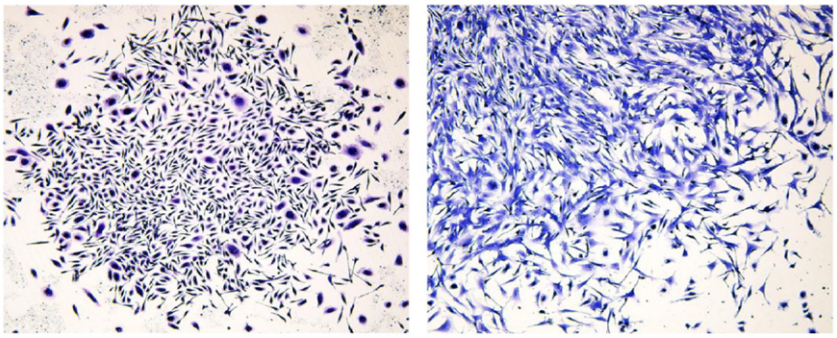

Figure 1 Detection of adherent cells in peripheral blood. Mice were treated with saline or G-CSF for 5, 7, 9, or 11 days. The blood was collected using various methods: * retro-orbital sinus with capillary tube, # retro-orbital sinus with 26G needle, and intracardiac as described under Materials and methods. Following red blood cells lysis cells were cultured in α-MEM 20% FCS for 10 days, fixed, and stained with toluidine blue. (A) Number of mice from which at least 1 of the blood sample gave rise to plastic adherent colonies/total mice tested. Typical colony derived from (B) blood and (C) bone marrow cells.

response to G-CSF administration. The bone marrow CFU-Fs are normally in a quiescent state and are not proliferating (Ben-Ishay et al., 1986). The increase in colony number could be due to a stimulation of the proliferation of the clonogenic CFU-F within the bone marrow. To first investigate this, bone marrow cells from G-CSF or saline-treated mice were treated with 200 μg/ml hydroxyurea (HU), the agent specifically cytotoxic to cycling cells under the conditions described under Materials and methods, washed twice, and plated for CFU-F assay. As shown in Figure 3, for both G-CSF and saline-treated mice, the number of colonies formed after treatment was not significantly different from untreated cells. This suggests that the cells responsible for the formation of the colony are not cycling at the time they are placed in culture.

Furthermore some of the authors have shown that in humans, G-CSF does not directly stimulate the proliferation of human MPC in a serum-free assay (Gronthos and Simmons, 1995). We used a similar serum-free assay to assess the effect of G-CSF on mouse MPC proliferation. As shown in Figure 4, unlike PDGF-AB, EGF or basic-FGF which are well-established mitogens for MPC (Gronthos and Simmons, 1995; Doucet et al., 2005), G-CSF did not stimulate the proliferation of mouse MPC in a serum-free assay. Based on these data we conclude that G-CSF does not act by increasing the proliferation of mouse MPC in the marrow of G-CSF-treated mice and that the observed increase in CFU-F numbers following G-CSF administration therefore represent an indirect effect of the cytokine on mouse stromal progenitor cells.

CFU-F from the marrow of G-CSF-treated mice exhibit enhanced osteogenic commitment

The bone turnover is the result of a balance between bone resorption and bone synthesis. Previous studies have shown that in addition to inducing mobilization of hematopoietic stem and progenitor cells, G-CSF administration also results in enhanced bone turnover that is accompanied by increased numbers of osteoclasts in the bone marrow (Takamatsu et al., 1998) and the chronic overexpression of G-CSF in transgenic mice leads to osteoporosis due to an increase of osteoclast mediated bone resorption (Takahashi et al., 1996). We therefore hypothesized that the increase in CFU-F number observed in G-CSF-treated mice might be causally linked to the bone remodeling response elicited as a consequence of cytokine administration. To investigate this

