Bone Modulation in Sustained Hematopoietic Stimulation in Mice

By Minako Y. Lee, Rikiro Fukunaga, Theodore J. Lee, Joan L. Lottsfeldt, and Shigekazu Nagata

To understand the etiology of bone modulation and hypercalcemia observed in granulocytosis of a tumor-bearing animal model and to gain insight into the implication of sustained hematopoietic stimulation on the bone tissue, in vivo responses of normal mouse hematopoietic and bone tissues to long-term injections of recombinant human and murine granulocyte colony-stimulating factor (G-CSF), murine granulocyte-macrophage CSF (GM-CSF), and human erythropoietin were quantitatively analyzed. Osteoclast activation was estimated by the osteoclast-endosteal ratio, determined by morphometric analyses of femoral sections. Medullary and bone areas were measured on transverse ground bone sections of the tibia. Recombinant murine G-CSF provoked marked granulocytosis associated with significant increases in the number of marrow granulocytes and their progenitors, and caused expansion of granulopoietic marrow into fatty marrow. The bone of G-CSF-treated mice showed a significant increase in endosteal osteoclast numbers with medullary area enlargement and a reduction in the bone thickness; indicative of endosteal bone resorption. Although GM-CSF had little effect on granulopoiesis, it caused peritoneal macrophages to increase and induced similar bone changes as those observed in G-CSF treatment. Enhanced erythropoiesis stimulated by erythropoietin was also associated with evidence of endosteal bone resorption. Bone changes induced by these growth factors were not associated with hypercalcemia. These animal studies document association of bone modulation in sustained stimulation of hematopoiesis, and implicate important physiologic effects of hematopoietic growth factors on skeletal tissue in vivo.

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MARKED GRANULOCYTIC hyperplasia in the bone marrow of mice bearing a murine tumor (CE mammary carcinoma) has been shown to be associated with bone modulation and hypercalcemia.1 Recently, we have found that the tumor produces at least two known colony-stimulating factors (CSFs), namely, granulocyte CSF (G-CSF) and macrophage CSF (M-CSF),2 and have suggested that G-CSF is primarily responsible for the granulocytosis observed in CE tumor-bearing mice and that M-CSF acts synergistically with G-CSF to enhance this granulocytosis.2 The etiology of bone modulation observed in this animal model of granulocytosis has been unclear. Although it has been suggested that bone marrow development is closely associated with bone modulation,3,5 the relationship between these two tissues has not been well explored. To clarify if a potent granulopoietic agent alone will produce any bone changes such as we observed in CE tumor-bearing mice, we have quantitatively assessed the bone marrow and bone tissue of mice that were treated with long-term injections of recombinant G-CSF. Furthermore, we extended our studies to characterize the effect of other hematopoietic growth factors on bone tissue in vivo to gain further insight into the physiologic implications of marrow hematopoietic activity on bone modulation.

MATERIALS AND METHODS

Mice. Twelve-week-old female C57Black6 mice were used. These mice were obtained from Jackson Laboratory (Bar Harbor, ME) or Simonsen Laboratories (Gilroy, CA), the former for G-CSF and the latter for GM-CSF and erythropoietin (Epo) studies. All mice were cared for in the vivarium of University of Washington, and fed a standard diet (Wayne Rodent Bldx; Premier Laboratory Diets, Bartonville, IL) and water ad lib.

Recombinant growth factor injections. Mice were divided into two groups, experimental and control, for each growth factor treatment. Mice in the former group received recombinant G-CSF, GM-CSF, or Epo according to the following schedule for a period of 3 weeks while those in the latter group received the same volume of respective diluent solution as controls. All mice received intraperitoneal injections of tetracycline (15 mg/kg; Lederle, Wayne, NJ) on day 21 and were killed 24 hours later. The body weights of the animals were recorded on days 0 and 22.

