

## **The development of bone changes induced in rats by recombinant human granulocyte colony-stimulating factor is suppressed by bisphosphonate**

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**Summary.** We have previously demonstrated that high doses of recombinant human granulocyte colony-stimulating factor (rhG-CSF) induce bone changes characterized by osteoclastic bone resorption and osteogenesis due to intramembranous ossification in rats. In this communication we examined the effects of a pretreatment with 3-amino-1-hydroxypropylidene-1,1-bisphosphonate (AHPPrBP), which is a powerful inhibitor of osteoclastic bone resorption, on bone changes induced by rhG-CSF in order to investigate the relation between osteoclastic bone resorption and osteogenesis. AHPPrBP (5 mg/kg/day) was subcutaneously given to 6-week-old rats for 2 days. From the following day of the final injection of AHPPrBP, rats received a subcutaneous injection of rhG-CSF (1,000 µg/kg/day) for 14 days, and the femur and tibia were evaluated histopathologically. By the analysis of peripheral blood leukocyte counts, spleen weights and bone marrow findings, the pretreatment with AHPPrBP had no effect on the activation of hematopoiesis related to the major pharmacological activity of rhG-CSF. In the rats treated with rhG-CSF alone, accelerated osteoclastic bone resorption and osteogenesis due to intramembranous ossification were observed in the trabeculae of metaphyseal spongiosa. The accelerated osteoclastic bone resorption induced by rhG-CSF was suppressed by the pharmacological activity of AHPPrBP. Furthermore, the osteogenesis induced by rhG-CSF was also suppressed by AHPPrBP. These results suggest that the osteogenesis induced by rhG-CSF is a sequential reaction of accelerated osteoclastic bone resorption, and moreover that the main action of rhG-CSF on bone is an acceleration of osteoclastic bone resorption.

**Key words:** Granulocyte colony-stimulating factor, Osteoclastic bone resorption, Osteogenesis, Intramembranous ossification, Bisphosphonate

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### **Introduction**

A large number of studies have suggested that colony-stimulating factors (CSFs) regulate the proliferation and differentiation of hematopoietic progenitor cells and that granulocyte CSF (G-CSF) acts on the granulocyte lineage and enhances some functions of mature granulocytes (Demetri and Griffin, 1991).

Recently, accelerated osteoclastic bone resorption and/or increased in number of osteoclasts have been reported in rats (Keller and Smalling, 1993; Soshi et al., 1996; Suzuki et al., 1997a-c) and mice (Lee et al., 1991) exogenously administered with recombinant human G-CSF (rhG-CSF), and in human G-CSF-transgenic mice (Takahashi et al., 1996). These observations suggest that G-CSF plays a role in the process not only of hematopoiesis but also of bone modulation. Furthermore, Keller and Smalling (1993) and the authors (Suzuki et al., 1997a-c) have demonstrated that osteogenesis due to intramembranous ossification was also observed in rats exogenously administered with rhG-CSF. In addition, we reported previously that bone resorption was observed in almost all lesions, and some of which accompanied osteogenesis. Lesions showing osteogenesis alone were observed in only a few areas (Suzuki et al., 1997b,c). This result suggests that the osteogenesis due to intramembranous ossification might be a coupling phenomenon after bone resorption. However, the relation between bone resorption and osteogenesis in bone changes induced by rhG-CSF is still obscure, and the role of G-CSF in bone modulation is poorly understood.

The present experiment was therefore undertaken to determine whether the rhG-CSF-induced osteogenesis is dependent upon bone resorption by testing the effects of bisphosphonate, which has a high affinity for hydroxyapatite crystals and is a powerful inhibitor of osteoclastic bone resorption (Boonekamp et al., 1986; Fleisch, 1987; Lowik et al., 1988; Flanagan and Chambers, 1989), on the rhG-CSF-induced bone changes.

## *Bone changes induced in rats by rhG-CSF*

### **Materials and methods**

#### *Animals*

Twenty male Sprague-Dawley rats were purchased from Japan SLC Inc. (Shizuoka, Japan) and subjected to the experiments at the age of 6 weeks. The animals were housed in cages (5/cage) in an animal room maintained at a temperature of  $23\pm 2$  °C, a relative humidity of  $55\pm 10\%$  and a 14-hour light and 10-hour dark cycle, and they were fed pelleted chow (MF; Oriental Yeast Co., Ltd., Tokyo) and tap water ad libitum.

