Calcif Tissue Int (1990) 47:158–163

Calcified Tissue International
© 1990 Springer-Verlag New York Inc.

A Model for Investigating the Local Action of Bone-Acting Agents *In Vivo:* Effects of hPTH(1–34) on the Secondary Spongiosa in the Rat

T. Takano-Yamamoto and G. A. Rodan

Department of Bone Biology and Osteoporosis Research, Merck Sharp & Dohme Research Laboratories, West Point, PA, USA

Summary. Cytokines and other local factors are likely to play an important role in bone remodeling. The purpose of this study was to develop an experimental model for evaluating the local action of various hormones and other agents on rat femur trabecular bone *in vivo*. Through a 1 mm diameter hole (1 mm deep) on the lateral aspect of the distal cortex of a rat femur (0.9 cm above the joint), a polyethylene tube was inserted and glued onto the bone. This tube was connected to a vascular-access-port which was implanted subcutaneously in the hip area of male Sprague-Dawley rats weighing 190–210 g. To evaluate this model, a single 50 μl injection of 10^{-8}, 10^{-10}, or 10^{-12} M hPTH(1–34) was given 1 day after catheter implantation and the number of osteoclasts was estimated 30 hours later. Bones were fixed, embedded, and stained with Masson's Trichrome stain and subjected to histomorphometric analysis. The single local parathyroid hormone (PTH) injection caused a dose-dependent increase in osteoclast number from 1.7 ± 0.3 for phosphate-buffered saline (PBS) controls to 3.4 ± 0.5, 5.8 ± 0.9, and 5.4 ± 1.0/mm for 10^{-12}, 10^{-10}, and 10^{-8} M PTH, respectively. There was no increase in osteoclast number in the femurs of PBS-injected control rats, in the femurs of sham-operated rats, or in the untreated contralateral femur of the PTH-injected rat. The local injection of hPTH(1–34) did not change serum calcium, serum phosphate, or the urinary phosphate/creatinine ratio. This system can provide a useful model for investigating the direct physiological and pathological effects of hormones and other factors on bone *in vivo*.

Key words: hPTH(1–34) — Local action — Histomorphometry — *In vivo*.

The regulation of bone formation and resorption is governed by complex interactions among systemic and local factors [1]. Calcium (Ca)-regulating hormones and other systemic growth regulatory hormones can act on the skeleton directly as well as indirectly via the local or systemic production of growth factors. The role of local factors in bone remodeling *in vivo* has been difficult to elucidate because the available approaches are limited and are often confounded by systemic effects.

Isaksson et al. [2] observed a direct growth-promoting effect of growth hormone on the epiphyseal plate *in vivo* following its injection into the proximal tibial epiphyseal plate of hypophysectomized rats. Lidor et al. [3] reported that cholecalciferol injected locally *in vivo* into the proximal epiphyseal growth plate of the tibia of rachitic chicks could cure unilaterally the rachitic lesions. Recently, Schlechter et al. [4] developed an experimental system that permits the delivery of a substance to one leg of an animal via its arterial blood. The object of this study was to develop means for studying the effect of very small amounts of bone-active substances on trabecular bone *in vivo*.

Parathyroid hormone (PTH) is a very potent regulator of bone resorption and formation *in vivo* and *in vitro* [5]. Studies on the time course of PTH action have shown a correlation between its effect on osteoclast number and increases in plasma Ca levels *in vivo* [6], as well as Ca release *in vitro* [7]. We developed an experimental model for investigating the local and direct action of various hormones and other factors on rat femur trabecular bone *in vivo*

Send reprint requests to T. Takano-Yamamoto, Department of Bone Biology and Osteoporosis Research, Merck Sharp & Dohme Research Laboratories, West Point, PA 19486, USA.

