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Hypercalcemia, Excessive Bone Resorption, and Neutrophilia in Mice Bearing a Mammary Carcinoma (41582)

MINAKO Y. LEE AND DAVID J. BAYLINK[1]

Department of Medicine, University of Washington School of Medicine, Seattle, Washington 98195

Abstract. In an attempt to gain insight into the relationship between bone marrow and bone tissue, studies of bone metabolism and quantitative analysis of bone structure were carried out in mice following a transplantation of a granulocytosis-inducing mammary carcinoma. With the progression of the tumor growth and development of granulocytosis, there was a sharp increase in plasma calcium and urine calcium, both reaching over 200% of control values. Hypercalcemia was associated with a significant increase in urinary hydroxyproline ($P < 0.005$), an increase in marrow medullary area ($P < 0.05$), and an increase in number of endosteal osteoclasts ($P < 0.005$), together indicating that the cause of hypercalcemia was an increase in bone resorption. In parallel with hypercalcemia and hypercalcuria, there was an increase in urinary cyclic AMP excretion. The removal of the tumor normalized both blood neutrophil counts and plasma calcium levels, suggesting that a humoral agent from the tumor tissue, rather than tumor metastasis to bones, may be responsible for the phenomena. These studies documented the association of excessive bone resorption in this animal model of tumor-induced neutrophilia; the model may prove useful for studies of tumor-associated hypercalcemia as well as studies of marrow and bone interactions.

Mice bearing a transplantable CE mammary carcinoma develop a striking increase in the blood granulocyte counts (1). We have previously demonstrated that this tumor-induced granulocytosis is due to a major expansion of granulocytopoietic bone marrow throughout the skeletal marrow. There is a marked increase in marrow neutrophil production rate and blood neutrophil turnover rate (2). Furthermore, the number of granulocyte–macrophage colony forming cells (CFU-c) nearly double in tumor bearing mice (3). The present study was prompted by the histological observation that the development of marrow neutrophilic hyperplasia is accompanied by the appearance of abundant osteoclasts along the endosteum (4). Since the bone marrow is confined in the cavity surrounded by bone, and the hemopoietic cells not only share the common blood supply with the bone tissue (5) but may also be derived from common precursor cells (6), it may be speculated that the extensive proliferation of the hemopoietic cells could influence bone cells and their metabolism. We therefore have examined bone structure in this model of stimulated granulocytopoiesis in an attempt to gain some insight into the metabolic relationship between these tissues.

Materials and Methods. *Mice.* Approximately 200 (BALB/c × CE)F_1 hybrid mice (bred in the Vivarium, University of Washington, Seattle) of both sexes were used at the age of 14–15 weeks. This hybrid was used because the tumor, CE mammary carcinoma, grew well in this strain, invariably produced marked neutrophilia, and marrow cellular changes were essentially identical to those of CE mice. The parental female BALB/c and male CE mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). At the age of 8–9 weeks, mice were splenectomized in order to confine hemopoiesis to the bone marrow (2). Mice were maintained under standard laboratory conditions and fed a standard diet (Wayne, Mouse Breeder Blox, Allied Mills, Ill.) until the beginning of the experimental protocol, at which time mice were fed a semisynthetic diet as described below.

Tumor cells. We used CE mammary adenocarcinoma, described by Delmonte *et al.* (1) as in our previous studies (2). Tumor cell suspensions in Hanks' balanced salt solution (Gibco) were prepared as described previously (2). Viability of tumor cells was tested with

[1] Present address: Jerry L. Pettis Memorial V. A. Hospital, 11201 Benton Street, Loma Linda, Calif. 92357.

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trypan blue and approximately 5×10^5 viable tumor cells in 0.2 ml of cell suspension were injected subcutaneously into the flank of each mouse.

Experimental protocol. Mice were divided into two groups, control and tumor-bearing groups. Each group consisted of at least eight males and eight females by the end of the experimental period. Since the mortality rate of tumor-bearing animals beyond 14 days after tumor inoculation was usually around 50%, the tumor-bearing group contained twice as many mice at the beginning of the experiment. Mice were placed in metabolic cages, four to five per cage, for collection of urine at various times, bled periodically for blood cell counts and chemical determinations, and received tetracycline injections for labeling of bone forming surfaces (7). They were fed a semisynthetic calcium control diet containing 0.6% calcium and 0.6% phosphorus (8) and given distilled water to drink. In some experiments, mice were fed a calcium-deficient diet containing 0.01% calcium and 0.6% phosphorus (8).