#### *Injection of rhG-CSF and bisphosphonate*

Recombinant human G-CSF (250  $\mu\text{g}/\text{ml}$ ) produced by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan) was used for this study. Bisphosphonate (3-amino-1-hydroxypropylidene-1,1-bisphosphonate, AHPPrBP) was purchased from Ciba-Geigy Pharmaceutical Co. (Basel, Switzerland), and was dissolved in saline to a final concentration of 1.25 mg/ml.

AHPPrBP was subcutaneously injected into rats (over the upper back) at the dose level of 5 mg/kg once a day for 2 days. From the following day of the final injection of AHPPrBP, rats received subcutaneous injection of rhG-CSF at the dose level of 1,000  $\mu\text{g}/\text{kg}$  once a day for 14 days.

#### *Study design*

The rats were divided into 4 groups of 5 animals each, receiving: 1) saline for 16 days (totally negative control), 2) rhG-CSF after 2-days saline treatment (rhG-CSF-treated group), 3) saline for 14 days after AHPPrBP treatment (AHPPrBP-treated group), and 4) rhG-CSF after AHPPrBP treatment (rhG-CSF+AHPPrBP-treated group). All animals were weighed on days 0 (first dosing day), 2, 5, 9, 12 and 16, and the doses of rhG-CSF and AHPPrBP were weight-adjusted. During the injection period, clinical signs of all animals were observed every day. On the day following the final injection, all animals were sacrificed by exsanguination under ether anesthesia. Before necropsy, peripheral blood leukocyte (white blood cell (WBC)) counts were measured in blood samples obtained from the abdominal aorta using a multiparameter auto-cell counter (electrical resistance detection, CC-780; Toa Medical Electronic Co. Ltd., Hyogo, Japan). Furthermore, serum samples were obtained from the abdominal aorta, and calcium (Ca) and inorganic phosphorus (IP) were measured using the o-cresolphthalein complexon and modified Taussky method, respectively.

#### *Bone histopathology*

At necropsy, the femur (distal metaphysis), tibia (proximal metaphysis), and spleen were removed from each animal, and the spleen was weighed. The bones

were fixed in 10% neutral-buffered formalin and embedded in paraffin after ethylenediaminetetraacetic acid (EDTA) decalcification. Longitudinal thin sections were made and stained with hematoxylin and eosin (HE).

For enzyme histochemical study, the tibia (proximal metaphysis) was fixed in periodate-lysine-paraformaldehyde fixative (containing 4% paraformaldehyde) (McLean and Nakane, 1974) overnight at 4 °C. The specimens were decalcified in EDTA-glycerol solution (Mori et al., 1988) for 5 weeks at -5 °C, and embedded in paraffin by AMeX methods (Sato et al., 1986). Sections of the specimens were deparaffinized in xylene and acetone, and enzyme histochemically stained. The tartrate-resistant acid phosphatase (TRAP) activity was detected according to the procedure using naphthol AS-BI phosphate (Sigma Chemical Co., St. Louis, MO) as substrate and hexazotized pararosaniline (Sigma Chemical Co.) as coupler, and the substrate solution was completed by adding 100 mM L(+)-tartaric acid (Barka and Anderson, 1962).

#### *Statistical analysis*

Group mean values and standard deviations were calculated for body weight, WBC counts, serum Ca, serum IP and spleen weight. These data were analyzed for statistical significance of any differences between the control group and each drug-treated group, using Student's or Welch's t-test at levels of 1 and 5%.

### **Results**

#### *Clinical signs and body weights*

No abnormal clinical signs were observed in any group. Figure 1 illustrates the body weights. The body weights of the rhG-CSF- and the AHPPrBP-treated groups were judged not to be different from those of the control group. The body weight at day 16 was significantly lower in the rhG-CSF+AHPPrBP-treated group than in the control group.