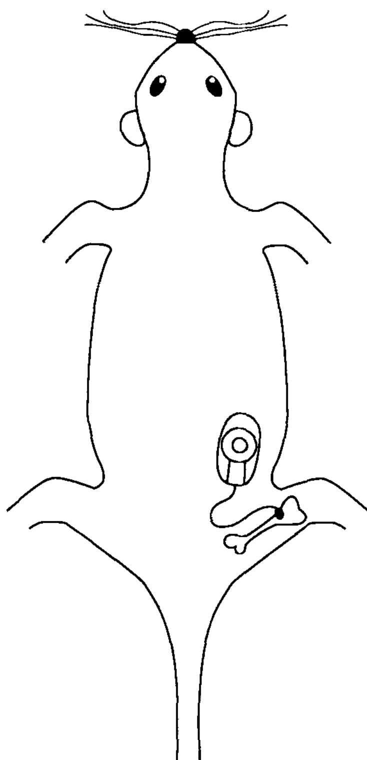

Fig. 1. Diagram of the surgical field for catheterizing the distal metaphyseal region of the right femur of rats. Solid line shows the subcutaneous tunnel connecting the catheter to a vascular-access-port in the right hip region of the rat.

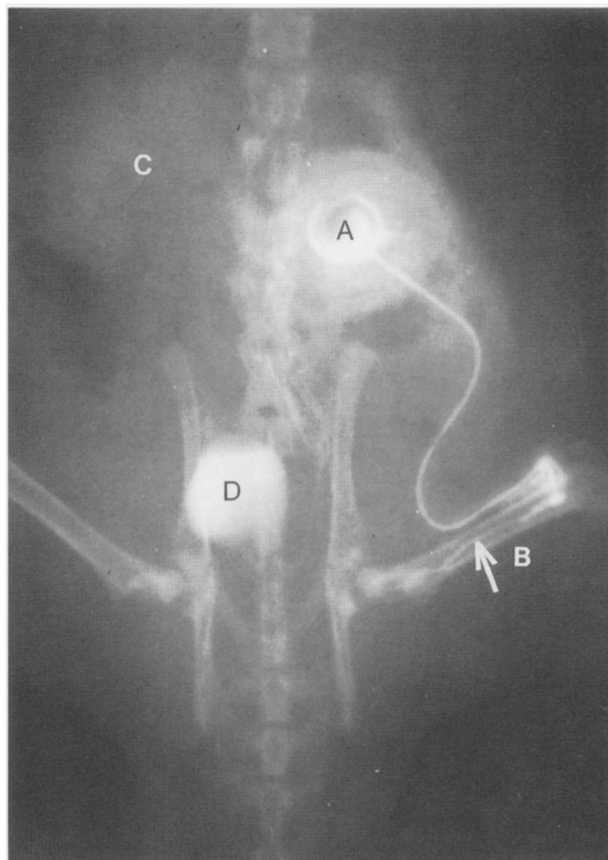

Fig. 2. Radiograph of rat after injection of radioopaque material into right femur through a vascular-access-port. The longitudinal vessel running through the center of the bone is indicated by the arrow. (A) Vascular-access-port; (B) right femur; (C) kidney; (D) urinary bladder.

and evaluated this model by examining the effects of PTH on osteoclast number on the secondary spongiosa.

Materials and Methods

Surgical Procedures

Male Sprague-Dawley rats weighing 190–210 g were anesthetized with ketamine hydrochloride and acepromazine malate (3:1 v/v). A small skin and muscle incision was made over the right femur. A hole 1 mm in diameter and 1 mm deep was drilled with a dental bur on the lateral aspect of the distal cortex of a rat femur (0.9 cm above the joint). A 2 mm piece of Intramedic PE-50 tube (Clay Adams, Parsippany, NJ) was inserted 1 mm deep and glued with cyanoacrylate on the outside of the bone. This tube was connected to a vascular-access-port (Harvard Apparatus, Boston, MA) which was implanted subcutaneously in the right hip area of the rat, as shown in Fig. 1. The filling space of the catheter was approximately 50 μl. The catheter was secured by a Dexon 3-0 ligature in an adjacent tissue. The contralateral left limb remained intact. Control rats were sham-operated.

To survey the distribution space and flow of the injected solution in the femur, several injections of 50 μl Renografin-76 (Squibb) were given 1 day after catheter implantation and radiographs were taken as shown in Fig. 2.