On Day 0, mice in the tumor group were inoculated with CE mammary carcinoma and mice in the control group received a subcutaneous injection of Hanks' balanced salt solution. Mice in both groups then received oxytetracycline (Pfizer, New York, N.Y.), 15 mg/kg of body weight, ip. On Day 5, blood samples were taken from one half of the animals in each group and on Day 8 the other half of the animals were bled. On Day 12 after the blood drawing, all mice received tetracycline (Lederle, Wayne, N.J.), 15 mg/kg, ip. On Day 16, all mice received demeclocycline (Lederle, Wayne, N.J.), 15 mg/kg, ip, and on Day 17, all mice were bled and sacrificed for bone measurements. On occasions when the number of tumor-bearing mice surviving up to Day 17 was in excess of that needed for the particular experiment, the tumor was removed from these extra mice under pentobarbital anesthesia (sodium pentobarbital, 0.06 mg/g body weight) and the subsequent ensuing changes in blood neutrophils and plasma calcium were determined serially by killing the animal on various days following the tumor excision.

Urine collection. Twenty-four hour urine samples were collected per cage on Days 5, 8, 12, and 16. Urine samples were spun at 250*g* at 4°C for 10 min to remove precipitates and stored at −20°C.

Blood drawing. Blood samples were collected from the lateral orbital sinuses by inserting heparinized microcapillary tubes. Approximately 200 μl of blood (about 13% of mouse blood volume) was withdrawn from each mouse at a time. From each tube the first drop of blood was used for leukocyte counts and the remaining blood in the capillary tube was centrifuged to obtain plasma which was pooled by group and stored at 4°C.

Bone area measurement. Quantitative histological measurements of bone substance were made from undecalcified ground-bone sections prepared from the tibio-fibular junction of left hind limbs of mice by modification of the method described for rats by Baylink *et al.* (7). Briefly, after fixation in 10% (v/v) neutral buffered Formalin for 24 hr, each tibia was divided at right angles to its long axis at the fibular junction. Three consecutive 50-μm-thick crosscut sections were prepared using a thin-sectioning machine. The sawed sections were then hand ground to a final thickness of 30 μm and mounted on glass slides. Bone length and area measurements were made from microscopic images, projected onto a digitizing tablet interfaced with a PDP-8 computer (7). Bone parameters measured were total area, medullary area, and medullary surface (7). The bone forming surface was identified by tetracycline fluorescence (7).

Bone and bone marrow histology. Histological sections were prepared from the right femora and the first lumbar vertebrae. These bones were fixed in 10% neutral buffered Formalin for 9 hr at 4°C, decalcified with 5% tetrasodium EDTA in 0.2 *M* phosphate buffer (pH 7.4) for 14 days at 4°C, dehydrated with ascending concentration of ethanol, and embedded in plastic embedding medium (JB-4, Polyscience, Warrington, Pa.). Two micrometer-thick sections were cut and stained for histochemical demonstration of acid phosphatase in osteoclasts (9) and of naphthol-chloroacetate esterase in neutrophilic granulocytes (2, 10). Osteoid was demonstrated according to Goldner's modification of Masson's trichrome staining (11).

Osteoclast enumeration. The number of osteoclasts were counted along the endosteal surface in the diaphyseal region in longitudinal sections of the femur. The results were

expressed as number of osteoclasts per millimeter of endosteal surface. Identification of osteoclasts were assisted by the presence of acid phosphatase activity in the cytoplasm. Enumeration was performed by one observer on duplicate serial sections from four of the control and four of the tumor-bearing mice (17-day tumor). The count on duplicate sections agreed with an error of less than 10%.

Blood cell counts. The blood leukocyte concentration was determined by Coulter counter and 200 leukocytes were scored to obtain differential counts on Wright–Giemsa-stained smears.