#### *WBC counts, spleen weights and bone marrow findings*

Marked increase in WBC counts and prominent splenomegaly were observed in the rhG-CSF- and the rhG-CSF+AHPPrBP-treated groups (Table 1). In addition, no significant difference was observed in the WBC counts and spleen weight between the rhG-CSF-treated and the rhG-CSF+AHPPrBP-treated groups. In the bone marrow, an increase of granulopoietic cells was observed in the rhG-CSF- and the rhG-CSF+AHPPrBP-treated groups, and marrow cavities were fully occupied by hematopoietic cells.

#### *Serum Ca and IP values*

No significant changes in serum Ca and IP levels

## Bone changes induced in rats by rhG-CSF

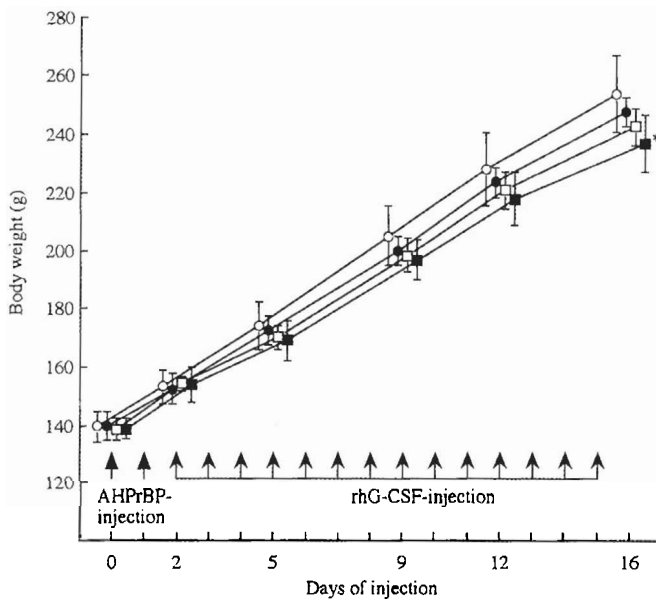
were observed in all groups (Table 1).

### Histopathological nature of bone changes

The histopathological nature of bone changes is summarized in Table 2.

In the rhG-CSF-treated group, bone changes characterized by accelerated osteoclastic bone resorption and osteogenesis due to intramembranous ossification were observed in the metaphyseal spongiosa of femur and tibia (Figs. 2b, 3-5). The changes were distributed in the area from the secondary spongiosa to the interface of primary spongiosa and secondary spongiosa. In the spongy trabeculae, a large number of osteoclasts and bone resorption bays (Howship's lacunae) were observed on the bone surface (Fig. 3). The medullary cavity around or beside resorptive bones was replaced by mesenchymal tissues composed of various types of stromal cells. Numerous osteoclasts and round- to polygonal-shaped mononuclear cells showing TRAP activity (preosteoclast/osteoclast precursor) were also observed on the bone surface and in the mesenchymal tissues (Fig. 4). In addition to the aforementioned findings, newly formed bones showing irregular shape, and woven bone structures that had no spicules of calcified cartilage were observed in and/or near the areas replaced by mesenchymal tissues (Fig. 5). These bones were filled with osteoid osteocytes, and their surfaces were covered with mono- or multilayered formative osteoblasts.

In the AHPPrBP-treated group, the amount of trabeculae in the metaphysis was greatly increased over



**Fig. 1.** Body weights of rats injected with saline (control; white circle), 1,000  $\mu\text{g}/\text{kg}$  of rhG-CSF (black circle), 5 mg/kg of AHPPrBP (white square), and both 1,000  $\mu\text{g}/\text{kg}$  of rhG-CSF and 5 mg/kg of AHPPrBP (black square). Each value represents the mean  $\pm$  SD ( $n=5$ ). \*: Significantly different from the control ( $p<0.05$ ).

controls, and the area of spongiosa extend further towards the diaphysal region (Fig. 2c). Spicules of calcified cartilage were observed in the middle of the trabeculae (Fig. 6a). On the trabecular bone surfaces, a large number of osteoclasts were observed (Fig. 6a, 7a), whereas ruffled border of osteoclasts and bone resorption bays on bone surface were not found.