To evaluate the physiological response of bone in this model, a single 50 μl injection of 10^{-8}, 10^{-10}, or 10^{-12} M hPTH was given 1 day after catheter implantation and its effects were compared to those of 50 μl phosphate-buffered saline (PBS).

Histology

Thirty hours after injection, three animals from each treatment group were sacrificed, and the femora were removed and fixed in 70% ethanol. The bone was dehydrated in an ethanol series and embedded in methyl methacrylate. Coronal sections (6 μm thick) were cut parallel to the long axis of the femur and stained with Masson's trichrome. Histomorphometric quantification of osteoclast number per mm of trabecular surface was carried out with a semiautomatic real-color image analyzer Magiscan (Joyce-Loebl, Newcastle upon Tyne, England) at ×488. A Sony model DXC-M3AP color video camera attached to the microscope was used to detect and transfer images to the image analyzer. Osteoclasts were identified as multinucleated cells, stained light red with Masson's trichrome stain, containing round nuclei located immediately adjacent to the bone surface. Trabecular bone surfaces (stained blue) were quantified using the software package

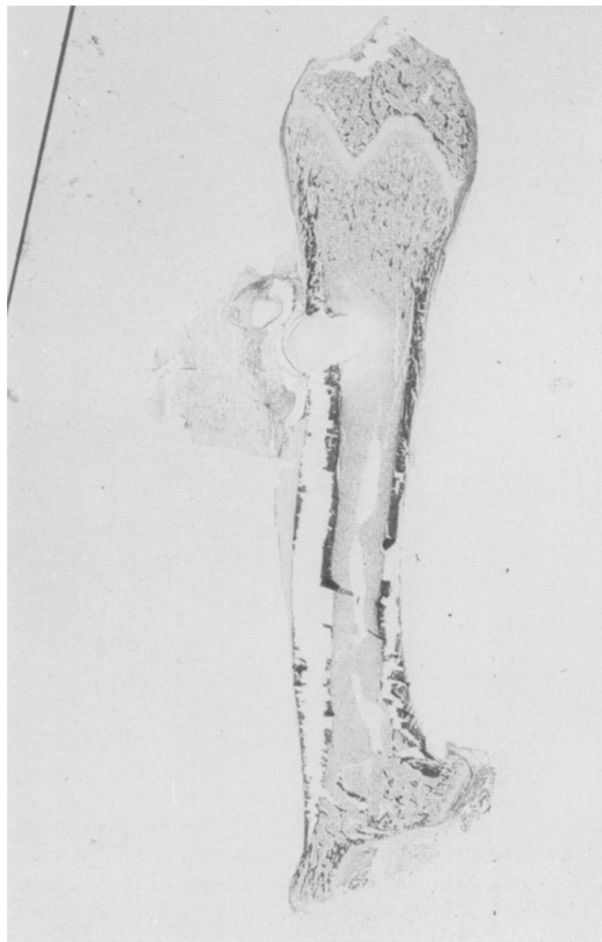

Fig. 3. Low power of photomicrograph of right femur of the operated rat. A 1 mm diameter hole was drilled with a dental bur on the lateral aspect of the distal cortex of a rat femur (0.9 cm above the joint). Note, there is no traumatic destruction in the secondary spongiosa of the distal metaphyseal region due to surgery or injection.

"colormenu" developed for bone histomorphometry (Joyce-Loebl). The distal femur metaphyseal region analyzed was located in the secondary spongiosa extending between 1.5 and 2.8 mm mesial to the epiphyseal growth plate. For each condition, slides from three animals were examined, a total of 10–40 osteoclasts per slide, counted in six fields.

Serum and Urine Chemistry

Urine samples and blood samples from the tail vein were obtained at the time of injection (time 0) and at 3 and 30 hours after the injection. Serum Ca (mg/dl) was determined by atomic absorption spectrophotometry. Phosphorus (mg/dl) in serum and urine was determined using the Gemstar inorganic phosphorus U.V. procedure. Urinary creatinine was analyzed with the Jaffe method by using the Gemstar creatinine system.

hPTH(1–34) was purchased from Bachem, Torrance, CA (activity 3,000 U/mg) and dissolved in PBS.