Recombinant murine G-CSF (mg-G-CSF) was produced by mouse C1217 cells transformed with a bovine papilloma virus expression vector containing a hybrid gene in which mouse G-CSF cDNA was placed under the SV40 early promoter,6 and purified to homogeneity as described elsewhere (manuscript in preparation). Biologic activity of G-CSF was assayed by stimulation of 3H thymidine incorporation into mouse NFS-60 cells.7 The purified recombinant mG-CSF had a specific activity of 2 × 106 U/mg. The purified protein was stored at −80°C in phosphate-buffered saline (PBS) containing 0.002% Tween 20 and 0.02% sodium azide. This protein solution was diluted to a concentration of 25 μg/mL in 10% (vol/vol) normal syngeneic fresh mouse serum in PBS, dialyzed overnight against PBS (Spectrapor, 12,000 to 14,000; Spectrum Medical Industries, Los Angeles, CA) at 4°C, and sterilized (Millipore GV, 0.22 μm filter unit; Millipore Product Division, Bedford, MA). Recombinant human G-CSF (hG-CSF) was prepared in the same manner as above. Mice in the G-CSF group were subcutaneously injected with human or murine G-CSF, 2.5 μg in 0.1 mL solution per mouse, once a day for 21 days, and on day 22, 24 hours after the last injection, they were killed.

Recombinant murine GM-CSF (mgGM-CSF; Genzyme, Boston, MA) was dissolved in PBS containing 5% syngeneic fresh mouse serum to a concentration of 150 ng GM-CSF in 0.2 mL.8 Mice were injected with 150 ng GM-CSF intraperitoneally, three times a day as described7 for 21 days. On day 22, 10 hours after the last injection, they were killed.

Recombinant human Epo (hEpo) (Epoetin alpha; Amgen, Thousand Oaks, CA) was diluted in PBS containing 0.025% mouse albumin (Fraction V; Sigma, St Louis, MO).9 It was used at 1,500 U/kg body weight in 0.1 mL solutions by intraperitoneal injections.

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three times a week as described.9 On day 22, 24 hours after the last injection, mice were killed.

**Blood samples.** On days 0, 7, 10, 14, 17, and 21 of G-CSF or GM-CSF injections, 40 μL orbital blood samples were obtained from each animal 1 hour before growth factor injections. White blood cells (WBC) were counted by a Coulter counter (Coulter, Hialeah, FL) and differential counts were made on 200 white cells from Wright-Giemsa stained blood smears. On days 0, 4, 11, and 18 of Epo injections, WBC counts, reticulocyte counts, and hematocrit values were determined from orbital blood samples.

**Hematopoietic cellularity and progenitors.** Bone marrow cells were extracted by grinding the whole femur; spleen cells were obtained by teasing the whole organ, and the cellularity and differential counts of nucleated cells were obtained as described.10 The number and the type of progenitors (colony-forming unit [CFU]) in these tissues were determined by analysis of colony formation in semisolid agar culture system.10 A conditioned medium of CE mammary carcinoma that contains G-CSF and M-CSF was used as a colony stimulator for the in vitro assay. Progenitors were classified into CFU of neutrophils (CFU-N), of macrophages (CFU-M), and of neutrophil-macrophages (CFU-NM) by specific esterase staining of colonies.10

**Serum calcium.** Blood was obtained by the cardiac puncture at the time of killing and serum calcium was measured by atomic absorption spectrophotometry.

**Osteoclast-endosteum ratio (OER).** A femur from each animal was cleaned and the femoral neck and the greater trochanter were removed. Bones were fixed in 10% neutral-buffered formalin, decalcified in 5% tetrasodium ethylenediamine tetra-acetic acid (pH 7.4), dehydrated in ethanol, and embedded in plastic medium ensuring that the plane of the section would be randomly oriented to the longitudinal axis of the femur. Two longitudinal sections through the center of the femur, each being 3 μm thick and at least 50 μm apart from each other, were sectioned using a microtome. Sections were stained to demonstrate the tartrate-resistant acid phosphatase (TRAP) property of osteoclasts and counter stained with methylene blue.11 By this method, the osteoclasts lining the endosteum were easily identified by their bright red color. Sections were examined under the microscope using a micrometer reticle (10 mm² divided into 0.5 mm²), using a modification of the method previously described.12 The number of intersections of the reticle grid with the endosteum lined by osteoclasts (A) or the endosteum without osteoclasts (B) were scored for the full length of the femoral diaphysis. The OER, (A)/(A + B), was expressed in percentages.