In the rhG-CSF+AHPPrBP-treated group, histopathological findings of bone were the same as those of the AHPPrBP-treated group (Fig. 2d, 6b, 7b). Namely, trabeculae with spicules of calcified cartilage were more numerous in the metaphysis, and extended further towards the diaphysal region. On the trabecular bone surfaces, a large number of osteoclasts without ruffled border were observed, and bone resorption bays were not found.

### Discussion

Recently, we have demonstrated that bone changes characterized by accelerated osteoclastic bone resorption and osteogenesis due to intramembranous ossification were observed in rats administered with 100 and 1,000  $\mu\text{g}/\text{kg}/\text{day}$  of rhG-CSF for 28 days (Suzuki et al., 1997b). By the analysis of incidence and distribution of bone resorption and osteogenesis induced by rhG-CSF, it was suggested that osteogenesis might be a coupling phenomenon after bone resorption (Suzuki et al., 1997b). It has been established that bone resorption and osteogenesis are sequential processes. Bone resorption occurs first, and osteogenesis occurs after bone resorption. These activities are coupling, such that under normal circumstances increased bone resorption is followed by increased osteogenesis, and suppression of bone resorption suppresses osteogenesis (Jaworski, 1984). In addition, suppression of bone resorption by calcitonin, estrogen, and bisphosphonates causes a decrease in osteogenesis, and increased bone resorption, as seen after ovariectomy, causes an increase in osteogenesis (Wronski et al., 1988a,b, 1991; Hayashi et al., 1989; Seedor et al., 1991; Stein et al., 1991). If the osteogenesis induced by rhG-CSF is coupled to increased bone resorption, osteogenesis will be suppressed by suppression of bone resorption. Thus, we examined the effects of pretreatment of AHPPrBP on bone changes induced by rhG-CSF.

**Table 1.** Peripheral blood leukocyte counts, blood biochemical values, and spleen weight of rats injected with rhG-CSF and AHPPrBP.

GROUP	WBC ( $\times 10^3/\mu\text{l}$ )	CA (mg/dl)	IP (mg/dl)	SPLEEN (mg)
Control	6.7 $\pm$ 0.8	10.49 $\pm$ 0.13	9.67 $\pm$ 0.52	694 $\pm$ 114
rhG-CSF	49.4 $\pm$ 8.7*	10.29 $\pm$ 0.37	9.32 $\pm$ 1.00	2,121 $\pm$ 184*
AHPPrBP	6.9 $\pm$ 1.2	10.44 $\pm$ 0.49	8.58 $\pm$ 0.72	605 $\pm$ 51
rhG-CSF + AHPPrBP	60.9 $\pm$ 14.9*	10.18 $\pm$ 0.34	8.94 $\pm$ 0.70	2,230 $\pm$ 297*

Each value represents the mean  $\pm$  SD ( $n=5$ ). \*: significantly different from the control ( $p<0.01$ ).