Student's t test was used for statistical comparison of means and P value of less than 0.05 were considered statistically significant.

Table 1. Effect of hPTH(1–34) injection on the number of osteoclasts apposed to trabecular bone of femur in rats

	Osteoclasts/mm trabecular surface	
Treatment	Right femur	Left femur
Sham-operated	2.0 ± 0.2	1.8 ± 0.2
PBS injection	1.7 ± 0.3	2.0 ± 0.4
hPTH(1–34) injection		
10^{-12} M	3.4 ± 0.5[a]	2.1 ± 0.1
10^{-10} M	5.8 ± 0.9[a]	2.2 ± 0.2
10^{-8} M	5.4 ± 1.0[a]	1.9 ± 0.4

[a] $P < 0.05$ compared to right and left femurs of the sham-operated and PBS-injected rats

The number of osteoclasts and the trabecular surface were determined for each condition in the distal femoral metaphysis of sham-operated and operated rats after 30 hours of injection with PBS or the indicated concentrations of hPTH(1–34). Results are presented as the mean of three samples and SEM

Results

The flow of radioopaque material injected into the femur is shown in Fig. 2. The vascular-access-port, the catheter tube, and the longitudinal vessel running through the center of the femur could be visualized immediately after injection of radioopaque material into the vascular-access-port in the right hip of the rat. One minute after injection, the radioopaque material disappeared from the central venous sinus of the right femur, but remained in the bone around the catheterizing tube near the insertion hole. The radioopaque material was visualized in the kidney and urinary bladder 5 minutes after injection. Figure 3 shows a low-power histological photomicrograph of the operated right femur. A small hole was drilled through the distal cortex of the rat femur 0.9 cm above the joint, where there is no trabecular bone. No traumatic destruction and no inflammatory response due to surgery or injection were seen in the secondary spongiosa of the distal metaphyseal region.

The effects of a single injection of hPTH(1–34) on the number of octeoclasts in the secondary spongiosa of the distal metaphysis of the rat femur is shown in Table 1. A local injection of 50 μl 10^{-12}, 10^{-10}, or 10^{-8} M hPTH(1–34) caused a dose-dependent increase in osteoclast number to 3.4 ± 0.5, 5.8 ± 0.9, and 5.4 ± 1.0/mm trabecular bone surface, respectively, compared to 1.7 ± 0.3 for PBS. There was no increase in osteoclast number in the trabecular bone of PBS-injected rats and in the