Chemical Analysis. Plasma calcium, phosphorus, creatinine, and urine creatinine were measured by standard autoanalyzer methods. Urine calcium was measured by atomic absorption spectroscopy. Urinary hydroxyproline was measured by the method described by Prockop and Udenfriend (12). Urinary cyclic AMP was measured by the competitive protein binding assay of Gilman (13).

Results. The transplanted tumor grew rapidly and reached the size of approximately 2 cm in diameter in 2–3 weeks after transplantation. The tumor growth was localized; metastasis of this tumor was never found in any organ examined. As previously shown (1, 2), with the progression of tumor growth, blood neutrophil counts rose sharply (Fig. 1). There was a significant ($P < 0.005$) elevation of plasma calcium reaching over the 200% of the control value on Day 12. The calcium level then declined slightly (N.S.) on Day 17, but was still significantly higher than the control ($P < 0.01$) (Fig. 1). Hypercalcemia was associated with slight but nonsignificant reduction in plasma phosphorus (Table I). Urinary calcium excretion, corrected for variations in urine creatinine, similarly increased reaching the peak value on Day 12 ($P < 0.005$, compared with the control value). By Day 17, urine calcium excretion declined ($P < 0.005$ when compared with Day 12) but it was still elevated when compared with the Day 17 controls ($P < 0.005$). The blood neutrophil counts and plasma calcium levels returned to near normal within a few days following the excision of the tumor. As the tumor regrew, the neutrophilia and hypercalcemia reappeared as shown in Fig. 1.

As shown in Table I, plasma creatinine was within normal range in tumor-bearing mice.

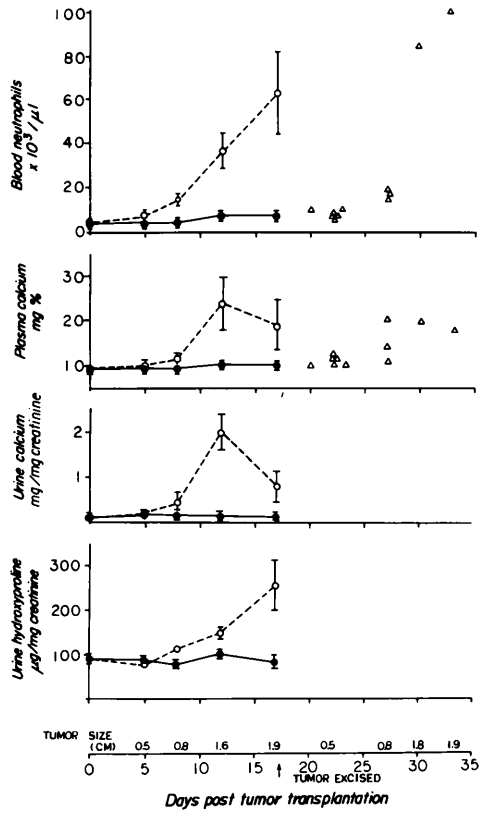

FIG. 1. Blood neutrophil count, plasma calcium, urine calcium, and urine hydroxyproline as a function of time and as a function of tumor size. Closed circles represent control mice and open circles represent tumor-bearing mice. Vertical bars indicate ±1 SD. The tumor was removed at 17 days as indicated by an arrow on abscissa. Triangles indicate values obtained from individual mice killed at various times after removal of the tumor.

The urinary creatinine concentration of tumor-bearing mice was found to be about one-third of the controls. However, there was no difference in the creatinine excretion per day of tumor-bearing mice and control mice, thus eliminating renal failure as a cause of hypercalcemia. Urinary hydroxyproline excretion gradually increased starting on Day 8 and then increased progressively until the end of study on Day 17 at which time it was significantly higher ($P < 0.005$) than the control (Fig. 1, Table I).