### Bone changes induced in rats by rhG-CSF

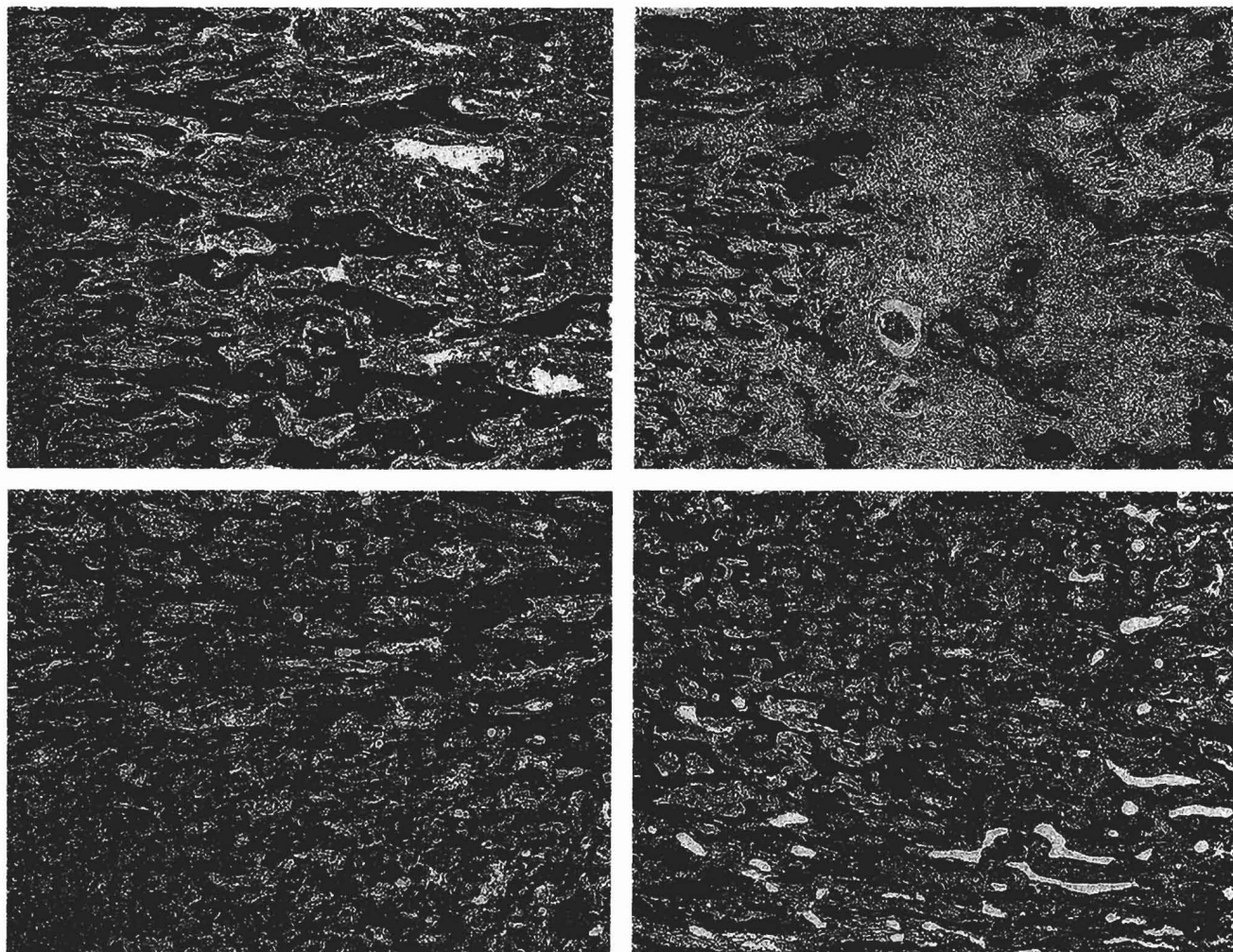
In the present study, rhG-CSF was given to rats for 14 days at dose level of 1,000  $\mu\text{g}/\text{kg}$ . Marked increase in number of WBC counts, prominent splenomegaly with extensive extramedullary hematopoiesis (Keller and Smalling, 1993) and granulocytic hypercellularity of bone marrow, which are related to the major pharmaco-

logical activity of rhG-CSF (Demetri and Griffin, 1991; Keller and Smalling, 1993), were observed in the rhG-CSF- and rhG-CSF+AHPPrBP-treated group. There were no differences in the aforementioned findings between the rhG-CSF-treated group and the rhG-CSF+AHPPrBP-treated one. These results indicated that the pretreatment

**Table 2.** Histopathological findings of bone in rats injected with rhG-CSF and AHPPrBP

FINDINGS	CONTROL		rhG-CSF		AHPPrBP		rhG-CSF + AHPPrBP	
	Femur	Tibia	Femur	Tibia	Femur	Tibia	Femur	Tibia
rhG-CSF-related changes								
Accelerated osteoclastic bone resorption	0*	0	4	4	0	0	0	0
Osteogenesis due to intramembranous ossification	0	0	3	3	0	0	0	0
AHPPrBP-related change								
Increase in metaphyseal spongy trabeculae	0	0	0	0	5	5	5	5

Each group is composed of 5 rats. \*: number of rats with changes.