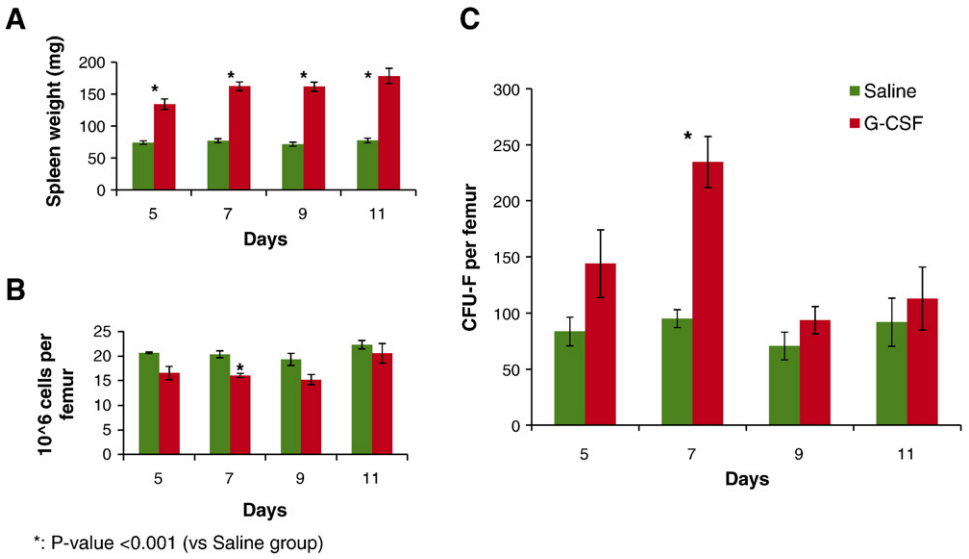

Figure 2 Time course of the increase of CFU-F numbers in the bone marrow of mice treated with G-CSF. Mice were treated with saline or G-CSF for 5, 7, 9, or 11 days. (A) The spleens of the mice were harvested and weighted as an indicator of HSC mobilization. (B) Bone marrow cells were flushed from the femur and the number of mononucleated cells was counted. (C) Cells (2×10^6) from the bone marrow of saline and G-CSF-treated mice were plated in 6-well plates. Cells were cultured in α-MEM 20% FCS for 10 days, fixed, and stained with toluidine blue. Colonies with a size greater than 1 mm were scored. The average results from 5 independent experiments are presented.

hypothesis we first examined whether CFU-F in the bone marrow of G-CSF-treated mice exhibited altered levels of osteogenic commitment compared to that of CFU-F in control mice. Bone marrow cells were harvested from saline and G-CSF-treated mice and plated to generate CFU-F and maintained in osteogenic medium for 5 weeks as described under Materials and methods. Colonies were then fixed and double-stained to detect both alkaline phosphatase activity (AP) and mineral deposition using Von Kossa staining (VK). Colonies were then scored according to the following scheme: colonies lacking both AP activity and VK staining (AP^-VK^-) were classified as being derived from uncommitted progenitors, immature osteoprogenitors as AP+colonies lacking VK staining (AP^+VK^-), and differentiated osteoprogenitors as giving rise to colonies demonstrating both AP activity and mineral deposition (AP^+VK^+) (Figs. 5A–C). When colonies falling into each of these three categories were

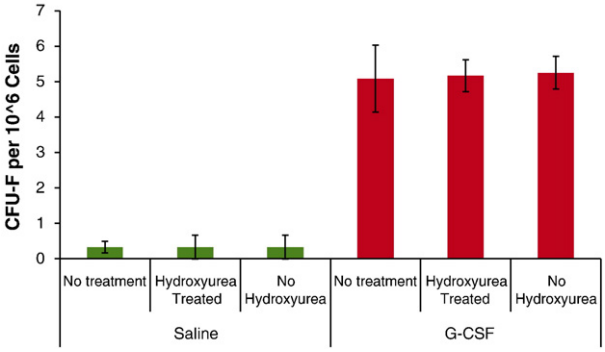

Figure 3 CFU-F-initiating cells are not cycling in the bone marrow. Bone marrow cells from saline and G-CSF-treated mice plated without manipulation, or incubated for 30 min at 37 °C in the presence or absence of 200 μg/ml of hydroxyurea (HU) prior to plating in α-MEM 20% FCS. After 10 days cells were fixed and stained with toluidine blue and colonies greater than 1 mm diameter were scored.

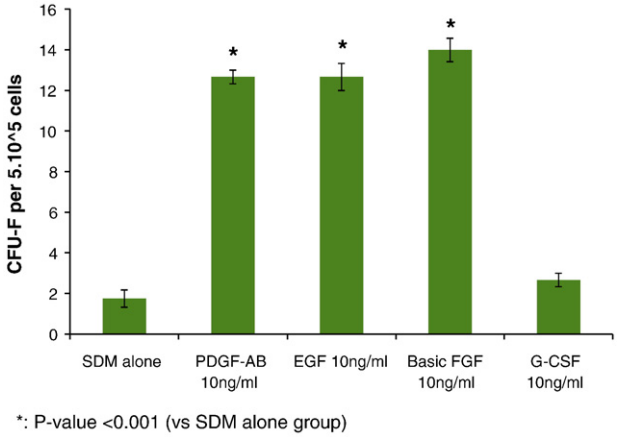

Figure 4 G-CSF does not induce *in vitro* CFU-F formation. Bone marrow cells (5×10^5 to 4×10^6) from untreated mice were plated in 6-well plates in triplicates in SDM supplemented with PDGF-AB, EGF, bFGF, or G-CSF. After 10 days cells were fixed and stained with toluidine blue and colonies greater than 1 mm diameter were scored.