To evaluate the possibility that the increased plasma calcium was due to increased intestinal absorption of calcium in tumor-bearing mice, we examined the plasma and urine calcium of mice fed a diet almost cal-

TABLE I. BLOOD AND URINE CHEMICAL ANALYSIS AND BONE MEASUREMENT

Growth and chemical parameters	Control mice	Tumor-bearing mice (14- to 16-day tumor)
Body weight (grams)	29.3 ± 3.51	23.1 ± 3.4
Tumor weight (grams)	—	2.9 ± 1.2
Plasma calcium (mg/100 ml)	9.53 ± 0.32 (16)	21.92 ± 4.77**
Plasma phosphorus (mg/100 ml)	7.2 ± 1.23 (16)	6.28 ± 2.36 (16)
Plasma creatinine (mg/100 ml)	0.46 ± 0.14 (8)	0.53 ± 0.08 (8)
Urine creatinine (mg/100 ml)	64.25 ± 9.00 (4)	22.75 ± 17.0 (4)**
Urine creatinine (mg/24 hr)	0.464 ± 0.092 (4)	0.442 ± 0.057 (4)
Urine hydroxyproline (μg/ml)	26.19 ± 7.22 (24)	51.07 ± 18.04 (19)**
Urine hydroxyproline/creatinine (μg/mg)	81.33 ± 14.18 (24)	302.19 ± 125.47 (19)**
Bone parameters		
Total area (mm^2)	0.949 ± 0.093 (8)	0.978 ± 0.056 (9)
Medullary area (mm^2)	0.199 ± 0.029 (8)	0.240 ± 0.021 (9)*
Endosteal surface (mm)	1.651 ± 0.114 (8)	1.800 ± 0.074 (9)
Osteoclast numbers/mm	1.46 ± 0.32 (4)	14.61 ± 3.63 (4)**

Note. The results shown represent the mean ± 1 SD of the values obtained from the indicated number (n) of samples.

* Significant difference between control and tumor group ($P < 0.05$).

** Significant difference between control and tumor group ($P < 0.005$).

cium-free. Twenty mice were divided into normal and tumor-bearing groups. One-half of each group was fed a diet containing low calcium (0.01% calcium) and the other half was fed a control diet (0.6% calcium). On Day 0, the tumor was transplanted into mice in the tumor group and an injection of Hanks' balanced salt solution was given to mice in the normal group. Mice were kept on this diet program for 14 days, at which time, they were bled for plasma calcium determinations and urine samples were collected. The results of this experiment is summarized in Table II. In the normal group, urinary excretion of calcium was significantly reduced in mice fed a low-calcium diet. Plasma calcium, however, was not lowered by a low-calcium diet, the change was rather in the opposite direction. In tumor-bearing mice, there was no significant difference in plasma or urine calcium levels between control diet and low-calcium diet groups indicating intestinal absorption of calcium was not the major determinant of the hypercalcemia of tumor-bearing mice.

The quantitative measurements of bone area is summarized in Table I. At the sampling site of the tibio-fibular junction, there was a significant enlargement of the medullary area ($P < 0.05$) of tumor-bearing mice compared with control mice. An increased medullary area is due to either decreased bone formation or increased resorption. Serial tet-

TABLE II. EFFECT OF LOW-CALCIUM DIET ON PLASMA AND URINE CALCIUM LEVELS

Mice	Diet	Plasma calcium (mg/100 ml)	Urine calcium (mg/mg creatinine)
Normal	Control	9.36 ± 0.36 (5)	0.55 ± 0.07 (3)
Normal	Low calcium	10.60 ± 0.23 (5)*	0.12 ± 0.03 (3)*
Tumor-bearing	Control	19.30 ± 6.67 (5)**	1.15 ± 0.37 (3)**
Tumor-bearing	Low calcium	18.68 ± 8.99 (5)***	1.31 ± 0.90 (3)***

Note. The results shown represent the mean ± 1 SD of the values obtained from the indicated number (n) of samples.

* $P < 0.005$ by t test as compared with normal mice with control diet.

** $P < 0.005$ by t test as compared with normal mice with control diet.

*** $P < 0.05$ by t test as compared with normal mice with low-calcium diet.

racycline labels given to measure bone formation at the endosteum revealed, in both control and tumor-bearing groups, an absence of tetracycline labels. Subsequent studies revealed that in this strain of mice, endosteal bone formation halts after approximately 5 weeks of age, which explains why our 15-week-old mice had no indication of bone formation at this site. Thus, the observed increase in medullary area could have arisen only through an increase in bone resorption rather than alteration in bone formation. Similarly, when vertebral bone sections were stained for osteoid, there was very little evidence of formation in either the control or the tumor-bearing animals, whereas in the tumor-bearing mice the number of osteoclasts was markedly increased as shown in Table I.