**Fig. 2.** Femur sections of rats injected with saline (a; control), 1,000  $\mu\text{g}/\text{kg}$  of rhG-CSF (b), 5 mg/kg of AHPPrBP (c), and both 1,000  $\mu\text{g}/\text{kg}$  of rhG-CSF and 5 mg/kg of AHPPrBP (d). b. Bone changes involving the replacement of medullary cavity by mesenchymal tissues are observed in the metaphysis. c and d. The amount of trabecular in the metaphysis is greatly increased. HE, x 40.

### Bone changes induced in rats by rhG-CSF

with AHPPrBP did not affect the major pharmacological activity of rhG-CSF.

In the AHPPrBP-treated group, the amount of trabeculae in the metaphysis was greatly increased. The fact that spicules of calcified cartilage were observed in the trabeculae indicated that the trabeculae occupying the metaphysis were cartilage bone due to enchondral ossification from growth plate. On the bone surfaces, an increase in number of osteoclasts was observed, whereas ruffled border of osteoclasts and bone resorption bays on bone surface were not found. These findings suggested that osteoclastic bone resorption was suppressed, and then, unresorbed enchondral bone (also called cartilage bone) trabeculae occupied the metaphysis. Therefore, it was confirmed that AHPPrBP exhibited its effect of resorption-suppression in this study.

In the rhG-CSF-treated group, bone changes

characterized by accelerated osteoclastic bone resorption and osteogenesis due to intramembranous ossification were observed. The histopathological nature of bone changes was similar to that in our previous studies (Suzuki et al., 1997b,c). In the rhG-CSF+AHPPrBP-treated group, histopathological findings of bone were the same as those in the AHPPrBP-treated group. The increase in number of osteoclasts was observed in the rhG-CSF- and rhG-CSF+AHPPrBP-treated groups. It is known that, in growing rats treated with AHPPrBP, the increase in number of osteoclasts is a secondary reaction occurring after the increase in amount of bone tissues due to suppressed osteoclastic bone resorption (Miller and Jee, 1975, 1977, 1979; Reitsma et al., 1980; Flanagan and Chambers, 1989). It is considered that the increase in number of osteoclasts in the rhG-CSF+AHPPrBP-treated group involved both the effect of rhG-CSF and the secondary reaction of AHPPrBP-treatment.

In the rhG-CSF+AHPPrBP-treated group, the accelerated osteoclastic bone resorption induced by rhG-CSF was suppressed by the pharmacological activity of AHPPrBP. Furthermore, the osteogenesis due to intramembranous ossification induced by rhG-CSF was also suppressed by pretreatment with AHPPrBP. These results suggest that the osteogenesis induced by rhG-CSF is dependent on the presence of a coupling mechanism, and moreover that the osteogenesis is a sequential reaction of accelerated osteoclastic bone resorption induced by rhG-CSF. The main action of rhG-CSF on bone, therefore, seems to be the acceleration of osteoclastic bone resorption.

It has been established that osteoclasts are derived from hematopoietic stem cell population and that the CSFs, especially macrophage CSF, regulate the

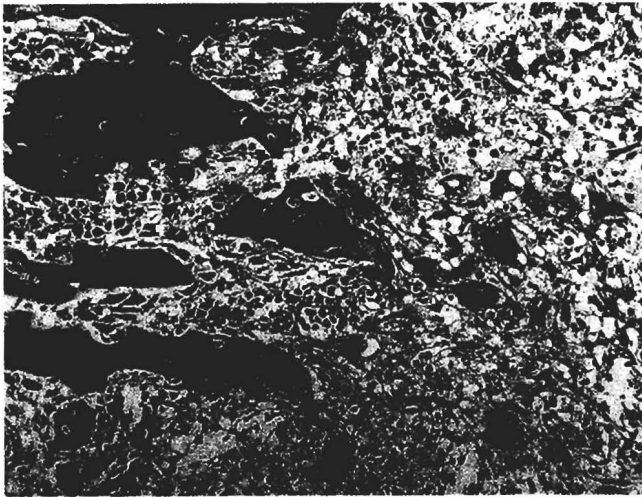


Fig. 3. Femur section of a rat injected with rhG-CSF. In the trabeculae, a large number of osteoclasts and resorptive bays (Howship's lacunae) are observed. HE, x 160