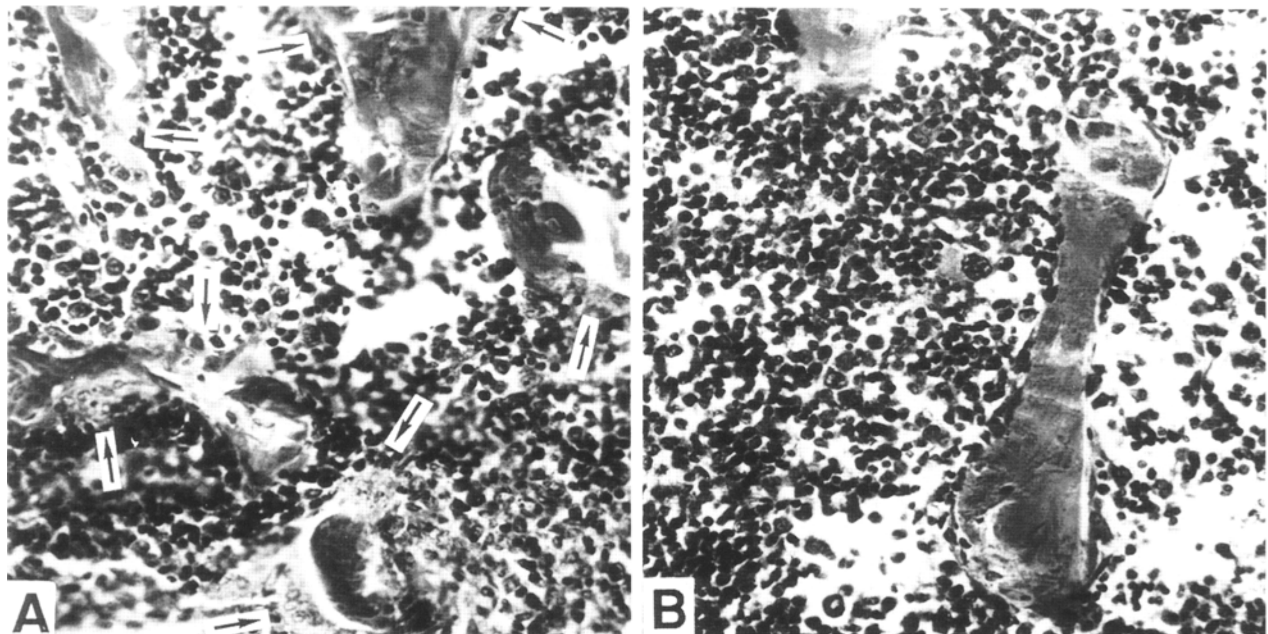

Fig. 4. Photomicrographs of Masson's trichrome-stained 6 μm sections from the distal femoral metaphyses of rats injected with hPTH(1–34) or PBS. Injection of peptide at 10^{-10} M results in abundant multinucleated osteoclasts (arrows) on trabecular surfaces of right femur (**A**), whereas there was no increase in osteoclast number in the untreated contralateral femur of the PTH-injected rats (**B**). (**A**) Right femur of rat injected with 50 μl hPTH(1–34) at 10^{-10} M. (**B**) Left (contralateral) femur of the PTH-injected rat (×575).

untreated contralateral femurs of the PTH-injected rats compared to the femurs of sham-operated rats. Furthermore, no increase in osteoclast number was seen in the proximal region of the PTH-injected femur. The numerous multinucleated osteoclasts on almost all subepiphyseal trabecular surfaces of distal metaphyses of PTH-injected femurs are shown in Fig. 4. The cortical bone of animals injected with 10^{-8} M hPTH(1–34) also contained osteoclasts at the endosteal surface near the hormone-injected area.

The serum Ca, serum phosphate, and urinary phosphate/creatinine ratio of PTH-injected rats did not differ significantly from PBS-injected or sham-operated rats 0, 3, and 30 hours postinjection (Table 2). The body weight of the rats was measured at the time of surgery, at the time of the injection of hPTH(1–34) or PBS, and at the time of sacrifice. The surgery and local injection of hPTH(1–34) had no significant effect on body weight and each group showed almost the same body weight gain at the time of sacrifice (data not shown).

Discussion

We present here an experimental model for investigating the local action of hormones and other factors on rat femur trabecular bone *in vivo*. As shown in Fig. 2, radioopaque material injected into the distal cortex of the rat femur diffuses into the area around the site of injection; thereafter it flows from the medullary sinusoids into the longitudinal central venous sinus and into the systemic circulation, finally being secreted in the urine. The radiographic evidence showing that the injected solution diffuses locally and enters the blood vessels suggests that this method can be used for the direct quasi-physiological local application of hormones and other factors to bone. However, no traumatic destruction and no inflammatory response were seen in the secondary spongiosa of the distal metaphyseal region due to surgery or injection (Figs. 3, 4). All animals recovered without difficulty and appeared to have full usage of the operated limb. There was no difference in body weight gains between sham-operated and PBS- or PTH-injected rats.

Parathyroid hormone (PTH) plays a major role in the maintenance of Ca homeostasis and the regulation of bone remodeling [8]. Its main target organs are the kidney and the skeleton. It has been shown long ago that PTH increases bone resorption and elevates plasma Ca in the absence of the kidneys, indicating direct action of PTH on bone. Local application of parathyroid tissue onto bone *in vivo* showed a substantial increase in the number of osteoclasts [9]. The direct effect of PTH on bone has

Table 2. Serum and urine chemistry from sham-operated and experimental rats before, 3, and 30 hours after injection of hPTH(1–34) or PBS