enumerated, approximately half of the colonies formed from the bone marrow of saline-treated, control mice fell into the undifferentiated (AP^-VK^-) category (Fig. 5D). In marked contrast, colonies derived from the bone marrow of G-CSF-treated mice show a marked reduction in the proportion of undifferentiated colonies. This was observed in mice treated with G-CSF for 5, 7, and 9 days where undifferentiated colonies represented only 30, 25, and 27%, respectively, of total colonies compared to 52% in saline control bone marrow. Of note, mice receiving G-CSF continuously over 11 days did not demonstrate a significant reduction in the proportion of undifferentiated colonies.

We have recently reported that the vast majority of bone marrow stromal precursors are located in the compact bone compartment (Short et al., 2009). Therefore, these results prompted us to hypothesize that the osteogenic colony-forming cells in the G-CSF-treated mouse bone marrow could be released from the bone due to increased bone resorption by higher osteoclast activity. We investigated the effect of G-CSF treatment on the CFU-F content in the compact bone compartment after 5 and 7 days of treatment with G-CSF. There were no statistically significant differences in the total number of CFU-F in the compact bone fraction of the femur from the mouse treated with G-CSF for 5 or 7 days as compared with a similar fraction of femur from the saline-treated mouse (873 ± 164, 1013 ± 301, and 947 ± 154 CFU-F per femur, respectively (Fig. 6). These results suggest that only a minor fraction of CFU-F is recruited into bone marrow by G-CSF.

The increase in the marrow CFU-F number by G-CSF is achieved through osteoclast functions

We then addressed more directly whether the increase in CFU-F number in the marrow by G-CSF was dependent on osteoclast activation, using an osteoclast inhibitor: osteoprotegerin (OPG). The maturation of osteoclast capable of bone resorption is dependent on a cell-to-cell interaction between osteoclast precursors and osteoblasts, which is mediated by RANK (receptor) on osteoclast precursors and RANKL (RANK ligand) on osteoblasts. OPG is a soluble ligand for RANKL, and the binding of OPG to RANKL blocks the RANKL action leading to the maturation of osteoclast (for review, see Khosla (2001)). Mice were treated with OPG at a concentration originally used by Simonet et al. (1997) for 2 days prior to and during the G-CSF administration. As presented in Figures 7B–D, OPG administration alone had no significant effect on the bone marrow cellularity, blood cell count, or CFU-F numbers in the marrow. A slight increase in the spleen weight of the OPG-treated mice (107 ± 0.5 mg) compared to that of the saline-treated mice (77 ± 3.2 mg) was statistically significant ($P<0.001$), which may reflect partial transition of hematopoiesis to spleen due to decrease

