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Effect of the Interaction of Parathyroid Hormone and Cyclosporine A on Bone Mineral Metabolism in the Rat

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Abstract. Cyclosporine A (CsA) induces high turnover osteopenia in the rat and there is evidence for this in humans. Recent studies suggest that increases in parathyroid hormone (PTH) may be involved in posttransplantation bone loss. However, human studies are difficult to interpret since transplant patients usually receive a cocktail of immunosuppressants and have underlying disease. Our aim was to try to resolve the influence of the absence or presence of PTH on CsA-induced bone disease. Male Sprague Dawley rats aged 7–9 months, either sham operated or parathyroidectomized (PTX), were randomly divided into vehicle and CsA groups. All PTX rats were given oral calcium supplementation *ad libitum.* The rats were divided into groups: basal, sham/vehicle, sham/CsA, PTX/vehicle, and PTX/CsA. Serial biochemistry was performed 0, 14, and 28 days after the start of the experimental period; bone histomorphometry was performed 28 days after the start of the experimental period. Statistical analysis consisted of group comparisons and factorial analyses. The results showed that CsA alone produced a high turnover osteopenia consistent with previous studies. In the PTX animals there was an increase in bone mass. PTX also decreased osteoblast activity and recruitment, and serum $1,25(OH)_2D$ levels. Serum levels of osteocalcin (BGP) were unaffected by PTX. The combination group (PTX/CsA) did not differ statistically from the controls in most of the histomorphometric parameters measured, with the exception of reduced mineral apposition and bone formation rates, reflecting the effects of PTX. Serum BGP and $1,25(OH)_2D$ levels did not differ, but PTH was reduced from the control. Explanations for these results are (1) CsA and PTX exert their effects via separate mechanisms, negating each other; (2) in the absence of PTH, CsA managed to cause bone loss, and thus PTH may not be essential for CsA-induced bone loss; or (3) the profound accelerated bone loss produced by CsA in normal rats requires PTH. These findings may help explain the discrepancies found in clinical studies where bone loss occurs with either elevated or normal PTH levels.

Key words: Cyclosporine A — Parathyroid hormone — Osteoporosis — Histomorphometry — Transplantation

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Many patients throughout the world suffer from chronic and life-threatening diseases of the heart, liver, kidney, bone marrow, and pancreas; organ transplantation has become the management of choice in treating these patients. Immunosuppressants in various combinations, have significantly altered the outcome of organ transplantation and survival [1–4]. However, they are not ideal drugs, as they possess numerous side effects and are known to play a major role in post-transplantation bone disease, most notably, osteoporosis [5].

Cyclosporin A (CsA) is used extensively in the prevention of organ rejection in transplantation. It is generally accepted that CsA causes bone loss in the human [3,6–12] contributing to posttransplantation bone disease. *In vivo* experiments by our laboratory have shown that CsA in the rat produces severe osteoporosis [5, 13, 14] that is dose and duration dependent [15]. Bone histomorphometry shows decreased percent trabecular bone volume, increased osteoclast number (increased resorption), and increased parameters of bone formation. Serum osteocalcin is also raised, reflecting the increased histomorphometric parameters of bone formation [15]. In addition, our laboratory has found that CsA increases 1α-hydroxylase activity thereby producing an increase in serum $1,25(OH)_2D$ [16].

It is thought that CsA-induced osteoporosis is mediated primarily via T-lymphocytes [17]. In addition, the critical role of the T lymphocyte has been recently confirmed by Zahner at. el. *in vitro* [18]. In rat bone, CsA has been shown to increase mRNA expression of IL-1 and IL-6, cytokines known to be involved in bone resorption [19].

