PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 170, 305–312 (1982)

Plasma Calcium, Plasma and Thyroidal Calcitonin, and Histomorphometric Bone Changes in Parathyroidectomized Rats (41435)

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Abstract. This study examined physiological and bone morphological changes resulting from parathyroidectomy (PTX) in male rats. Two diets were provided: one contained 1.2% calcium, 1% phosphorus; the other 0.4–0.6% calcium, 0.6% phosphorus. However, at 5½ months post-PTX all rats were transferred to a diet containing 0.3% calcium. The daily ration for each rat was 16 g. The parameters examined were: (1) Plasma calcium concentrations; (2) plasma and thyroidal calcitonin levels; and (3) histomorphometry of metaphyseal trabecular bone. When maintained on the lower calcium diet, fasted plasma calcium concentrations of PTX rats stabilized between 5 and 6 mg/dl. In contrast, in rats fed the higher calcium diet, these values gradually rose to between 7 and 8 mg/dl (5 months post-PTX). After transfer to the 0.3% calcium diet, plasma calcium values fell to <6 mg/dl. Thyroidal calcitonin content following PTX rose to values three times those of age-matched controls regardless of the daily calcium intake. The volume of trabecular bone in the tibial metaphysis increased threefold by 6½ months after PTX; however, there was a decrease in osteoid on these bone surfaces. The static parameters of bone resorption and formation in PTX rats were not statistically different from controls; however, the ratio of osteoblasts to lining cells on trabecular surfaces increased following PTX. The cause of the increase in thyroidal calcitonin following PTX is as yet unknown, but appears to be unrelated to the changes in plasma calcium in rats fed a high calcium diet. This rise in plasma calcium is attributed to accumulation of calcium in the bone surface exchangeable compartment which is reversible. The increase in volume of trabecular bone may be due to slight changes in rates of bone turnover which are not detectable in analysis of static parameters. There was no evidence that the epiphyseal growth plate, or the rate of enchondral bone formation was affected by PTX. The effect of high dietary calcium on post-PTX plasma calcium values points out the need for close control of calcium content of rat chow if the rat is to be used as a model for studying calcium homeostasis or hormonal effects on bone remodeling.

Gittes *et al.* (1) reported over 10 years ago that chronically parathyroidectomized rats maintained on a 1% calcium diet accumulated calcitonin in the thyroid gland. More recently, these earlier studies were extended by Peng and Garner (2) in parathyroidectomized Fischer and Holtzman rats kept on Wayne Lab Blox diet (1.2% calcium content) for up to 13 months. They reported a progressive rise in the levels of serum immunoreactive calcitonin, an increase in thyroidal calcitonin concentrations, a hyperplasia of the thyroidal C cells, and a gradual return of plasma calcium toward normal levels. The change in plasma calcium values required 3 or more months to be significantly elevated. In another study, Kemm (3) reported that plasma calcium concentrations in rats maintained on a low calcium diet (0.2%) remained low following parathyroidectomy for at least 6 weeks. Also, in preliminary reports, Crenshaw and Peng (4, 5) reported that gross histological examination indicated that parathyroidectomy in rats was followed in time by an increase in amount of trabecular bone in the metaphysis of long bones. The rats used in their study were maintained on the laboratory chow containing 1.2% calcium.

It is quite possible that both the anatomical and physiological changes reported to occur following parathyroidectomy might

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0037-9727/82/070305-08$01.00/0

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be influenced greatly by the amount of calcium in the diet; in fact, all observations reported were made using rats on a high calcium diet. Therefore, we examined the changes in plasma and thyroid gland calcitonin concentrations with increasing age in chronically parathyroidectomized rats maintained on diets different in their calcium content. Our purpose was to determine whether the chronic effects of parathyroidectomy on plasma calcium levels and on thyroidal calcitonin concentrations were influenced by the dietary content of calcium. In addition, the metaphyseal trabecular bone mass was subjected to histomorphometric analysis in an attempt to determine the cause of the increase in bone mass produced by parathyroidectomy.

