ARTICLE

G-CSF Indirectly Induces Apoptosis of Osteoblasts During Hematopoietic Stem Cell Mobilization

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The objective of this study was to explore the mechanism underlying osteoblast suppression in the process of hematopoietic stem cells mobilization induced by granulocyte colony-stimulating factor (G-CSF). The apoptosis of human and mouse osteoblasts was examined by detecting caspase 3. The levels of serum DKK1 and osteocalcin in the supernatant of co-culture of mouse osteoblasts and mouse bone marrow nucleated cells were measured. The number of mouse osteoblasts co-cultured with mouse bone marrow nucleated cells was measured and the osteocalcin mRNA level was also measured. The G-CSF-induced decrease in osteoblast function was partly due to the apoptosis of osteoblasts. There was no significant difference in the level of serum DKK1 in healthy donors before and 5 days after mobilization. The osteocalcin gene and protein expression was significantly different in co-cultured osteoblasts with bone marrow nucleated cells treated with and without G-CSF. Osteoblasts undergo apoptosis during mobilization and G-CSF affects osteoblasts through bone marrow nucleated cells.
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Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
☑ Short-term G-CSF treatment leads to a reduction of osteoblasts.
WHAT QUESTION DID THIS STUDY ADDRESS?
☑ How G-CSF impacts osteoblasts during stem cell mobilization.

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE
☑ Osteoblasts undergo apoptosis during mobilization and G-CSF affects osteoblasts through bone marrow nucleated cells.
HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE
☑ These data will have important future implications for clinical trials for the purpose of increasing the number and effectiveness of HSPC therapies via targeting osteoblasts.

Hematopoietic stem cell transplantation has become one of the most effective methods for the treatment of systemic diseases such as blood diseases. Stem cell mobilization has been widely used in clinics because it has many advantages. Full mobilization of hematopoietic stem/progenitor cells to the peripheral blood as well as smooth homing and implantation of bone marrow are the keys to success. However, enough CD34+ cells cannot be collected from some patients and normal donors in clinics. Therefore, understanding the mobilization mechanism of hematopoietic stem cells, and developing new mobilization agents according to its mechanism, will help solve the problem of poor mobilization.

Our preliminary results suggest that short-term administration of granulocyte colony-stimulating factor (G-CSF) can result in a decreased number and activity of metaphyseal osteoblasts in both human and mouse mobilization models, and changes in osteoblasts occur prior to mobilization. Further studies have shown that a decrease in the number

of osteoblasts could lead to reduced expression of SDF-1, SCF, OPN, and other proteins, resulting in the occurrence of mobilization.[1] At present, the mechanism of the G-CSF mobilization process is not fully elucidated. In particular, the cause of osteoblast suppression is still controversial. In addition, the results of previous studies are mostly based on animal models. In this study, we further explored the mechanism of osteoblast suppression in both healthy donors and mouse models.

METHODS
Collection of blood serum and biopsy specimens from healthy donors
Peripheral blood and bone marrow biopsy specimens were collected from 21 healthy donors for allogeneic hematopoietic stem cell transplantation before and at 5 days of mobilization by subcutaneous injection of G-CSF (Kirin Kunpeng Biopharmaceutical, China) at 5 μg/kg per dose, twice a day.

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Serum samples were isolated within 30 min after collection of peripheral blood, aliquoted according to need, and stored at –80°C. Biopsy specimens were immediately fixed with 10% formalin for 24 h, subjected to conventional decalcification, gradient ethanol dehydration, transparent, paraffin-embedded, prepared as 5 μm paraffin sections and placed on polylysine-coated slides. The study was approved by the Hospital Ethics Committee and all healthy donors were informed and gave written consent before sample collection.