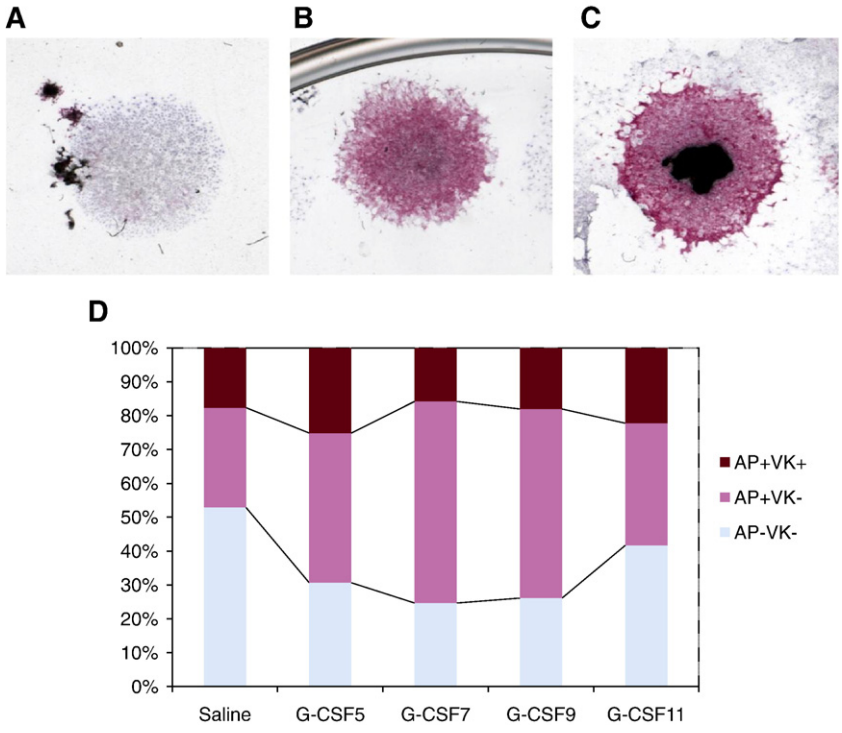

Figure 5 Increased commitment to the bone fate in CFU-F from G-CSF-treated mice. Mice were treated with saline or G-CSF for 5 to 11 days. Cells from the bone marrow of saline and G-CSF-treated mice were plated in medium as described under Materials and methods for 5 weeks. The cells were fixed and stained for alkaline phosphatase activity, mineral deposition (Von Kossa), and with toluidine blue. Colonies with a size greater than 1 mm were scored as (A) undifferentiated: AP^-VK^-, (B) committed to bone differentiation: AP^+VK^-, (C) fully committed bone cells: AP^+VK^+. (D) The average results from two independent experiments are expressed as colony per 10^6 cells plated.

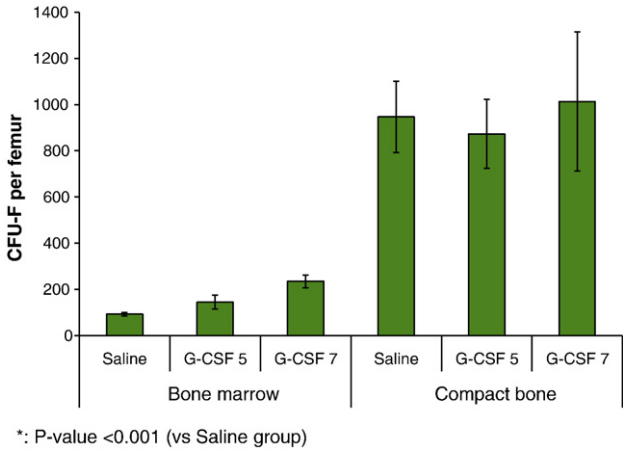

Figure 6 Localization of the CFU-F in the mouse femur. Mice were treated with saline or G-CSF for 5 to 7 days. The bone marrow and compact bone cells were obtained as described under Materials and methods. Cells (2×10^6) from the bone marrow and compact bone cells (20×10^3) of saline and G-CSF-treated mice were plated in triplicates in 6-well plates. Cells were cultured in α-MEM 20% FCS for 10 days, fixed, and stained with toluidine blue. Colonies with a size greater than 1 mm were scored. Average results from three independent experiments are presented.

in the bone marrow space by OPG. The increase in the spleen weight and blood cell count in the G-CSF-treated mice compared to those in the saline-treated mice indicates HSC mobilization. This increase is not inhibited by the administration of OPG (Figs. 7A and C), indicating that the reduction of osteoclast activity did not inhibit HSC mobilization by G-CSF. On the other hand, the CFU-F number in the marrow of mice treated with both OPG and G-CSF is not significantly different from that of the saline-treated group (135.2 ± 39.7 and 95.2 ± 9.3 CFU-F per femur, respectively, Fig. 7D). These results suggest that the increase in CFU-F number in the marrow of the G-CSF-treated mouse is a consequence of increase in osteoclast activity. Nevertheless, we conclude that the increase in the CFU-F number in the bone marrow of G-CSF-treated mice is triggered by the increase in the osteoclast activity in the bone marrow.

Discussion

The growing interest for MPCs as therapeutic tools in multiple hematological and nonhematological disorders raised the need to investigate alternative source for these cells. As for HSC that can be mobilized from the marrow to the peripheral blood, we investigated the possibility of releasing MPC in the circulation.

Detection of MPCs in the circulation

The origin of MPCs in peripheral blood is poorly documented. It has recently been established that perivascular cells