Some investigators have shown that IL-6 is modulated by PTH [20–23], thus providing a possible link among parathyroid hormone (PTH), IL-6, CsA, and osteoporosis. However, there is controversy over IL-6's effect on osteoclastic resorption *in vitro* and *in vivo.* PTH has been proposed to be a major factor in posttransplantation bone disease in humans. Huang et al. [24] demonstrated that trabecular bone loss in human posttransplantation was associated with a statistically significant increase in serum PTH. Compston

et. al. [25] demonstrated a highly significant increase in plasma PTH levels at 1 and 2 months post-liver transplantation. However, an increase in PTH alone cannot be responsible for the early rapid bone loss as Thiebaud et. al. [11] demonstrated that after an initial decrease at 2 months postcardiac transplantation, there was a 90% increase in PTH levels at 18 months as compared with pretransplantation levels. In addition, Shane et al. [26] did not find an association between precardiac transplantation intact PTH levels and fractures. Human studies are difficult to interpret since transplant patients usually receive a cocktail of immunosuppressants, most commonly glucocorticoids, CsA, and azathioprine.

Thus, because of the debate surrounding the role of secondary hyperparathyroidism in posttransplantation bone disease, this study was designed to ascertain if there is, independent of other immunosuppressants, an interaction between CsA and PTH. We postulated that by decreasing PTH we would modify bone loss in CsA-treated rats, especially as PTH acts in combination with other cytokines, e.g., IL-6 which is a known bone resorber.

Materials and Methods

Animals

Fifty-nine male Sprague Dawley rats, aged 7–9 months and weighing between 510 and 610 g were purchased from Taconic Inc. (Germantown, NY, USA). All rats were housed, two to a cage, under similar conditions at 18–24°C in a 12-hour dark-light cycle and maintained on a diet of Agway Prolab RMH 3000 (Agway, Syracuse, NY, USA), containing 0.75% calcium, 0.85% phosphorus and vitamin-D_3 1045 IU/kg, and tap water *ad libitum.* Twenty-four rats underwent parathyroidectomy (PTX) by Taconic Inc. Calcium (Ca) supplementation (3 g/100 ml) in tap water was given to the parathyroidectomized animals *ad libitum* throughout the study.

Cyclosporine-A

CsA was kindly provided by Novartis Pharmaceuticals (East Hanover, NJ, USA) in a solution containing 100 mg CsA/ml and 10% alcohol by volume in olive oil. The CsA solution was diluted with pure alcohol-olive oil vehicle to yield a concentration of 10 mg/ml. CsA-treated rats received 10 mg/kg of this solution and vehicle-treated rats received 1 ml/kg of alcohol in olive oil by daily gavage.

The CsA dose is based on the therapeutic dose range for CsA in rats for heart, kidney, and liver transplantation and graft-versus-host disease [27], and previous studies showing that CsA at a dose of 7.5–15 mg/kg causes high turnover osteopenia [15, 28].

Experimental Protocol

All rats were randomized prior to surgery and, in addition, after the 10-day acclimatization period, the sham-operated and parathyroidectomized animals were divided randomly into vehicle and CsA groups. The experimental period of the study extended over 28 days. However, because of the surgery being performed by the vendor and the need for an acclimatization period, the surgery (sham and parathyroidectomy) was performed on day -18.

The rats were divided into five groups:

1. Basal Group: Normal rats that received none of the experimental modalities (n = 10)
2. Group A (Sham/Vehicle): Sham operated 18 days prior to the start of the experiment, and received CsA vehicle in equivalent volume per kilogram of body weight by daily oral gavage for 28 days (n = 12)
3. Group B (Sham/CsA): Sham operated 18 days prior to the start of the experiment, and received CsA 10 mg/kg by daily oral gavage for 28 days (n = 13)
4. Group C (PTX/Vehicle): Parathyroidectomized 18 days prior to the start of the experiment, and received CsA vehicle in equivalent volume per kilogram body weight by daily gavage for 28 days (n = 8)
5. Group D (PTX/CsA): Parathyroidectomized 18 days prior to the start of the experiment, and received CsA 10 mg/kg by daily oral gavage for 28 days (n = 9).

Note, in addition to the above animals, seven rats from the parathyroidectomized groups were eliminated after the conclusion of the experiment because of incomplete PTX, this was determined by serum PTH and Ca values.