**Medullary and bone area measurements.** Both tibias from each animal were used to prepare ground bone sections.1 Briefly, transverse sections (375 μm thick) were cut perpendicular to its long axis at the fibula-tibia junction using an Isomet low speed saw and Isocut wafering blade (No 11-4263; Buehler Ltd, Lake Bluff, IL). Sections were further ground to 100 μm thick and mounted on slides. Using a microscope equipped with a drawing tube, the image of the cross section was traced onto a digitized tablet, and the total cross sectional area (TA) and the medullary area (MA) were measured using a Sigmascan computer program. The bone area (BA) was TA – MA. The thickness of the cortical bone (BT) was calculated by the formula: $BT = R - r$, where the outer radius $R = \sqrt{TA/\pi}$ and the inner radius $r = \sqrt{MA/\pi}$. The bone forming surface was identified by tetracycline fluorescence.1

**Statistical analysis.** Means and SD were obtained for each experimental and corresponding control group of mice. Student’s t-test was used for comparison of means.

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**RESULTS**

**Blood cells.** Daily subcutaneous injections of recombinant G-CSF into normal mice induced marked granulocytosis in which blood neutrophil counts reached greater than 30,000/μL, approximately 20 times that of controls on day 21 (Fig 1). No significant changes were observed in the number of other blood leukocytes, including blood monocytes. This degree of neutrophilia is similar to that induced by CE mammary tumor in mice,1,2,10,12,13 and confirms the potent neutrophilic effects of mG-CSF in vivo. Although there is great degree of homology between human and murine G-CSF by in vitro bioassays,14,15 the same dose of recombinant hG-CSF induced much less neutrophilia with the highest level reaching approximately 10,000/μL on day 21 (Fig 1).

**Granulopoiesis.** In Table 1, the bone marrow changes induced by 3-week injections of recombinant mg-CSF are summarized. Twice as many granulocytes were present in the femurs of G-CSF-injected mice. This increase was primarily attributed to a threefold increase in the number of postmitotic neutrophilic granulocytes. Lymphocytes and erythroblasts were greatly decreased in numbers. The number of total myeloid progenitors (CFU-total) per femur was significantly (P < .005) increased in the G-CSF group of mice. Among these progenitors, a significant (P < .01) increment was noted in CFU-N, but not in CFU-M, indicating preferential stimulation of neutrophilic granulocyte lineage by G-CSF injections. These bone marrow data were similar to our previous studies in CE mammary tumor-induced neutrophilia,10,11 and confirmed a profound marrow granulopoietic effect of mg-CSF in vivo. In the bone marrow of hG-CSF–treated mice, similar but less conspicuous cellular changes were observed (data not shown). There was marked splenomegaly with extensive extramedullary hematopoiesis with increased numbers of progenitors of all cell lines in both murine and human G-CSF–treated animals. Under extended stimulation of marrow granulopoiesis, there was histologic evidence of

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Fig 1. Effect of daily injections of recombinant murine or human G-CSF on blood neutrophil counts in mice. (●) mG-CSF (2.5 μg/d/mouse) (n = 5). (○) hG-CSF (2.5 μg/d/mouse) (n = 4). (△) Control (n = 13). Vertical bars: SEM.
active granulopoietic marrow extending into the fatty marrow of the peripheral appendicular skeleton, a phenomenon we observed previously in augmented granulopoiesis in CE tumor-bearing mice. 