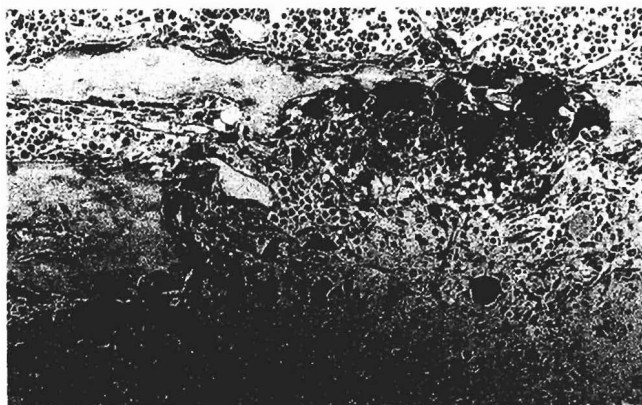


Fig. 4. Tibia section of a rat injected with rhG-CSF. TRAP-positive osteoclasts and mononuclear cells are observed on the bone surface and in the mesenchymal tissues. x 125

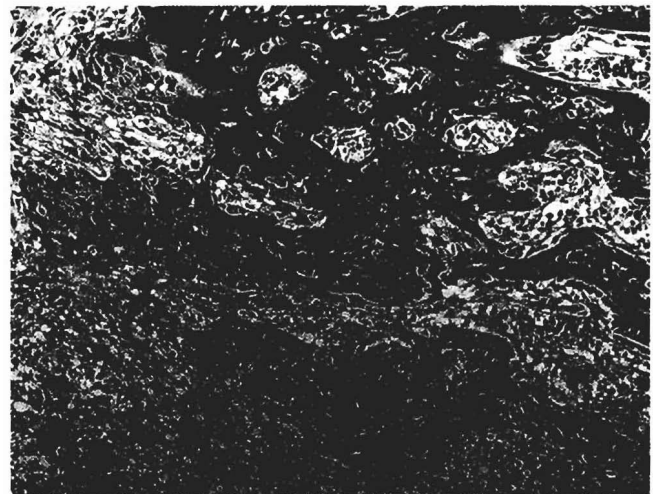


Fig. 5. Femur section of a rat injected with rhG-CSF. Irregular-shaped woven bones are seen near the area replaced by mesenchymal tissues. The bones are filled with osteoid osteocytes, and their surfaces are covered with formative osteoblasts. HE, x 125