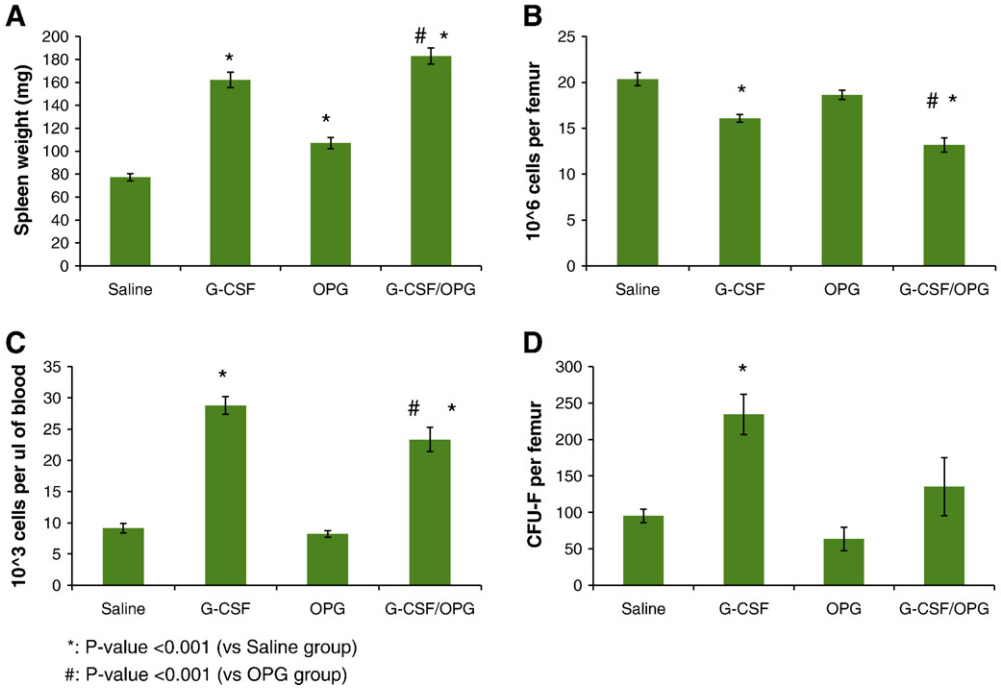

Figure 7 Osteoprotegerin treatment prevents the increase of the number of CFU-F in the bone marrow of G-CSF-treated mice. Mice were treated with saline for 7 days or G-CSF for 7 days or OPG and G-CSF for 7 days, starting OPG administration 2 days prior to the start of G-CSF administration. (A) The spleens of the mice were harvested and weighted as an indicator of HSC mobilization. (B) Bone marrow cells were flushed from the femur and the number of mononucleated cells was counted. (C) Blood was harvested and the number of mononucleated cells was counted. (D) Cells (2×10^6) from the bone marrow of saline and G-CSF-treated mice were plated in 6-well plates. Cells were cultured in α-MEM 20% FCS for 10 days, fixed, and stained with toluidine blue. Colonies with a size greater than 1 mm were scored. Average results from two independent experiments are presented.

(pericytes) are a source of CFU-F (da Silva Meirelles et al., 2009; Crisan et al., 2008). In support, CFU-Fs are also detected in nonskeletal organs such as dermis (Toma et al., 2001) and adipose tissue (Wagner et al., 2005; Sakaguchi et al., 2005; Zuk et al., 2002). The low frequency of CFU-F in peripheral blood and the blood vessel as a source for CFU-F have therefore raised a possibility that the low level peripheral blood CFU-F could be a contaminant generated from blood vessels and surrounding tissues by needle puncture when blood is collected.

We have observed that the cells forming adherent cell colonies from blood samples differed in morphology from those forming colonies derived from bone marrow samples (Figs. 1A and B). Adherence to plastic is often used as a crude method to enrich for MPCs but fibroblasts are not the only cells with such adherent properties. The analysis of the cellular composition of the adherent layers from long-term bone marrow culture showed the presence of endothelial like cells and macrophages (Dexter, 1979). More recently, Gutierrez-Rodriguez and colleagues have shown that adherent layers generated with human umbilical cord blood contain only macrophages but no fibroblasts (Gutierrez-Rodriguez et al., 2000). In the colonies formed from our blood samples, we did not detect significant phagocytic activity assayed using Indian ink (data not shown).

There are conflicting reports related to the detection of MPC in the peripheral blood of patients receiving G-CSF. These studies have shown that when peripheral blood cells are plated directly onto plastic, no CFU-Fs are detected (Kassis et al., 2006; Lazarus et al., 1997) or at very low frequency (Lund et al., 2008). Furthermore Kassis et al. show that only with fibrin microbeads MPC-like cells forming adherent layers are detected in the peripheral blood of G-CSF-treated patients. Fibrin may provide an appropriate substrate for the adhesion of circulating MPC. In our study, we use conditions that we determined optimal for the growth of mouse bone marrow MPCs (i.e., selected batch of fetal calf serum and 5% O2). It is possible that circulating MPC have different cell culture requirements. Previous studies have shown that colony formation with bone marrow stromal cells *in vitro* could be modulated by the presence of feeder cells (Kuznetsov and Gehron Robey, 1996), and bone marrow cells themselves can provide the feeder effect. In this sense, it is possible that some adherent cells from the peripheral blood sample might have a suppressive effect. In order to test this hypothesis we performed CFU-F assay for compact bone cells in the presence or absence of blood cells. We did not detect a reduction of the CFU-F numbers in the presence of blood cells, ruling out this hypothesis (data not shown).