Animal grouping and specimen collection

A total of eight female C57Bl/6 mice 6–8 weeks old, weighing 20–25 g were assigned to the negative control group (untreated group) and an experimental group with eight per group. Mice were obtained from the Institute of Laboratory Animal Science and were housed in sterilized micro-isolator cages and received autoclaved food and water. There was no significant difference in the body weight between these two groups. The mice in the experimental group were given 250 μg/kg G-CSF (Filgrastim) dissolved in 250 μl phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) (Roche, Nutley, NJ), pH 4.55, by subcutaneous injection for consecutive 5 days. Mice in the control group were subcutaneously injected with an equal volume of PBS. At 6 h after the last injection, blood was collected from the orbital vein to isolate serum. Mice were sacrificed by cervical dislocation to isolate femur specimens, which were used in future experiments.

Collection of mouse bone marrow nucleated cells

Mice were sacrificed by cervical dislocation and the femoral and tibial specimens were isolated. The bone marrow cavity of the femur and tibia of mouse C57Bl/6 mice was washed repeatedly with PBS containing 2% fetal bovine serum several times with a 5 ml syringe until the bone marrow cavity was white. The cell suspension was collected, mixed with red cell lysate (0.15 M NH4Cl, 1.0 mM KHCO$_3$, 0.1 mM EDTA, pH 7.4) to remove red blood cells and filtered through a nylon mesh to collect mouse bone marrow nucleated cells.

Measurement of serum DKK1 in healthy donors before and after G-CSF mobilization and osteocalcin in the co-culture supernatant using enzyme-linked immunosorbent assay (ELISA)

Human DKK1 in serum samples collected from healthy donors before and after G-CSF mobilization and mouse osteocalcin in the supernatants of co-cultured osteoblasts and bone marrow cells were measured using a human DKK1 protein ELISA kit (Enzo Life Sciences, Farmingdale, NY) and mouse osteocalcin protein ELISA kit (Biomedical Technologies, Stoughton, MA) according to the instructions of the manufacturers. The measured absorbance values were used to calculate the concentrations of DKK1 (pg/ml) and osteocalcin (ng/ml) using Advanced Grapher 2.01.

Real-time quantitative polymerase chain reaction (RQ-PCR) for detecting expression of osteocalcin in mouse osteoblasts in the co-culture system

The adherent mouse osteoblasts at day 4 of co-culture were routinely digested. Total RNA was extracted from these cells using Trizol (Invitrogen, Carlsbad, CA) and reverse transcribed into cDNA. Primers for amplification of osteocalcin were 5'TCTCTCTGCTCACTCTGCTGGCC3' and 5'TTTGTCAGACTCAGGGCCGC3' designed according to its sequence in GenBank using the software GENE RUNNER. β-actin was used as an internal control. RQ-PCR was performed on an ABI 7500 fluorescence quantitative PCR instrument (Applied Biosystems, Foster City, CA) in a 20 μl reaction system consisting 10 μl of the Power SYBR Green PCR Master mix (Applied Biosystems), 0.5 μl of each primer at conditions of 95°C for 15 min followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. The expression level of osteocalcin relative to controls was calculated as $\triangle\triangle$CT values.

In vitro culture of osteoblasts

Skulls of C57Bl/6 mice at age 3–4 days old were harvested, cut into pieces, and digested with 50 mg/mL type II collagenase (Gibco, Gaithersburg, MD) for 60 min. The digestive solution was discarded and the bone pieces were washed with PBS and cultured in flasks with α-MEM (Hyclone, Ogden, UT) differentiation medium containing 10% fetal bovine serum (Gibco), 100 μg/ml ascorbic acid (Sigma-Aldrich, St. Louis, MO), and 5 mM β-phosphoglycerol(Sigma-Aldrich). When cells reached 80% confluence, cells were passaged and used for experiments at passages 2 and 3.

In vitro co-culture of osteoblasts with bone marrow nucleated cells

Osteoblasts at passages 2 or 3 were mixed with 2 × 10^7 bone marrow nucleated cells and cultured in 10 ml α-MEM (Hyclone) differentiation medium supplemented with or without 0.5 μg/ml G-CSF (Kirin Kunpeng Biopharmaceutical) for 4 days. The culture supernatant was collected and stored at –80°C. The adherent cells were washed twice with PBS and digested with 0.125% trypsin-EDTA to collect osteoblasts.