Materials and Methods. *A. General procedures.* Male Sprague–Dawley rats were monitored over a 7-month period, beginning at 5 weeks of age (body weight between 130 and 150 g), at which time the majority were parathyroidectomized under light ether anesthesia. Control, intact rats were maintained concurrently. Several days after surgery, blood samples were obtained for calcium analysis after the rats were fasted overnight. Only rats with a plasma calcium concentration less than 7.0 mg/dl were included in the parathyroidectomized group for the remainder of the experiment.

At 6 weeks of age, both control and parathyroidectomized rats were divided into two groups. Both groups were provided food daily at 1400 hr, but were permitted to feed *ad libidum*. Group 1 was maintained on standard laboratory chow (1.2% calcium), but each rat was restricted to a 16-g daily ration (approximately 195 mg of calcium and 160 mg of phosphorus daily). Group 2 was fed a diet having 0.4 to 0.6% calcium. The 16-g daily ration provided 65 to 90 mg calcium and 90 mg P daily. After 5 months on these diets, several rats from each group were sacrificed. For the remaining rats, the calcium provided in the daily ration (both groups) was reduced so as to provide only 50 mg of calcium daily (0.3% calcium); the phosphate content was maintained at 90 mg P daily. The experiment was terminated 6 weeks later, at which time the 8-month-old rats had been parathyroidectomized for approximately 6½ months.

At stated intervals throughout the experimental period blood samples were obtained from the tail for measurement of plasma calcium levels. Calcium was analyzed by fluorometric titration with EGTA (6). When rats were sacrificed, sufficient plasma was obtained for calcitonin analyses by radioimmunoassay (7), and the thyroids were removed either for calcitonin analysis or for immunohistochemical identification of C cells (8). The thyroid glands used to determine calcitonin content were homogenized in 10^{-3} *N* HCl in 0.15 *M* NaCl (7, 9). At the conclusion of the experiments (8-month-old rats), bones were obtained for histological preparation as described below.

B. Quantitative histomorphometry of bone. Tibiae were removed immediately upon sacrifice of the animal and dissected free of soft tissue. A piece of cortical bone was removed transversely from the anterior tuberosity of the proximal tibia to allow for a quicker penetration of the fixatives. Bones were then placed in a phosphate-buffered modified Millonig's fixative at pH 7.4 and 37°C. Samples were dehydrated with increasing concentrations of acetone and embedded in methyl methacrylate. Undecalcified bone sections were cut at a thickness of 5 μm on a Jung Model K microtome, collected on 0.5% calfskin gelatin-coated slides, and stained with a modified Goldner stain.

Light microscopic evaluations were made on a Leitz microscope with a Merz grid on an eyepiece reticule. Objective magnification was 16× and eyepiece magnification was 12.5×. Measurements were made until either 100 fields or 1000 intersections were counted. All fields were located in the epiphyseal–metaphyseal area of each tibia. The morphometric analysis described by Merz and Shenk (10), and terminology used by Malluche *et al.* (11) were used in the study.

Results. *Plasma calcium values.* Plasma calcium values were obtained at intervals

after parathyroidectomy. In each case, blood was obtained 4 hr prior to feeding, representing a fasting value. The data are summarized in Fig. 1. As reported by Peng *et al.* (2), when rats are maintained on a high calcium diet (1.2% calcium or 190 mg daily), plasma calcium values in parathyroidectomized rats gradually rose. In our animals, by 5 months after parathyroidectomy, the 4-hr prefeeding plasma calcium value had risen to 8.0 mg/dl. To demonstrate the role of dietary calcium, these rats (5 months parathyroidectomized) were transferred to a diet containing 0.3% calcium, which provided slightly less than 50 mg calcium daily. Within 3 weeks after this reduction in calcium intake, the plasma calcium concentration (prefeeding values) had fallen approximately 2 mg/dl. In contrast to the parathyroidectomized rats kept on high calcium diet, the plasma calcium values in animals maintained during the first 5-month period on 0.4 or 0.6% calcium diet (60 to 90 mg daily) remained in the 6 to 7 mg/dl range. There appeared to be little or no increase during this period in rats on this calcium intake and only a small fall occurred when the daily calcium intake was reduced further to <50 mg daily. Plasma calcium values in control, intact rats remained between 10 and 11 mg/dl on both diets until they were transferred to the lower calcium intake (0.3% calcium), at which time even these plasma calcium values fell slightly to the low normal range (between 9 and 10 mg/dl).