Urinary cyclic AMP of control mice, pooled from eight normal mice on Days 5, 8, 12, and 17, was 231.0 ± 67.7 n*M*/mg creatinine (mean ± 1 SD, $n = 4$) and that of urine samples pooled from eight tumor-bearing mice was increased 2- to 4-fold; 587.7, 1017.9, 1155.7, and 393.7 n*M*/mg creatinine on days 5, 8, 12, and 17 post-tumor transplantation, respectively.

Discussion. Experimentally a close association of hemopoiesis and bone remodeling has been repeatedly observed (14, 15). Furthermore, bone environment may provide a favorable condition for the proliferation of hemopoietic stem cells (16). Clinically, extensive marrow hyperplasia is known to result in skeletal abnormalities and altered bone metabolism in thalassemia (17), and hypercalcemia has been associated with exacerbation of leukemia (18). With a desire to understand the relationship between marrow and bone tissue, we have examined the animal model of granulocytosis with a special emphasis on bone metabolism.

We have found that mice bearing CE mammary carcinoma not only had remarkable granulocytosis but also had severe hypercalcemia. The sharp increase in plasma calcium was associated with an increase in urinary calcium excretion. After reaching the peak value on Day 12 post tumor transplantation, both urine and plasma calcium declined without evidence of renal failure. An increase in intestinal absorption of calcium as the sole cause of the hypercalcemia was excluded by the finding that a calcium-deficient diet did not correct the hypercalcemia. Thus, the cause of hypercalcemia of tumor-bearing mice was traced to bone origin. An elevated urinary hydroxyproline and increased medullary cavity in the absence of endosteal bone formation clearly indicate increased bone resorption. With the progression of the tumor growth, there was continued increase in urinary hydroxyproline indicating persistent bone resorbing activity. Although it seems likely that the increased osteoclast number contributed to the hypercalcemia, further studies are needed to document whether other types of cells such as marrow granulocytes or macrophages also contributed to increased bone resorption.

The normalization of plasma calcium upon removal of the tumor suggests that a humoral factor from the tumor is responsible for the hypercalcemia rather than tumor metastasis to the bone. Various factors have been considered responsible in tumor-induced neutrophilia (19, 20). Also factors and hormones have been reported as mediators of hypercalcemia associated with tumors; osteoclast activating factor in lymphosarcoma cell leukemia (21), prostaglandin-producing tumors (22, 23), and secretion of parathyroid hormone or parathyroid hormone-like substances (23).

The elevated urinary cyclic AMP in our tumor mice is suggestive of hormonal mediation. Somewhat against this latter possibility is the finding that whereas blood neutrophils and urine hydroxyproline continued to increase throughout the 17-day study, urine cyclic AMP reached a peak value on Day 12 and then dropped almost to control level on Day 17. On the other hand, serum and urine calcium exhibited the same pattern as that of urine cyclic AMP. Our results suggest that the tumor tissue produces a humoral agent that directly or indirectly stimulate the production of osteoclasts and that this results in the increase in plasma calcium. The fall in plasma calcium following removal of the tumor is consistent with this interpretation. Whether a single factor is responsible for the stimulation of neutrophilia and osteoclast production remains to be determined.

We have documented in these experiments that a marked neutrophilia of mice bearing CE mammary carcinoma was associated with severe hypercalcemia resulting from excessive

bone resorption. The model may prove useful for the study of pathophysiology of neutrophilia and of hypercalcemia associated with cancer as well as for studies of regulatory mechanisms involved in marrow and bone interactions.