Treatment	Hours after injection	Serum		Urine
		Ca mg/dl	Pi mg/dl	Pi/Creatinine
Sham-operated	0	10.0 ± 0.9	8.9 ± 0.9	2.0 ± 0.5
	3	10.2 ± 0.2	9.2 ± 0.4	1.9 ± 0.3
	30	10.0 ± 0.5	8.8 ± 0.3	2.3 ± 0.5
PBS injection	0	10.2 ± 0.6	9.1 ± 0.2	1.7 ± 0.7
	3	10.6 ± 0.2	9.7 ± 0.4	2.4 ± 0.5
	30	10.8 ± 0.8	9.7 ± 0.6	2.3 ± 0.5
hPTH(1–34) injection	0	10.2 ± 0.6	9.3 ± 0.2	2.1 ± 0.4
10^{-12} M	3	10.0 ± 0.5	9.4 ± 0.2	2.5 ± 0.3
	30	10.3 ± 9.7	9.6 ± 0.4	2.0 ± 0.3
10^{-10} M	3	10.4 ± 0.7	9.4 ± 0.5	2.3 ± 0.4
	30	10.7 ± 0.8	9.5 ± 0.5	2.4 ± 1.0
10^{-8} M	3	10.0 ± 0.9	9.0 ± 0.5	2.4 ± 0.6
	30	10.0 ± 0.5	9.5 ± 0.8	1.7 ± 0.3

Results are the mean ± SD from 4 rats per group

also been extensively studied *in vitro* [1, 7]. PTH can increase the size of the osteoclast population and the activity of individual osteoclasts [7]. PTH was therefore used to evaluate the suitability of this model for investigating the direct and local action of bone-acting agents *in vivo*.

A single local injection of 50 μl 10^{-12}–10^{-8} M hPTH(1–34) (0.05–5 fmol) caused a dose-dependent increase in osteoclast number on the secondary spongiosa of the distal metaphysis of rat femora 30 hours later (Table 1). The response was obtained at a concentration of injected hormone as low as 10^{-12} M, probably further diluted following injection. The maximum effect was seen at the injected concentration of 10^{-10} M which is probably around the physiological concentration of PTH in rats [10, 11]. The findings show that a single brief exposure to the hormone sufficed to elicit the *in vivo* response. A clear advantage of this system is the need for very small amounts of hormone or growth factors to investigate the direct effects of active agents on bone *in vivo*. Relatively large doses of PTH have been given systemically to experimental animals to investigate the effect of PTH on osteoblastic bone formation [12–15] or osteoclastic bone resorption [16–18] *in vivo*. Because the liver and the kidney metabolize and eliminate the intact and the aminoterminal fragments of PTH, it is still unclear whether the effect of systemically administered large doses of PTH on bone requires physiological modification of the molecule and whether different metabolites have a different potency or different effects on bone [19, 20]. This approach can be used to examine these questions for PTH and other substances.

The highly localized effect obtained in this system was evident from the lack of osteoclast changes in the untreated contralateral femurs of the PTH-injected rats (Table 1), and especially in osteoclast number in the proximal region of PTH-injected femora. This is consistent with the pattern of blood flow in the rat femur. As expected, no PTH effects on Ca or phosphate homeostasis could be detected in these experiments. The PTH doses injected locally did not significantly change the serum Ca, serum phosphate, or urinary phosphate/creatinine ratio compared with those of sham-operated rats and of PBS-injected rats (Table 2). By replacing the vascular-access-port with osmotic minipumps, this local application system should make possible the continuous infusion of very low doses of substances over extended periods without inducing systemic effects. This system should prove useful for investigating the direct physiological or pharmacological effects of hormones and other factors on bone *in vivo*.

Acknowledgments. The development of this experimental approach was stimulated by discussions with Drs. Mundy and Pfeilshifter. We thank Dr. Masaki Noda for his advice regarding the anatomy of the femur circulation, Drs. David Thompson and Miron Weinreb for guidance with the Joyce Loebl Image Analysis system, and Ms. Dianne McDonald for preparing the manuscript.