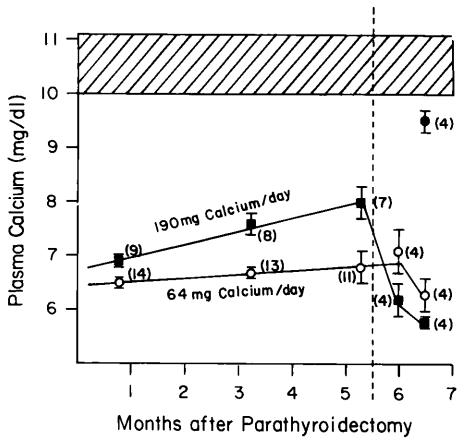

FIG. 1. Plasma calcium concentrations following parathyroidectomy. ○,■ = PTX rat values ± SE. Number in parentheses = number of rats. Shaded area = range of control values from animals randomly selected. ● = Control value after 5 weeks on 0.3% diet. Dashed vertical line indicates time at which all rats were transferred to 0.3% diet (45–50 mg calcium daily).

Plasma calcitonin values. Plasma calcitonin concentrations were determined in rats sacrificed both at 5 and 6½ months postparathyroidectomy. These values are summarized in Table I. Blood samples were obtained 4 hr prior to feeding (i.e., in the fasted state). At 6½ months of age (5 months PTX), neither dietary calcium or parathyroidectomy significantly affected fasting plasma calcitonin levels, although mean values from parathyroidectomized rats tended to be slightly higher. Transfer of all rats, normals and parathyroidectomized, to a 0.3% calcium diet (approximately 50 mg calcium daily) reduced plasma calcitonin levels to nondetectable levels (less than 0.12 ng/ml).

Thyroidal calcitonin concentrations. Thyroid glands were removed at sacrifice, weighed, and immediately frozen at −20°C. Later, calcitonin was extracted and measured. The data are presented in Table I as nanograms calcitonin per milligram wet weight of fresh thyroid tissue. The level of dietary calcium did not appear to influence thyroidal calcitonin content. However, in each case, glands from parathyroidectomized rats contained considerably more calcitonin than did those from control, thyroid-intact rats. Since thyroid weights were not different for intact and parathyroidectomized rats, both the total content and concentration of calcitonin increased in the thyroid after parathyroidectomy. The important observation here is that the elevated calcitonin content in parathyroidectomized rats, unlike the fasting plasma calcium level, did not appear to be influenced by the amount of calcium in the diet.

Thyroidal C-cell hyperplasia. The thyroids were removed from a few parathyroidectomized rats maintained on 0.4% calcium diet for 5 months, and processed histologically for immunohistological iden-

TABLE I. PLASMA AND THYROIDAL CALCITONIN

(A) Rats sacrificed at age 6½ months (5 months PTX)	
(1) Fasted plasma calcitonin (ng/ml)	
(1) Normal on Wayne (3)	0.24 ± 0.06
(2) Normal on 0.6% (2)	0.23
(3) PTX on Wayne (2)	0.29
(4) PTX on 0.6% (3)	0.28 ± 0.08
(5) All normals (5)	0.23 ± 0.05
(6) All PTX (5)	0.28 ± 0.07
(2) Thyroidal calcitonin (ng/mg)	
(1) Normal on Wayne (3)	176 ± 38
(2) Normal on 0.6% (2)	282
(3) PTX on Wayne (3)	882 ± 286
(4) PTX on 0.6% (3)	868 ± 135
(5) All normals (5)	218 ± 36.4
(6) All PTX (6)	876 ± 142
Statistical significance groups 5 to 6	$P < 0.001$
(B) Rat sacrificed at age 8 months (6½ months PTX)	
(1) Fasted plasma calcitonin—1½ months on 0.3% calcium diet	
All values nondetectable	(0.12 ng/ml)
(2) Thyroidal calcitonin—1½ months on 0.3% calcium diet	
(1) Normal, from Wayne (1)	234
(2) Normal from 0.6% (3)	255 ± 37
(3) PTX, from Wayne (3)	773 ± 63
(4) PTX, from 0.6% (4)	865 ± 143
(5) All normals (4)	249 ± 27
(6) All PTX (7)	826 ± 82
Statistical significance groups 5 to 6	$P < 0.001$