During the acclimatization period, the basal group received double labeling, for histomorphometry, of demeclocycline (Sigma, St. Louis, MO, USA) 15 mg/kg subcutaneously (SQ) on days -10 and -9, and calcein (Sigma) 10 mg/kg SQ on days -2 and -1. These rats were sacrificed on day 0 of the study to obtain basal values for histomorphometric analysis. All the experimental animals received double labeling of demeclocycline (*Sigma*) 15 mg/kg SQ on days 18 and 19, and calcein (*Sigma*) 10 mg/kg SQ on days 26 and 27, for histomorphometric determination of kinetic parameters of bone turnover. The rats were weighed and bled on days 0 and 14 under ketamine HCI (Fort Dodge Laboratories Inc., Fort Dodge, IA, USA) 100 mg/kg and acepromazine maleate (Fermenta Animal Health Co., Kansas City, MO, USA) 1 mg/kg anesthesia. The rats were sacrificed by cardiac puncture on day 28 under ketamine HCI anesthesia. Xylazine and acepromazine anesthesia were avoided since it has been shown that the combination of ketamine with xylazine and ketamine with acepromazine in rats can cause a marked elevation of serum PTH levels [29]. On days 0 and 14, blood was obtained by retroorbital venous sinus puncture and on day 28, the rats were bled by cardiac puncture. Blood was centrifuged and the serum stored at −70°C.

In order to confirm the successful outcome of the surgery, and the lack of parathyroid hyperplasia post-parathyroidectomy, the parathyroidectomized rats did not receive supplemental calcium 12 hours prior to bleeding on days 0 and 28. They did, however, receive supplemental calcium prior to bleeding on day 14.

Experimental procedures were reviewed and approved by the institutional animal care committee in accordance with the policy of the NIH, and the animals were cared for according to the principles outlined in the NIH guide for the care and use of laboratory animals.

Assays

Serum osteocalcin (BGP) was measured by polyclonal radioimmunoassay (RIA) using a modification of a previously described assay [30]. Antibody to rat BGP was raised in rabbits, immunized by multiple-site intradermal injections of purified rat BGP, and used in a final dilution of 1:50,000. Purified rat BGP was used for the preparation of standards and ^{125}I-labeled tracer. An equilibrium assay was performed and antibody/BGP complexes were separated from free iodine labeled BGP by using goat antirabbit gamma globulin (CalBiochem, La Jolla, CA, USA). These complexes were counted with a Beckman 5500 gamma counter (Beckman, Irvine, CA, USA). The lower limit for the detection of the assay is 0.5 ng/ml. We have used this assay extensively [31–34].

Serum 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) was assayed using a commercially available kit (Incstar Corporation, Stillwater, MN, USA). Vitamin D metabolites were extracted and purified using C_{18}OH and silica cartridges. The assay was then performed using a competitive RIA procedure which is based on a polyclonal antibody that is specific for both $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$. Coefficients of variation, quoted by the manufacturer, are 9.7–14.1% for intraassay precision and 14.7–26.0% for interassay precision.

Serum PTH was measured by a two-site immunoradiometric assay (IRMA) using a commercially available kit (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA), which utilizes two different goat antibodies to the N-terminal region (1–34) of rat PTH; one antibody immobilized onto plastic beads to capture the PTH molecules, and the other antibody radiolabeled for detection. The immobilized antibody binds both PTH (1–84) and N-terminal PTH (1–34). Coefficients of variation, quoted by the manufacturer, are 4.0–4.3% for intraassay precision and 4.3–4.7% for interassay precision. Our laboratory has previously confirmed the validity of this assay in the rat [35]. Serum blood urea nitrogen (BUN), creatinine, total Ca, phosphate, and alkaline phosphatase (ALP) were measured in serum samples by a photometric procedure using a Boehringer Mannheim/Hitachi 747-100 Automatic Analyzer (Boehringer Mannheim, Indianapolis, IN, USA). Serum BUN was analyzed by a coupled urease and GLDH enzymatic procedure. Serum creatinine was analyzed by employing a substrate triggered, rate method, utilizing a modification of the Jaffé reaction. Serum total Ca was determined by a sample blanked, endpoint method using EDTA reversal of the complexation of calcium with o-cresolphthalein complexone. Serum phosphate was measured by a sample blanked, endpoint method using acidic ammonium phosphomolybdate, and serum ALP was analyzed by a q-nitrophenylphosphate-triggered rate method.