**Bone parameters.** The endosteal lining of the femur in G-CSF–treated mice was apposed with many TRAP-positive mononuclear and multinuclear cells of various sizes (Fig 2C and D). As shown in Table 2, column A, the OER of femoral bones of mG-CSF–treated mice was significantly (P < .005) higher than that of the controls, suggesting an increased endosteal bone resorption in G-CSF–treated mice. Typical examples of tibia sections of a G-CSF–treated and a control mouse are shown in Fig 3. The medullary area of mG-CSF–treated mice was significantly (P < .005) larger than that of the controls (Table 2, column A). As the medullary area enlarged, the total cross-sectional area also increased. This enlargement was most likely due to periosteal bone formation, as was evidenced by the presence of a rim of tetracycline fluorescence along the periosteum in both experimental and control animals. Although no statistically significant difference was noted in the bone area between the control and G-CSF–treated mice, the bone thickness was significantly (P < .05) reduced in mG-CSF–injected mice (Table 2, column A). Bone changes in hG-CSF–treated mice showed a similar changes as those seen in mG-CSF–treatment (Table 2, column A). These findings suggested increased endosteal bone resorption coupled with periosteal bone formation in mice that received long-term G-CSF treatment. Serum calcium levels were 8.62 ± 0.15 mg% for G-CSF–treated mice and 8.90 ± 0.33 mg% for control mice; thus, G-CSF–induced bone changes were not associated with hypercalcemia.

To delineate granulopoietic and bone modulating changes observed in G-CSF–treated animals, we examined bones of mice in which hematopoiesis was stimulated by growth factors that are known to stimulate a different marrow cell population than that affected by G-CSF. Consistent with findings by others, 3-week injections of recombinant mGM-CSF into normal mice caused no significant changes in blood cell counts; however, the number of peritoneal macrophages increased to approximately 10 times that of the control animals, indicating the factor was indeed effective in vivo. In contrast to G-CSF treatment, but consistent with the observation of others, differential counts of bone marrow cells as well as the number of bone marrow progenitors of GM-CSF–treated mice were not significantly different from controls (data not shown). Nonetheless, we observed significant increase in the OER and the medullary area with the reduction in the bone thickness in GM-CSF–treated animal group when these values were compared with those in the corresponding control group (Table 2, column B). As expected, Epo injections increased the hematocrit of mice to 67.0% ± 2.7% on day 18 (control: 49.3% ± 1.2%), similar to previously reported values. A modest increase in bone leukocyte counts to 9,400 ± 2,167/μL without changes in differential counts was observed in Epo-injected mice. Marked erythropoietic responses were observed in the bone marrow and spleen of Epo-injected mice. As shown in Table 2, column C, bone parameters of Epo-injected mice also showed significantly increased OER associated with enlargement of medullary area. Other parameters showed a similar trend as for those mice with G-CSF treatment, but values were not statistically significant. In contrast to G-CSF–treated mice, infiltration of active granulopoietic marrow into the fatty marrow of metatarsal bones was not obvious in GM-CSF– or Epo-treated mice. Neither of the bone changes induced by GM-CSF or Epo injections were associated with hypercalcemia. There were no significant differences in the body weight of animals between experimental and control groups for any growth factors.