Note. Numbers in parentheses = no. of animals. Wayne diet contained 1.2% calcium; 0.6%, = percentage calcium in diet. Values given with SE except when group consisted of less than three animals.

tification of C cells. Peng *et al.* (2, 8) have reported a hyperplasia of the thyroidal C-cell population in both male and female rats, which increased with age in animals maintained on a diet containing 1.2% calcium. They also reported C-cell hyperplasia in parathyroidectomized rats on this high calcium diet. Our study confirms the hyperplasia in 6½-month-old rats (5 months parathyroidectomized) even when they were maintained on the *lower* dietary calcium.

Quantitative histomorphometry of the tibia. Specific trabecular bone parameters within the metaphyseal area (secondary spongiosa) of the tibia were quantified. The field counting technique of Kemmel and Jee (12) was followed using a Merz grid on an eyepiece reticule and a microscope slide holder moveable in only X and Y axes. Because Peng and Crenshaw (4, 5) have recently reported an increase in the extent of trabecular bone in tibia of rats following parathyroidectomy, it was important in our study to evaluate this bone quantitatively.

The morphometric measurements were made on both tibias from each rat. Since this experiment was designed primarily for analysis of calcium and calcitonin, the only rats taken for histomorphometric analysis were those sacrificed at the conclusion of the study. The only bone samples examined were from rats maintained on the lower dietary content (0.4–0.6% calcium) for the entire study.

The data summarized in Table II show that by 6 months after parathyroidectomy, there was a significant *increase* in the total amount of mineralized trabecular bone, but a *decrease* in the osteoid, or nonmineralized bone, located in the tibial metaphysis. In more technical terms, parathyroidectomy produced a significant increase in the volumetric density of mineralized trabecular bone, in the surface density of this bone, and trabecular diameter compared to age- and sex-matched intact controls.

The volumetric density of the osteoid decreased in parathyroidectomized rats due primarily to a decreased surface density or

TABLE II. PARAMETERS MEASURING OSTEOID AND MINERALIZED TRABECULAR BONE

	Intact controls	*P* value	PTX
(A) Mineralized trabecular bone			
(1) Volumetric density (mm^3/cm^3)	83.8 ± 14.5	<0.01	243.5 ± 38.4
(2) Surface density (mm^2/cm^3)	433.5 ± 359.0	<0.005	8174.3 ± 812.5
(3) Mean trabecular diameter (μm)	76.2 ± 7.7	<0.05	118.5 ± 12.6
(B) Trabecular osteoid surfaces			
(1) Volumetric density (mm^3/cm^3)	3.7 ± 0.5	<0.05	1.9 ± 0.1
(2) Surface density (mm^2/cm^3)	641.9 ± 129.3	N.S.	261.1 ± 37.4
(3) Mean width of osteoid seams (μm)	6.2 ± 1.1	N.S.	7.5 ± 1.5
(4) Surface extent of osteoid seams (%)	15.1 ± 3.3	<0.05	3.2 ± 0.3
(C) Absolute volume of trabecular bone Mineralized and osteoid (%)	8.7 ± 1.5	<0.01	24.3 ± 3.8

Note. Controls have four rats per group, two tibia per rat. PTX group has three rats, two tibia per animal. N.S., not statistically different.

surface extent of osteoid on trabecular bone. The average width of the osteoid seam present was not different from the controls. Despite this decrease in the amount of osteoid, the absolute trabecular bone volume, mineralized bone plus osteoid, was significantly increased in parathyroidectomized rat tibiae due to the large increase in volume of mineralized trabecular bone.