Mobilization of MPCs into the circulation

Our study did not show a significant difference in the number of CFU-F detected between the peripheral blood of mice treated and untreated with G-CSF, indicating that in contrast to HSC, G-CSF may not be a suitable stimulus for MPC mobilization. In a recent report Liu et al. report an increase in the adherent cell number obtained from culture of peripheral blood cells of mice treated with rhuG-CSF (Liu et al., 2007). Other factors such as irradiation may be more efficient in mobilizing MPCs. Irradiation was in fact used to test the capacity of marrow stromal fibroblasts to circulate in a parabiosis setting (Maloney et al., 1985). However, in this case, too, circulating CFU-Fs are very rare: less than 1 "donor" CFU-F detected per femur in the irradiated parabiot partner.

In contrast, it has been shown that rats housed in hypoxic chambers contain a dramatically high level of CFU-F in the peripheral blood as compared to those housed under normoxic conditions (Rochefort et al., 2006). However, we have noted that no difference is detected in the number of CFU-F in bone marrow in these experiments.

Circulation of MPC could also be stimulated in a pathological context. In support, cells with MPC phenotype are detected at higher frequency in the peripheral blood of acute burn patients (Mansilla et al., 2006). MPCs migrated to the injury site. One of the possible mechanisms for it is a response to inflammation which is also thought to cause migration of MPCs toward tumors, where they contribute to the tumor microenvironment (Spaeth et al., 2008).

HSC mobilization is generally observed at Days 4–6 post G-CSF treatment (Sudo et al., 1997). In recent years several publications have pointed out the importance of bone as the HSC niche (Arai et al., 2004; Calvi et al., 2003). Since the bone-forming osteoblasts can be derived from CFU-Fs, the increase in the marrow CFU-F number 48 h after the mobilization of HSC (Fig. 2) suggests that this phenomenon reflects a mechanisms responsible for reconstituting the HSC compartment after HSC mobilization.

Increased number of MPC in the bone marrow as a consequence of increased bone turnover

While the G-CSF treatment did not induce the mobilization of CFU-F into the peripheral circulation, we observed an increase in the CFU-F number in bone marrow following G-CSF treatment, confirming a previous report (Liu et al., 2007), which hit maximum after 7 days of treatment (Fig. 2).

Using HU, we have demonstrated that the increase in the CFU-F number is not caused by a direct effect of G-CSF on the proliferation of the colony-forming cells. The report that G-CSF receptor is not detected on osteoblasts (Katayama et al., 2006) supports our conclusion.

It has been demonstrated that administration of G-CSF stimulates osteoclast-mediated bone resorption (Takamatsu et al., 1998; Takahashi et al., 1996). The balance between bone resorption and bone formation is tightly regulated in order to maintain bone homeostasis. Elevated osteoclastic activity therefore results not only in a high bone resorption rate but also in a high bone synthesis rate. Bone-forming osteoblasts are one of the progeny of CFU-Fs. The increase in the CFU-F numbers observed could therefore be related to an increase in the bone formation in response to the bone resorption induced by the osteoclast activity. Alternatively, the increase in CFU-F number is a consequence of the bone resorption by osteoclast. Our results suggest that both seem to be involved.

First, our *in vitro* bone nodule formation assay indicated that the G-CSF treatment resulted in a lower proportion of colonies composed of undifferentiated mesenchymal cells and increased proportion of colonies consisting of early osteoblastic cells (Fig. 5), suggesting that an increased number of MPCs may have activated the osteogenic differentiation pathway or that the MPC migration into the marrow is already committed

to the bone fate. Second, our *in vivo* experiments with OPG an inhibitor of osteoclast maturation indicated that OPG inhibited the G-CSF-induced increase in the marrow CFU-F number, while it did not inhibit HSC mobilization as indicated by the spleen weight and blood cell count increases (Fig. 7). This is in accord with a previous study with pamidronate, a bisphosphanate family of inhibitors for bone resorptive activity of osteoclasts, in that the G-SCF-induced mobilization of HSC is not affected (Takamatsu et al., 1998). Interestingly, a recent report has shown that the stimulation of osteoclasts by RANKL also induces HSC mobilization via a mechanism distinct from the mechanism involved in G-CSF-induced HSC mobilization (Kollet et al., 2006).