**DISCUSSION**

By quantitative and kinetic studies of neutrophils in mice, we have previously shown that the marked neutrophilia of CE tumor-bearing mice was due to a marked increase in the production rate of neutrophils from the bone marrow. In these animals, the increased neutrophil production was due to a significant increase in the postmitotic pool of marrow granulocytes, accelerated maturation of neutrophils, augmentation of committed neutrophilic progenitors, and expansion of granulopoietic marrow throughout the skeleton. Our studies here have demonstrated that long-term injections of mG-CSF, which is now known to be produced from the tumor, essentially reproduce the bone and bone marrow pictures of CE tumor-bearing mice and confirmed that this factor is primarily responsible for the marked granulocytosis observed in CE tumor-bearing mice. Our studies have also documented selective stimulation of neutrophilic committed progenitors by G-CSF in vivo; a finding that is consistent with a known function of G-CSF established by in vitro assays. These potent granulopoietic effects of the recombinant mG-CSF of our study are in agreement with a recent report by Chang et al, who observed the development of marked neutrophilia in mice transplanted with hematopoietic cells transfected with a mG-CSF gene. It is interesting to note that they described infiltrating codes of neutrophils in muscle adjacent to sternum marrow. We have also observed such granulocyte infiltration near the sternum in CE tumor-bearing mice (unpublished), which could be the results of extensive granulocyte proliferation and marrow expansion with bone resorption.
Fig 2. (A and B) Histologic sections of the mouse metatarsal bone treated with control diluent (A) or mG-CSF (B) for 21 days. Note extension of active granulopoietic marrow into normally fatty marrow of the metatarsal bone in G-CSF–treated marrow. (Original magnification ×480). (C and D) Histologic sections of the femur stained for TRAP of osteoclasts, taken from control-injected (C) and mG-CSF–treated (D) mice. Note increased TRAP-positive cells along the endosteum (arrows) and irregular endosteal lining in mG-CSF–treated marrow. (Original magnification ×190.)

Skeletal changes in mG-CSF–treated mice were consistent with enhanced endosteal bone resorption. There was a marked increase in the number of endosteal osteoclasts (about 200% of control) and an increase in the medullary area (about 120% of control). The medullary area enlargement was accompanied by an enlargement of the total cross-sectional area indicating periosteal bone formation, and a reduction in the bone thickness. The fact that similar bone changes were also observed in the bone marrow stimulated by GM-CSF, which does not cause granulocytosis but is considered to stimulate more immature myeloid progenitors than those stimulated by G-CSF, or in the bone marrow that was stimulated by Epo, suggests that the observed bone modulation is mediated through a mechanism that is common to all these growth factors, rather than a specific action of G-CSF on bone cells. Such common mechanisms may include activation of macrophages, or mediation of stromal cells in the marrow. In this study, the
Table 2. Bone Parameters in Growth Factor-Treated Mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>A (mG-CSF (5))</th>
<th>B (hG-CSF (4))</th>
<th>C (Control (13))</th>
<th>A (mG-CSF (5))</th>
<th>B (Control (5))</th>
<th>C (hEpo (10))</th>
<th>B (Control (10))</th>
</tr>
</thead>
<tbody>
<tr>
<td>OER (%)</td>
<td>35.9 ± 9.7*</td>
<td>22.1 ± 3.3*</td>
<td>16.1 ± 4.5</td>
<td>47.5 ± 6.0*</td>
<td>30.4 ± 11.3</td>
<td>34.4 ± 10.3*</td>
<td>20.9 ± 8.3</td>
</tr>
<tr>
<td>Medullary area (mm²)</td>
<td>0.280 ± 0.015*</td>
<td>0.279 ± 0.016*</td>
<td>0.230 ± 0.023</td>
<td>0.266 ± 0.026#</td>
<td>0.232 ± 0.015</td>
<td>0.252 ± 0.023#</td>
<td>0.230 ± 0.019</td>
</tr>
<tr>
<td>Total area (mm²)</td>
<td>0.804 ± 0.038#</td>
<td>0.801 ± 0.038#</td>
<td>0.766 ± 0.030</td>
<td>0.806 ± 0.024</td>
<td>0.800 ± 0.019</td>
<td>0.833 ± 0.053</td>
<td>0.810 ± 0.048</td>
</tr>
<tr>
<td>Bone area (mm²)</td>
<td>0.524 ± 0.026</td>
<td>0.522 ± 0.046</td>
<td>0.536 ± 0.039</td>
<td>0.539 ± 0.012#</td>
<td>0.567 ± 0.023</td>
<td>0.580 ± 0.042</td>
<td>0.580 ± 0.037</td>
</tr>
<tr>
<td>Bone thickness (mm)</td>
<td>0.207 ± 0.008#</td>
<td>0.207 ± 0.020#</td>
<td>0.224 ± 0.018</td>
<td>0.212 ± 0.008#</td>
<td>0.233 ± 0.010</td>
<td>0.232 ± 0.013</td>
<td>0.237 ± 0.011</td>
</tr>
</tbody>
</table>