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1. Delmonte L, Liebelt, AG, Liebelt RA. Granulopoiesis and thrombopoiesis in mice bearing transplanted mammary cancer. Cancer Res **26**:149–159, 1966.
2. Lee M, Durch S, Dale D, Finch C. Kinetics of tumor induced murine neutrophilia. Blood **53**:619–632, 1979.
3. Lee MY, Sperlin A, Dale D. Distribution of granulocytopoietic committed stem cells in mice with tumor induced neutrophilia. Exp Hematol **8**:249–255, 1980.
4. Lee MY, Rosse C. Replacement of fatty marrow by active granulocytopoietic bone marrow following transplantation of mammary carcinoma into mice. Anat Rec **195**:31–46, 1979.
5. DeBruyn PPH, Breen PC, Thomas TB. The microcirculation of the bone marrow. Anat Rec **168**:55–68, 1970.
6. Walker DG. Control of bone resorption by hematopoietic tissue: The induction and reversal of congenital osteopetrosis in mice through use of bone marrow and splenic transplants. J Exp Med **142**:651–663, 1975.
7. Baylink D, Stauffer M, Wergedal J, Rich C. Formation, mineralization and resorption of bone in vitamin D-deficient rats. J Clin Invest **49**:1122–1134, 1970.
8. Wergedal J. Enzymes of protein and phosphate catabolism in rat bone. I. Enzyme properties in normal rats. Calcif Tissue Res **3**:55–56, 1969.
9. Thompson ER, Baylink DJ, Wergedal JE. Increase in number and size of osteoclasts in response to calcium or phosphorus deficiency in the rat. Endocrinology **97**:283–289, 1975.
10. Deubelbeiss KA, Dancy JT, Harker LA, Cheney B, Finch CA. Marrow erythroid and neutrophil cellularity in the dog. J Clin Invest **55**:825–832, 1975.
11. Sherrard DJ, Baylink D, Wergedal J. Bone disease in uremia. Trans Amer Soc Artif Intern Organs **18**:412–415, 1972.
12. Prockop DJ, Udenfriend S. A specific method for analysis of hydroxyproline in tissue and urine. Anal Biochem **1**:228–239, 1960.
13. Gilman AG. A protein binding assay for adenosine 3':5'-cyclic monophosphate. Proc Nat Acad Sci USA **67**:305–312, 1970.
14. Amsel S, Maniatis A, Tavassoli M, Crosby WH. The significance of intramedullary cancellous bone formation in the repair of bone marrow tissue. Anat Rec **164**:101–112, 1969.
15. Patt HM, Maloney MA. Bone marrow regeneration after local injury: A review. Exp Hematol **3**:135–148, 1975.
16. Chan SH, Metcalf D. Local production of colony-stimulating factors within the bone marrow. Role of nonhematopoietic cells. Blood **40**:646–653, 1972.
17. Pootrakul P, Hungsprenges S, Fucharoen S, Baylink D, Thompson E, English E, Lee M, Burnell J, Finch C. Relation between erythropoiesis and bone metabolism in thalassemia. N Engl J Med **304**:1470–1473, 1981.
18. Haskell CM, DeVita VT, Canellos GP. Hypercalcemia in chronic granulocytic leukemia. Cancer **27**:872–879, 1971.
19. Burlington U, Cronkite EP, Laissue JA, Reincke U, Shadduck RK. Colony stimulating activity in cultures of granulocytosis-inducing tumor. Proc Soc Exp Biol Med **154**:86–92, 1977.
20. Miura Y, Suda T, Mizoguchi H, Kubota K, Takaku F. Hemopoietic stem cells in nude mice bearing CSF-producing tumors. Exp Hematol **8** (Suppl 7):68, 1980.
21. Mundy GR, Rick ME, Turcotte R, Kowalski MA. Pathogenesis of hypercalcemia in lymphosarcoma cell leukemia: Role of an osteoclast activating factor like substance and a mechanism of action for glucocorticoid therapy. Amer J Med **65**:600–606, 1978.
22. Tashjian AH Jr, Voelkel EF, Levine L, Goldhaber P. Evidence that the bone resorption-stimulating factor produced by mouse fibrosarcoma cells is prostaglandin E_2: A new model for the hypercalcemia of cancer. J Exp Med **136**:1329–1343, 1972.
23. Voelkel EF, Tashjian AH, Franklin R Jr, Wasserman E, Levine L. Hypercalcemia and tumor-prostaglandins: The VX_2 carcinoma model in the rabbit. Metabolism **24**:973–986, 1975.
24. Lafferty FW. Pseudohyperparathyroidism. Medicine (Baltimore) **45**:247–260, 1966.

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