Caspase 3 protein levels in osteoblasts in healthy donors and model mice detected by immunohistochemistry before and after G-CSF mobilization

The continuous paraffin sections were deparaffinized and hydrated. After repair with 0.01 mol/L sodium citrate solution for 12 min, the sections were blocked using goat sera for 10 min. Two consecutive sections were divided into two groups. One group was incubated with mouse anti-osteocalcin monoclonal antibody (Abcam, Cambridge, UK; ab8448, 1:400 dilution) and rabbit anti-caspase3 polyclonal antibody (Abcam; ab44976, 1:400 dilution), respectively, for 90 min. After washing with PBS, these sections were incubated in turn with biotinylated secondary antibody and streptavidin-peroxidase at room temperature for 30 min. After visualizing the positive proteins with DAB staining, the sections were counterstained with hematoxylin, observed under optical microscopy, and photographed. The murine femur continuous paraffin sections were prepared and subjected to immunohistochemical staining using rabbit anti-osteocalcin polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA; FL-95, 1:300 dilution) as described above. The number of osteoblasts (OB.N/B.s) was counted as the
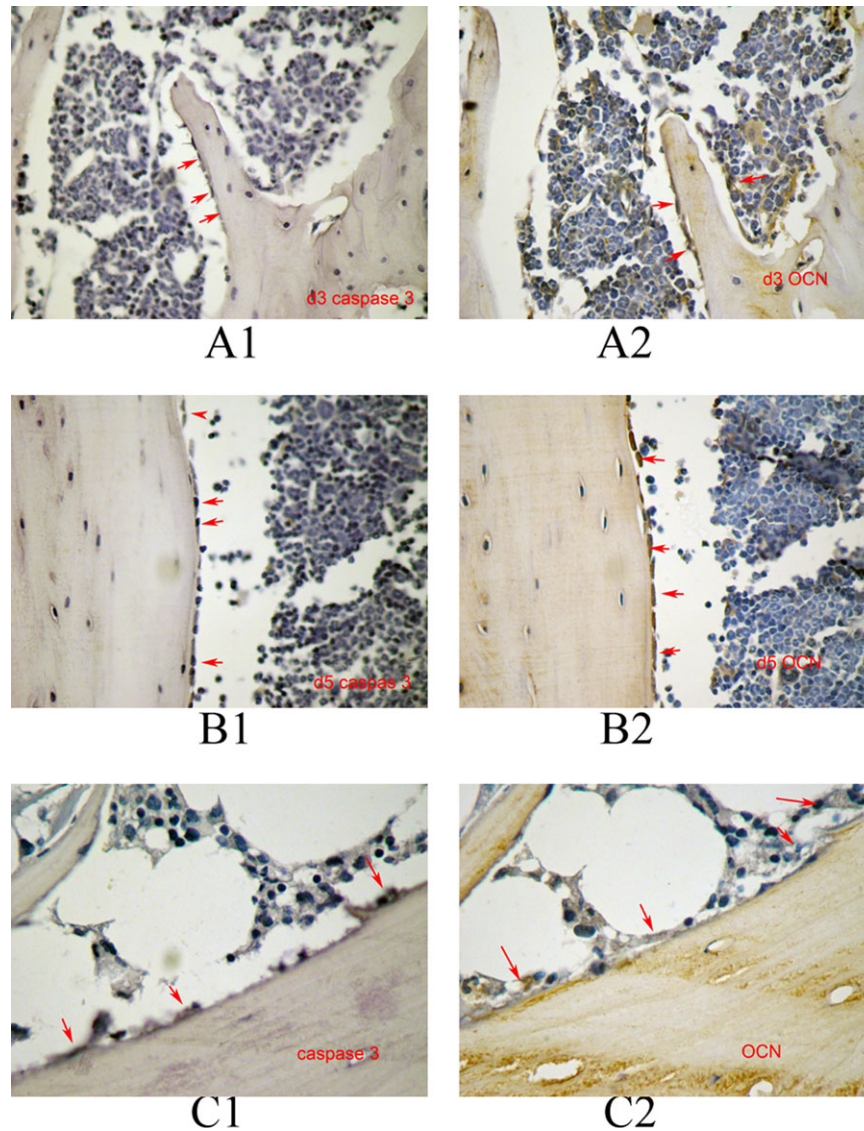
Figure 1 Caspase 3 and OCN staining in sequential representative sections. Anti-caspase 3 antibody staining of osteoblast in sequential representative sections of the femur from C57Bl/6 mouse on d3 and d5 (**a1-2,b1-2**, original magnification, 400×) and healthy donor mobilized state (**c1-2**, original magnification, 400×). Osteoblast (red arrows) was indicated by morphology and by OCN expression.