The standard static parameters of bone resorption and formation were determined. No data for the epiphyseal growth plate are provided because qualitatively (under light microscopy) this area appeared to be unaffected by parathyroidectomy. The data are shown in Table III, and they indicate that modeling of trabecular bone was relatively unaffected by parathyroidectomy. Because of the lack of statistically significant differences between the two groups, we suggest that the increased trabecular bone found in the metaphysis of the tibia in parathyroidectomized rats must result from small differences in the ratio of bone resorption to formulation, which are difficult to detect.

Because of the lack of significant differences in the data for bone formation and resorption between normal and parathyroidectomized rats, two additional parameters were measured. These parameters provided data on the ratio of osteoblasts to lining cells on specific trabecular surfaces. These ratios compared the populations of cells on osteoid and mineralized surfaces. The data for these two parameters are provided in Table III–B, and indicate that the ratio of osteoblasts to lining cells on mineralized surfaces was increased in parathyroidectomized rats. The mean value for this ratio on those surfaces in which osteoid still persisted was also increased, but the difference did not achieve statistical significance. One interpretation of the data provided by these two parameters is that bone is mineralized more rapidly in parathyroidectomized rats than in normal rats, since the decrease in surface osteoid was accompanied by an increase in the number of osteoblasts on mineralized surfaces.

Discussion. The pertinent observations from these experiments can be summarized as follows: (i) The gradual rise in plasma calcium levels of parathyroidectomized rats which occurred on a 1.2% calcium diet is a reflection of the high calcium content of the diet, because it does not occur in rats maintained on a lower amount of dietary calcium. (ii) In contrast, the C-cell hyperplasia occurring in the thyroid glands of parathyroidectomized rats does not appear to be related to either the calcium content of the diet or to the fasting plasma calcium level. (iii) The finding of an accumulation of unreabsorbed trabecular bone in the metaphyses of long bones in parathyroidectomized rats as reported by Crenshaw and Peng (4, 5) was confirmed; histomorphometric analyses in the present studies help to elucidate the cause.

The gradual rise in plasma calcium levels in parathyroidectomized rats maintained on

TABLE III. PARAMETERS OF TRABECULAR BONE FORMATION AND RESORPTION

	Intact controls	*P* value	PTX
(A) Trabecular bone formation			
(1) Fraction of active formation, osteoblast–osteoid interface per bone surface (%)	11.3 ± 3.2	N.S.	2.7 ± 0.1
Fraction of osteoblast-osteoid interface per osteoid surface (%)	72.2 ± 4.0	N.S.	84.9 ± 7.9
(2) Fraction of inactive formation, lining cell–osteoid interface per bone surface (%)	3.8 ± 0.4	<0.005	0.6 ± 0.2
(3) Surface extent of osteoblasts on bone (%)	58.7 ± 4.9	N.S.	73.6 ± 1.1
(4) Surface extent, lining cells on bone (%)	19.3 ± 2.1	N.S.	17.9 ± 1.2
(5) Relative activity of osteoblasts (%)	16.4 ± 4.8	N.S.	3.5 ± 0.2
(B) Ratio of osteoblasts to lining cells			
(1) On osteoid	2.9 ± 0.7	N.S.	5.4 ± 1.9
(2) On mineralized bone surfaces	2.6 ± 0.3	<0.025	4.1 ± 0.4
(C) Trabecular bone resorption			
(1) Surface density of active resorption, bone–osteoclast interface (mm^2/cm^3)	81.2 ± 32.6	N.S.	62.8 ± 24.0
(2) Surface density of inactive resorption empty Howship's lacunae on bone (mm^2/cm^3)	223.3 ± 16.7	<0.025	373.9 ± 50.7
(3) Osteoclastic index ($no./mm^2$)	0.5 ± 0.2	N.S.	0.4 ± 0.1
(4) Total extent of resorption, active and inactive (%)	6.9 ± 0.6	N.S.	5.3 ± 0.4