## Bone changes induced in rats by rhG-CSF

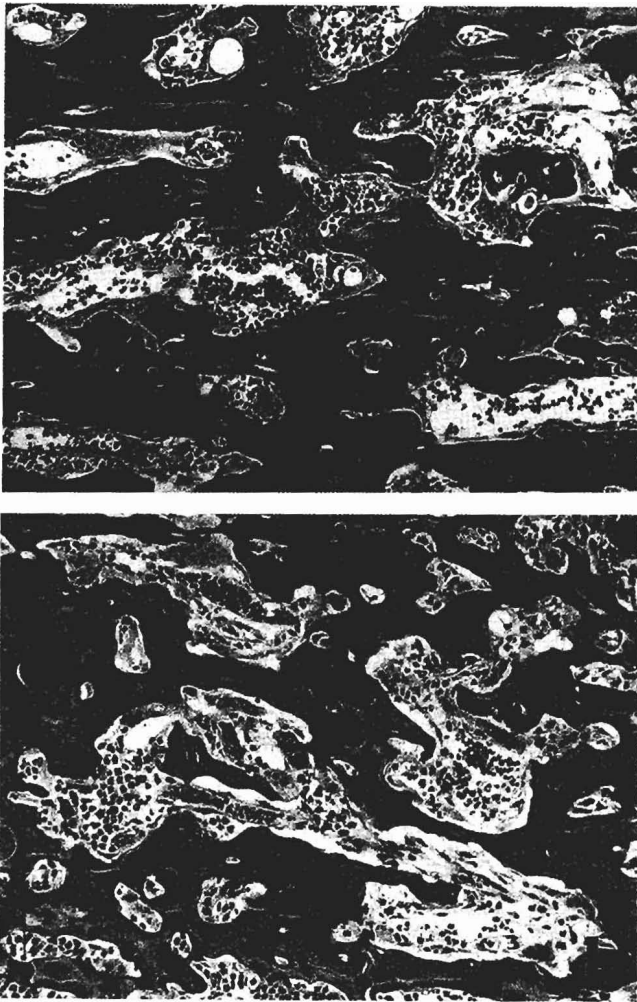


Fig. 6. Femur sections of rats injected with AHPrBP and rhG-CSF. Large amount of trabecular with spicules of calcified cartilage are observed in rats injected with AHPrBP alone (a), and both rhG-CSF and AHPrBP (b). HE, x 160

proliferation and differentiation of osteoclast progenitors (Kodama et al., 1993; Hofstetter et al., 1996; Suda et al., 1996; Odgren et al., 1997). At present, the role of G-CSF in osteoclastogenesis is poorly understood. However, it has been reported that G-CSF weakly stimulated growth of osteoclast progenitors in vitro (Takahashi et al., 1991). In addition, we have previously reported that the development of bone changes induced by rhG-CSF is closely related to the bone marrow hematopoietic activity and that the changes take place in the bone with active hematopoietic marrow (Suzuki et al., 1997a). Therefore, it is conceivable that high levels of rhG-CSF might result in stimulation of the proliferation and differentiation of osteoclast progenitors as well as hematopoietic progenitors.

In addition, it has been shown that G-CSF affects not only hematopoietic cells but also stromal cells

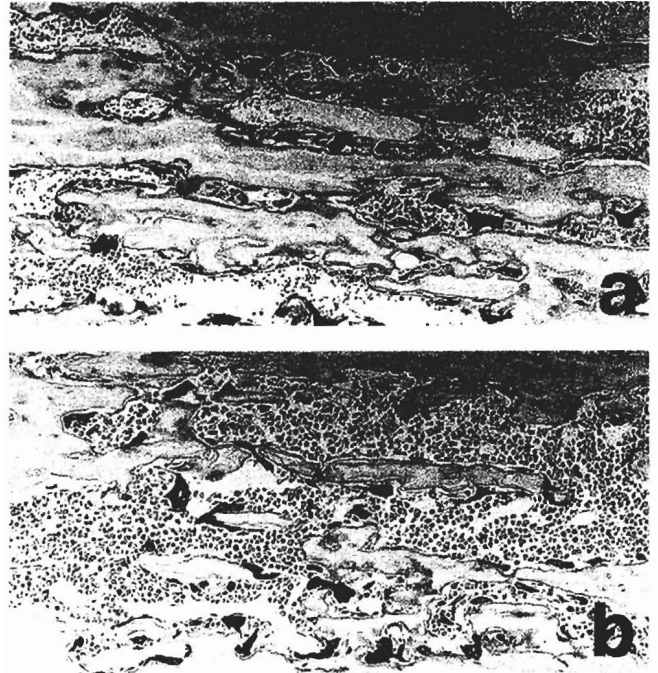


Fig. 7. Tibia sections of rats injected with AHPrBP and rhG-CSF. A large number of TRAP-positive osteoclasts are observed in rats injected with AHPrBP alone (a), and both rhG-CSF and AHPrBP (b). x 100

(Bussolino et al., 1989, 1994; Fukushima et al., 1992, 1994). These cells, which are components of marrow microenvironment, play an important role in modulating the hematopoiesis and the development of osteoclasts (Kukita et al., 1993; Shevde et al., 1994; Takahashi et al., 1995; Suda et al., 1996). It is possible that high levels of rhG-CSF may affect marrow microenvironment and bring about not only hematopoiesis but also osteoclast development. Now, further study on the mechanisms of rhG-CSF-induced bone changes is in progress.

*Acknowledgments.* We thank Ms. Masako Un-no, Ms. Yukiko Tabata, Ms. Yayoi Karasawa, Ms. Kyouko Aruga and Ms. Fusami Akahane at Chugai Pharmaceutical Co. for their skillful technical assistance.

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Accepted October 28, 1998