number of osteocalcin-positive cells per number of trabeculae under a light microscope at 400× magnification.

Statistical analysis
The data were analyzed using SPSS 15.0 (Chicago, IL) statistical software and described as means ± standard error, and the mean was compared using the nonparametric Mann–Whitney test. $P < 0.05$ was considered statistically significant. Each experiment was repeated four times.

RESULTS
Apoptosis is the major mechanism underlying osteoblast suppression during G-CSF mobilization
In previous studies, the mechanism underlying the decrease in the number of mature osteoblasts in the mobilization process is still controversial.[2] Our immunohistochemical exploration of the expression of caspase3 and osteocalcin in the

osteoblasts of bone marrow biopsy specimens of healthy donors and the femoral consecutive sections of mouse specimens showed that caspase3 was abundantly expressed in the healthy donor and mouse femoral osteoblasts (cubic cell close to the bone, osteocalcin positive) on the third and fifth days of G-CSF mobilization (**Figure 1a,b**), indicating that mature osteoblasts underwent apoptosis during the mobilization process.

No significant change in serum DKK1 level before and after mobilization
Another reason for the decrease in the number of mature osteoblasts is the inhibition of osteoblast differentiation. The Wnt pathway is an important pathway in the development of osteoblast differentiation.[3] Therefore, the expression level of DKK1, the key inhibitor of the Wnt pathway, was examined by ELISA. The results showed no significant changes in serum
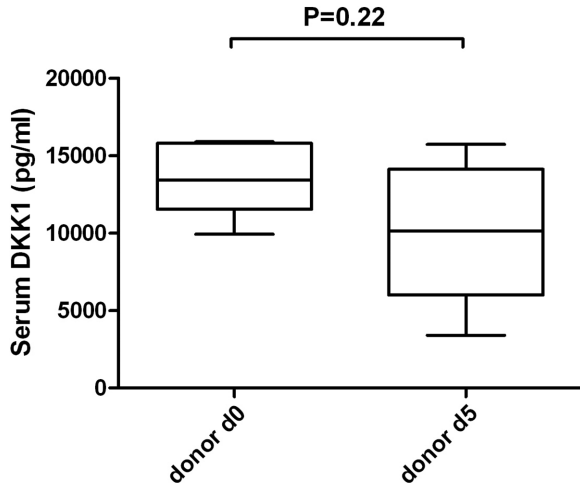
Figure 2 Serum DKK1 concentrations did not show a significant difference on day 0 and day 5 of G-CSF administration in human. Serum samples were collected from 12 donors and their serum DKK1 levels were measured by ELISA (data are means ± SD).

DKK1 level in 12 normal donors before and after mobilization ($P = 0.22$, **Figure 2**).