Note. Controls have four rats per group, two tibia per rat. PTX group has three rats, two tibia per animal. N.S., not statistically different.

a high calcium diet was reversible if the calcium content of the diet was reduced. It seems likely that, in the absence of parathyroid hormone, bone surfaces accumulate excess calcium (and phosphate) which is included in the "exchangeable" compartment of bone. These calcium deposits in parathyroidectomized rats are constantly equilibrating with plasma calcium thus influencing its concentration. The deposits will be removed gradually when the dietary calcium is lowered. This conclusion is reinforced by the histological appearance of trabecular bone surfaces. We interpreted the cell type present and the absence of osteoid as an indication of more rapid deposition of mineral on new osteoid in parathyroidectomized rats.

A phenomenon which has never been explained adequately is the underlying cause of the C-cell hyperplasia which occurs in the thyroid glands of parathyroidectomized rats. This cannot be attributed to the calcium content of the diet or the plasma calcium levels per se. One avenue might be worthy of further investigation. Cooper *et al.* (13) reported that when the parathyroid glands were transplanted to sites removed from thyroid, there were differences in the postprandial handling of phosphate and calcium and in the postprandial rise in gastrin secretion. They suggested that the normally close anatomical apposition of the thyroid and parathyroid glands may be of some special significance for calcium control. Whether the hyperplasia of the C cells is due to the absence of parathyroid hormone per se or simply occurs after removal of the parathyroids to a site distant from the thyroidal C cells themselves remains to be determined.

The accumulation of trabecular bone in the tibial metaphysis in parathyroidectomized rats is of interest. Due to the continued activity of the epiphyseal plate in the rat long bone after sexual maturity is at-

tained, trabecular bone is continuously formed in these sites throughout most of the life of the animal. This bone is gradually remodeled by osteoclasts and osteoblasts. In normal rats the rate of osteoclastic activity exceeds that of osteoblastic activity so that the trabecular bone of the metaphysis is gradually resorbed and usually has remnants extending only to the junction of the metaphysis and the shaft of the long bone. Apparently, in the absence of parathyroid hormone, the remodeling process of metaphyseal trabecular bone continues, but with a sufficient change in ratio of resorption to formation to extend the life span of these trabeculae, including an increased width in those found in the primary and secondary spongiosa of the tibia. Unfortunately, changes in rates of bone resorption and bone formation were not studied. Additional studies using tetracycline markers should help determine these rates. Data are also needed to determine changes in activity of osteoclasts or osteoblasts, and the diagnosis of cell populations, as well as changes in rates of calcium influx and efflux from bone.

Therefore, one must be cautious in attributing the changes we observed solely to the absence of parathyroid hormone per se. The loss of parathyroid hormone causes a change in the calcium fluxes between the extracellular fluid and the bone fluid compartment which can change the calcium environment of the bone cells (14). Plasma phosphate levels also are higher. These two factors could change the activity of osteoblasts (15). Equally unknown is the relationship of calcitonin to these changes. Following parathyroidectomy, calcitonin accumulates in the thyroid and enters the blood despite lowered plasma calcium concentrations. How endogenous calcitonin influences bone cell function in the absence of parathyroid hormone is unknown. With these reservations, the skeletal changes observed after parathyroidectomy in the rat may provide a unique model for the study of the remodeling of trabecular bone.

Finally, our results demonstrate that control of the dietary calcium intake is necessary when calcium homeostasis or bone remodeling are studied. Rats adapt to a specific calcium intake by adjusting intestinal calcium absorption and by modifying internal processes regulated by parathyroid hormone, calcitonin and $1,25(OH)_2D_3$. However, controlled changes in dietary calcium intake provide an additional method for studying control of plasma calcium concentrations and for determining the effect of small changes in endogenous hormone concentrations on long-range changes in bone morphology.

This is to acknowledge the technical assistance of Dorothy B. Raneri and Johnny F. Obie. Thanks are also expressed to Linda Hobbs for her clerical assistance. The research was supported by grants NSF PCM-8007288 and NIH-AM-17743.

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Received December 28, 1981. P.S.E.B.M. 1982, Vol. 170.