Recruitment of MPC to bone marrow by G-CSF is potentially from the bone

Association of the increase in the marrow CFU-F number with higher bone turnover prompted us to speculate that the mobilized MPCs are originated from the bone. We have demonstrated that compact bone represents a large reservoir of MPC (Short et al., 2009). However, we did not detect a statistically significant reduction in the number of CFU-F in the compact bone fraction of G-CSF-treated mice. The increase in the marrow CFU-F number was approximately 130 CFU-F per femur (Fig. 6). Considering the number of CFU-F measured in the compact bone of saline or G-CSF-treated mice (873 ± 164, 1013 ± 301, and 947 ± 154 CFU-F per femur), it is unlikely that we were able to accurately measure the decrease in MPCs of this degree in the compact bone compartment. However in support of this hypothesis, it has been recently shown that the number of endosteal osteoblasts is significantly reduced in mice treated with G-CSF for 5 days (Semerad et al., 2005) and that the morphology of the osteoblasts in G-CSF-treated mice is modified (Katayama et al., 2006). The average length of osteoblast projection into bone as well as the thickness of the osteoblasts at the endosteum is reduced in G-CSF-treated mice as compared to PBS/BSA-treated animals. In addition we observed that the bones from G-CSF-treated mice were more prone to fracture when flushing the marrow, probably caused by osteoporosis induced by G-CSF. All these observations suggest that the increase in osteoclast activity loosens the structure of the bone at the endosteal region, releasing osteoblasts and osteoprogenitors in the marrow.

In conclusion, while an efficient and clinically applicable stimulus to release MPC in the circulation is still to be identified, we propose that G-CSF administration could be used to provide a temporary increase in MPC in the bone marrow. Furthermore the increase of bone commitment of the MPC obtained in such MPC enriched in bone marrow would make these cells suitable candidates for applications such as skeletal defect repair.

Material and methods

Cytokine and chemicals

G-CSF was purchased from Amgen Biologicals, (Australia). Osteoprotegerin was kindly provided by Amgen, Inc. (Thousand Oaks, CA).

Animals

Balb/c mice were housed and handled according to the Australian Welfare procedure. All methods and animal procedures were reviewed and approved by the Animal Ethics Committee.

Cytokine and drug administration to mice

For time-course experiments, 10- to 14-week-old male Balb/C mice were divided into two groups. The first group of mice received daily subcutaneous injection of human recombinant G-CSF at a dose of 250 μg/ kg (body weight)/day for 5, 7, 9, or 11 days. The second group received saline for 5, 7, 9, or 11 days. For each individual experiment each group contains 3–4 animals. For comparative studies, mice were divided into 4 groups of 4 animals and received daily subcutaneous injections of saline, human recombinant G-CSF at a dose of 250 μg/ kg of body weight/day for 7 days, osteoprotegerin (OPG) at a dose of 10 mg/ kg of body weight/day for 10 days, OPG, for 10 days, and G-CSF for the last 7 days.

Tissue collection

In the initial experiments, blood was harvested from the retro-orbital sinus with a capillary tube. In subsequent studies, blood collection was performed as follows in order to minimize possible contamination of the blood sample by cells from the blood vessel or surrounding tissues. Mice were anesthetized by inhalation of isoflurane (David Bull Laboratories, Mulgrave, VIC, Australia), and blood was collected from the retro-orbital sinus using a syringe equipped with 26 G needles which were prerinsed with sterile 10% EDTA solution. The mouse abdominal cavity was then opened and a second sample of blood was harvested with a new syringe and needle by intracardiac puncture. Red cells were lysed by adding 10 vol of ammonium chloride hypotonic lysis buffer, and cells were washed with PBS-2% fetal calf serum (FCS, Hyclone, Logan, UT, USA), prior to being plated for assay of CFU-F.

Twelve hours after the last injection mice were culled by cervical dislocation, the femurs were harvested under sterile conditions, and the outer surface of the bone was extensively cleaned of any remaining tissues by scraping with a scalpel blade. The ends of the femora were cut off with a scalpel blade and bone marrow cells were obtained by flushing with PBS–2% FCS. The cells obtained after flushing constitute the bone marrow fraction. Flushed bones were then cut in small pieces and incubated with collagenase I (3 mg/ml) (Worthington Biochemical Co., Freehold, NJ, USA) for 45 min at 37 °C under constant agitation as previously described (Short et al., 2009). Cells released after incubation constitute the compact bone fraction.

The spleens of the animals were harvested and weighed as a surrogate measure of mobilization of hematopoietic stem and progenitor cells.