G-CSF affects osteoblasts through an indirect pathway mediated by marrow mononuclear cells

Previous studies have shown that G-CSF receptors are not expressed in osteoblasts. Therefore, G-CSF inhibits osteoblasts indirectly.[4–6] We then examined whether G-CSF induced inhibition of osteoblasts is mediated by bone marrow mononuclear cells. Mouse osteoblasts were co-cultured with mouse bone marrow mononuclear cells and G-CSF was added on the first day of the culture. On day 4 of culture, the osteocalcin mRNA level in osteoblasts was measured using real-time quantitative PCR. The results showed significant changes between the control and G-CSF-stimulated cells ($P = 0.024$, **Figure 3**). Moreover, the ELISA results also showed that the osteocalcin protein level in the supernatant of co-culture was significantly different between the control and G-CSF-stimulated cells (1.53 ± 0.02 ng/mL vs. 0.77 ± 0.08 ng/mL, $P = 0.03$, **Figure 4**). These results suggest that G-CSF does not directly act on osteoblasts during mobilization, but is mediated by marrow mononuclear cells.

DISCUSSION

Osteoblasts are the main components of hematopoietic stem cell niches of endosteum. Most of the primary hematopoietic stem cells are distributed around osteoblasts.[7] The spatial closeness of osteoblasts and hematopoietic stem cells suggests that they may be functionally interactive and interdependent.[8] Although the effects of osteoblasts on hematopoietic stem cells are not completely understood, accumulating evidence has shown that osteoblasts are involved in hematopoietic stem cell mobilization.[2,4,9] Our previous studies have shown that the number of osteoblasts was reduced and osteoblast functions were inhibited during the G-CSF mobilization in a normal donor and mouse mobilization model. Continuous monitoring of osteoblasts
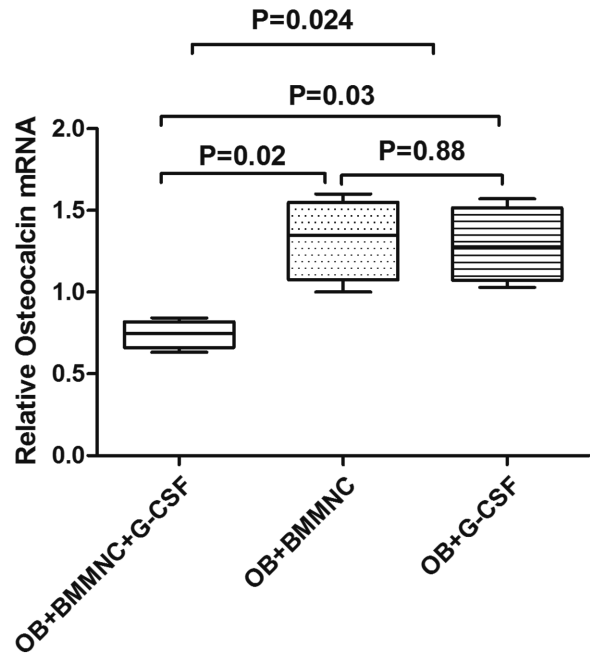
Figure 3 Mouse osteoblasts (OB about 5×10^5 osteoblasts) co-cultured with 2×10^7 bone marrow mononuclear cells (BMMNC) with or without G-CSF (0.5 μg/ml, Filgrastim) while OCN mRNA expression showed a significant difference between the three groups (means ± SD of four independent experiments).

and hematopoietic stem cells suggest that 3 days after mobilization, the number of peripheral blood LSK cells did not significantly increase, but the number of osteoblasts was significantly reduced. The results showed that the changes in
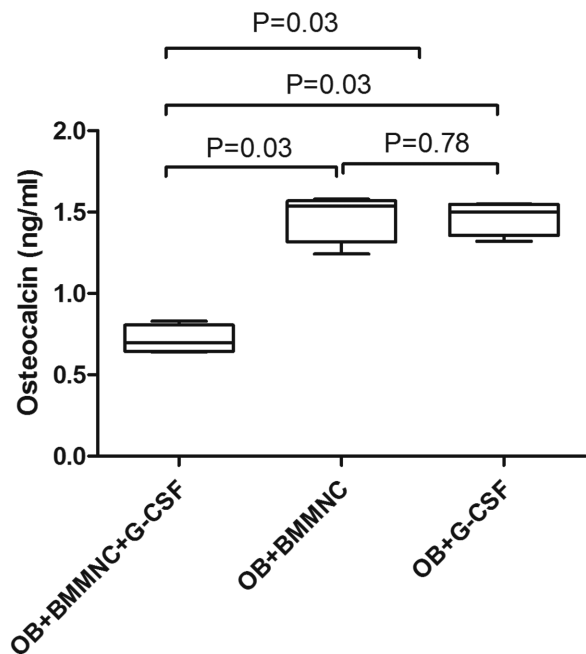
Figure 4 Supernatant liquid OCN concentrations of different groups with or without G-CSF were measured by ELISA. There was significant difference between the three groups (means ± SD of four independent experiments).