Values are means ± SD.
The number in parentheses indicates the number of mice for each group.
*P < .005 when compared with respective controls.
#P < .01 when compared with respective controls.
†P < .05 when compared with respective controls.

amount of recombinant mG-CSF and GM-CSF we could obtain was limited and, therefore, limited the number of experimental animals. However, in addition to data shown in Table 2, we also tested the effect of mG-CSF on another mouse strain, Balb/c × CE, and obtained similar findings. It should be mentioned that bone parameters, particularly the OER of control groups, vary for each growth factor experiment. These variations could be attributed to different diluents and injection schedules used for each growth factor to obtain maximum responses; however, when the experimental values are compared with respective control values, data are consistent; all indicating increased bone resorption.

The osteoclast, the major cell type responsible for bone resorption, is now believed to be derived from the hematopoietic stem cell population and hematopoietic growth factors have been implicated in controlling osteoclast development. It is conceivable that activation of stem cells by persistently high levels of hematopoietic growth factors might result in triggering as yet unknown endogenous mechanisms to activate osteoclasts, or activate primitive hematopoietic progenitors that can give rise to osteoclasts as well as hematopoietic cells. The receptor for mG-CSF has been detected on granulocytic cells and in leukemic cells, but not on other tissues. However, several in vitro studies have shown the proliferative effect of G-CSF or GM-CSF on a variety of nonhematopoietic cells including osteogenic sarcoma cells, marrow stromal fibroblasts, and endothelial cells. The implication of these in vitro studies of CSFs in an in vivo situation is unknown.

The fact that bone changes induced by mG-CSF injections were not associated with hypercalcemia, contrary to our previous observation in CE mammary tumor-bearing mice, suggests additional mechanisms for the CE tumor-induced hypercalcemia. M-CSF is another factor that is produced from the CE mammary tumor. Recently, Yoshida et al have reported that osteopetrotic (op/op) mouse fibroblasts are defective in producing functional M-CSF, and suggested a role of this factor in normal osteoclast development. In our previous studies, transplantation of a M-CSF-producing Bc66 tumor did not cause blood or marrow changes nor hypercalcemia. Moreover, bone parameters of Bc66 tumor-bearing mice did not show any significant changes when compared with sham operated controls (unpublished observation). However, these negative results do not exclude the possibility that M-CSF plays a role in osteoclast development as a local factor in the marrow.

Recombinant hG-CSF and GM-CSF have been clinically

![Fig 3. Transverse ground bone sections of tibia after 21 days of mG-CSF or control injections. (A) Tibia of a control mouse. (B) Tibia of a mG-CSF–injected mouse. (Original magnification ×50. )](image_url)
used for various myelogenous marrow disorders. Although no obvious side effects have been observed in clinical use of these factors, mild to moderate bone pain is the most common complaint of patients receiving CSF therapy. Admittedly, the dose of G-CSF in our study was chosen to maintain neutrophilia in mice for a long period and was considerably higher than that of hG-CSF used in patients; however, one should be aware of the bone modulating effect of these growth factors as a possible deleterious consequence of long-term treatment in humans. A similar situation may be seen in the case of thalassemia, in which chronic stimulation of erythropoietic tissue and the resulting expansion of marrow cavity can impair skeletal function.

In summary, G-CSF is a potent granulopoietic agent in vivo. Sustained stimulation of hematopoiesis by long-term administration of recombinant G-CSF, GM-CSF, or Epo not only induces hematopoietic cellular changes characteristic for their respective cell lineages, but also causes enhanced bone remodeling. The fact that such skeletal changes can be induced by pure hematopoietic growth factor injections strongly suggests that hematopoietic activity and bone remodeling process are physiologically related events. These observations may have important clinical implications.

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Bone modulation in sustained hematopoietic stimulation in mice

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