Standard CFU-F assay

For assay of CFU-F in peripheral blood samples, all mononuclear cells derived from 250 μl of blood were plated

in triplicate in medium comprising alpha-MEM supplemented with 20% of a lot-selected fetal calf serum (Hyclone, Logan, UT, USA), 5 mM sodium pyruvate, 1% L-glutamine 2 mM, 1% penicillin–streptomycin (α-MEM 20%FCS). CFU-F derived from BM were enumerated by plating cells under identical conditions in triplicate over the range 5×10^5 to 4×10^6 total bone marrow cells and at 10^4 to 2×10^4 cells for cells derived from compact bone. All cultures were maintained in 10% CO_2, 5% O_2, humidified air for 10 days and then colonies were fixed and stained in 0.01% toluidine blue in 2% formalin. Colonies larger than 1 mm diameter were scored. CFU-F numbers were expressed as the number of colonies per femur for the bone marrow and compact bone cell fractions, respectively.

Serum-free CFU-F assay

From 5×10^5 to 4×10^6 total bone marrow cells were plated in triplicate in 6-well plates in serum-deprived medium (SDM): IMDM supplemented with 1% BSA, recombinant human insulin (10 μg/ml), transferrin (100 μg/ml), β-mercaptoethanol (5×10^{-5} M), low-density lipoprotein (LDL) (20 μg/ml), and L-glutamine 2 mM. All cultures were maintained in 10% CO_2, 5% O_2, humidified air for 10 days. CFU-F were fixed and stained in 0.01% toluidine blue in 2% formalin. Colonies larger than 1 mm diameter were scored. The CFU-F numbers were expressed as number of colonies per 5×10^5 cells.

Hydroxyurea assay

To determine whether increases in CFU-F numbers occurring in the bone marrow compartment are due to proliferation of preexisting stromal progenitors, bone marrow cells from G-CSF-treated and control mice were treated with hydroxyurea. The optimal concentration for HU to kill dividing cells was determined on passaged bone marrow stromal cells (MSC). Briefly, bone marrow stromal cells were plated at low density in α-MEM 20% FCS in 75 cm^2 flasks and grown for 36–48 h to ensure a significant proportion were cycling at the time of harvest. The adherent cells were harvested by incubation with trypsin–EDTA, washed, and resuspended in medium and treated in suspension with HU over a dose range from 5 μg/ml to 1 mg/ml for 30 min at 37 °C. After washing twice in medium, cells killed as a consequence of HU treatment were identified by addition of 7-aminoactinomycin D (7-AAD) at a final concentration of 2.5 μg/ml for 5 min and flow cytometric analyses performed on a FACscan (Becton Dickinson). Data analysis was performed using Cell Quest software. The proportion of MSC killed by drug treatment was maximal at concentrations of HU of 200 μg/ml and above (data not shown). Based on these data, bone marrow cells were pooled from 3 G-CSF or saline-treated mice and incubated with HU at a final concentration of 200 μg/ml in culture medium for 30 min at 37 °C, washed twice with medium, and plated in triplicate as described above to assay the content of CFU-F. As a control for HU treatment, cells were incubated under identical conditions at 37 °C in the absence of HU and additional aliquots of cells also plated without any treatment.

In vitro bone nodule formation assay

Cells from the bone marrow of saline and G-CSF-treated mice were plated in triplicate over a range of cell concentrations in 6-well plates in α-MEM 20%FCS supplemented with 1.8 mM KH_2PO_4 (BDH Chemicals Ltd. Poole, Dorset, UK), 100 μM L-ascorbic acid 2-phosphate (ASC-2P; Novachem, Melbourne, Australia), 5×10^{-5} M β-mercaptoethanol, and dexamethasone sodium phosphate at a final concentration of 10^{-8} M (DEX; David Bull Laboratories). Cultures were maintained in 10% CO_2, 5% O_2 in humidified air and medium was replaced twice weekly from Day 7, for 5 weeks. Adherent cells were washed with PBS and formalin-fixed. Alkaline phosphatase activity (AP) was detected histochemically by incubation with 0.1 mg/ml of naphtol AS MX-PO4 (Sigma) and 0.6 mg/ml of Red Violet LB Salt (Sigma) in 0.1 M Tris-HCl, pH 8.3. Cells were then washed in distilled water and the mineral deposit was detected by staining with Von Kossa's reagent (VK) (2.5% silver nitrate in distilled water). Cells were counterstained with toluidine blue. Colonies larger than 1 mm diameter were scored according to the three categories indicated in the text.

Statistical analysis

Significant differences were determined using single factor ANOVA.

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