osteoblasts occurred prior to mobilization of hematopoietic stem cells, suggesting that a rapid reduction in osteoblast after application of G-CSF may lead to mobilization of hematopoietic stem cells.

Although research on the effects of the matrix composition on HSC mobilization has made great progress, the relationship between osteoblasts and matrix components and whether osteoblasts affect HSC mobilization by altering matrix composition are still not clear. Previous studies have shown that the destruction of the SDF-1-CXCR4 axis is a key mechanism for HSC mobilization.[10,11] However, a number of studies have shown that SDF-1 is mainly expressed in osteoblasts.[4,9,11] These studies suggest that the negative regulation of G-CSF on SDF-1 is due in part to the decrease in the number of osteoblasts. Our previous studies also suggest that the expression of SDF-1 in osteoblasts is reduced during HSC mobilization in both human and mouse models. Similarly, other factors, such as SCF and OPN, that are expressed in osteoblasts and important to HSC mobilization, are also significantly decreased due to the decrease in the number of osteoblasts. Therefore, it can be deduced that the occurrence of HSC mobilization is due in part to enzymatic degradation of the matrix components and that the decrease in the number of osteoblasts and reduction of osteoblastic functions are also important factors affecting HSC mobilization.

The decrease in the number of osteoblasts is possibly due to osteoblast apoptosis and inhibition of osteoblast differentiation.[2,9] Therefore, we examined the expression of caspase3 in serial sections of osteoblasts using an immunohistochemical method and showed that osteoblasts indeed underwent apoptosis after HSC mobilization. Inhibition of osteoblasts differentiation is another reason for the reduced number of mature osteoblasts. Because the Wnt pathway is an important pathway in osteoblast differentiation and development, the expression of the DKK1 protein, an important inhibitory factor of the Wnt pathway, in donor serum before and after HSC mobilization was also examined,[3] but showed no significant difference, indicating that osteoblast differentiation of the Wnt pathway is not suppressed in the HSC mobilization process. Therefore, the decreased number and function of mature osteoblasts during G-CSF-induced HSC mobilization is due, in part, to osteoblast apoptosis rather than Wnt pathway-mediated osteoblast differentiation.

It is unlikely that G-CSF directly acts on osteoblasts, leading to the above changes, because no mobilizing agent can bind directly to osteoblasts. G-CSF receptors were expressed on neutrophils, monocytes, and hematopoietic stem cells. But the RT-PCR results showed that the G-CSF receptor was not expressed in osteoblasts, and the SDF-1 expression level was not changed in osteoblasts after 5 days of culture in medium supplemented with G-CSF.[4] We co-cultured mouse osteoblasts and bone marrow mononuclear cells in medium with or without G-CSF and found a significant reduction in osteoblasts activities, indicating that bone marrow mononuclear cells partially mediate the effects of G-CSF on osteoblasts. Moreover, studies have suggested that G-CSF may act on the sympathetic nervous system, and eventually lead to osteoblast suppression and mobilization through adrenaline.[9] All these results suggest that G-CSF acts on cells that expresses G-CSF receptors, and through these cells ultimately leads to a decreased number and function of osteoblasts.

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Conflict of Interest. The authors declare no conflicts of interest.

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