References

1. Huffer WE (1988) Biology of disease, morphology and biochemistry of bone remodeling: Possible control by vitamin

D, parathyroid hormone, and other substances. Lab Invest 59:418–441
2. Isaksson OGP, Jansson J-O, Gause IAM (1982) Growth hormones stimulates longitudinal bone growth directly. Science 216:1237–1239
3. Lidor C, Atkin I, Ornoy A, Dekel S, Edelstein S (1987) Healing of rachitic lesions in chicks by 24R, 25-dihydroxycholecalciferol administered locally into bone. J Bone Min Res 2:91–98
4. Schlechter NL, Russell SM, Greenberg S, Spencer EM, Nicoll CS (1986) A direct growth effect of growth hormone in rat hindlimb shown by arterial infusion. Am J Physiol 250:E231–E235
5. Raisz LG (1976) Mechanism of bone resorption. In: Aurbach GD (ed) Handbook of physiology, sect. 7. American Physiological Society, Washington, DC 7:117–136
6. Holtrop ME, King GJ, Cox KA, Reit B (1979) Time-related changes in the ultrastructure of osteoclasts after injection of parathyroid hormone in young rats. Calcif Tissue Int 27:129–135
7. King GJ, Holtrop ME, Raisz LG (1978) The relation of ultrastructural changes in osteoclasts to resorption in bone cultures stimulated with parathyroid hormone. Metab Bone Dis Rel Res 1:67–74
8. Parfitt AM (1976) The actions of parathyroid hormone on bone: relation to bone remodeling and turnover, calcium homeostasis, and metabolic bone disease. Mechanisms of calcium transfer between blood and bone and their cellular basis: morphological and kinetic approaches to bone turnover. Metabolism 25:809–844
9. Barnicot NA (1948) The local action of the parathyroid and other tissues on bone in introcerebral grafts. J Anat 82:233–248
10. Kalu DN (1984) Evaluation of the pathogenesis of skeletal changes in ovariectomized rats. Endocrinology 115:507–512
11. Insogna KL, Stewart AF, Vignery Am-C, Weir EC, Namnum PA, Baron RE, Kirkwood JM, Deftos LM, Broadus AE (1984) Biochemical and histomorphometric characterization of a rat model for humoral hypercalcemia of malignancy. Endocrinology 114:888–896
12. Kalu DN, Pennock J, Doyle FH, Foster GV (1970) Parathyroid hormone and experimental osteosclerosis. Lancet 1:1363–1366
13. Tam C, Heersche J, Murray T, Parsons JA (1982) Parathyroid hormone stimulates the bone apposition rate independently of its resorptive action: differential effects of intermittent and continuous administration. Endocrinology 110:506–512
14. Tam C, Cruickshank B, Swinson DR, Anderson W, Little H (1979) The response of bone apposition rate to some nonphysiologic conditions. Metabolism 28:751–755
15. Gunness-Hey M, Hock JM (1984) Increased trabecular bone mass in rats treated with human synthetic parathyroid hormone. Metab Bone Dis & Rel Res 5:177–181
16. Heller M, McLean FC, Bloom W (1950) Cellular transformations in mammalian bones induced by parathyroid extract. Am J Anat 87(3):315–339
17. Walker DG (1971) The induction of osteopetrotic changes in hypophysectomized, thyroparathyroidectomized and intact rats of various ages. Endocrinology 89:1389–1406
18. McGuire JL, Marks SC (1974) The effects of parathyroid hormone on bone cell structure and function. Clin Orthop Rel Res 100:392–405
19. Herrmann-Erlee MPM, Nijweide PJ, van der Meer JM, Ooms MAC (1983) Action of bPTH ad bPTH fragments on embryonic bone in vitro: dissociation of the cyclic AMP and bone-resorbing response. Calcif Tissue Int 35:70–77
20. Martin KJ, Freitag JJ, Conrades MB, Hruska KA, Klahr S, Slatopolsky E (1978) Selective uptake of the synthetic amino terminal fragment of bovine parathyroid hormone by isolated perfused bone. J Clin Invest 62:256–261

Received June 7, 1989, and in revised form November 21, 1989.