Histomorphometry

At the time of sacrifice the right tibia was removed, dissected free of soft tissue, and fixed in 70% reagent alcohol. The proximal third of each tibia was stained with Villanueva bone stain (Polysciences Inc., Warrington, PA, USA), dehydrated in graded concentrations of ethanol, defatted in acetone, and then embedded undecalcified in methyl methacrylate (Eastman Organic Chemicals, Rochester, NY, USA). The bone was cut in the frontal longitudinal plane using a low-speed metallurgical saw, ground to a thickness of 20 μm, and then mounted on microscope slides for morphometric measurements. A digital image analysis system consisting of an epifluorescent microscope, a digitizing pad (Summagraphic, Fairfield, CT, USA) coupled to an Apple Macintosh SE computer, and the morphometry program Stereology (KSS Computer Engineers, Magna, UT, USA) was used for the quantitative measurements of the proximal tibial metaphysis. This measured area was restricted to the trabecular bone of the secondary spongiosa between 1 and 4 mm distal to the growth plate-metaphyseal junction. The following histological parameters were calculated as previously described [36, 37]: percent trabecular area (%Tb.Ar), trabecular width (Tb.Wi), trabecular number (Tb.N), trabecular separation (Tb.Sp), percent mineralizing surface (%MS), mineral apposition rate (MAR), bone formation rate (tissue volume referent)(BFR/TV), bone formation rate (bone volume referent) (BFR/BV), and percent eroded perimeter (%E.Pm).

Statistical Analysis

Statistical analyses were performed using the statistics package SPSS Release 7.0 for Windows 95 (SPSS Inc., Chicago, IL, USA). Statistical significance for body weight and biochemical parameters measured on days 0, 14, and 28 were determined by repeated measures multivariate analysis of variance (MANOVA) where "time" was the repeated factor. In certain circumstances (serum BGP, 1,25-$(OH)_2$vitamin D, BUN, total Ca, and phosphate) statistical interactions occurred between the groups being analyzed and the repeated factor "time", thus invalidating the repeated measures model. Therefore, in these circumstances, statistical significance was determined by using one-way ANOVA (with Bonferroni post hoc tests) at each of the time points (days 0, 14, and 28).

Serum PTH was measured on day 28 only and hence statistical significance was determined by using a one-way ANOVA (with Bonferroni post hoc tests). Similarly, the bone histomorphometric data were obtained on day 28 only. Analysis was performed using a two-step procedure. Firstly, the data obtained from the basal group (sacrificed on day 0) were compared with the experimental control group (Sham/Vehicle, sacrificed on day 28), by using independent samples *t*-tests, to ensure that the vehicle used did not modify baseline histomorphometric indices, thereby ensuring that the vehicle group was an adequate control. Secondly, each of the experimental groups was compared with control (Sham/Vehicle) using a one-way ANOVA (with Bonferroni post hoc tests) for each variable.

In addition to the above group analyses, 2 × 2 factorial analyses (FA) were performed to assess the main effects of PTX and CsA, as well as any statistical interaction between these two factors.

Further intergroup analyses were done for each variable by ANOVA (with Bonferroni post hoc tests) at each time point, comparing Sham/CsA with PTX/Vehicle groups, Sham/CsA with PTX/CsA groups, and PTX/Vehicle with PTX/CsA groups. The results from these analyses were noncontributory and have not been included.

Throughout all analyses, a P value of less than 0.05 ($P < 0.05$) was considered a statistically significant difference. All values and graphs are expressed as means ± standard error of the mean (SEM).

Results (Table 1)

Body Weight

There were no statistically significant differences between the groups. However, there was a general decrease in weight over the course of the experiment ($P < 0.0005$).

Serum Osteocalcin (BGP)

Compared with control (Sham/Vehicle), the Sham/CsA group had increased BGP levels on day 14 ($P < 0.0005$) and day 28 ($P < 0.01$). The Sham/Vehicle, PTX/Vehicle, and combination groups were statistically indistinguishable.

Serum 1,25$(OH)_2$D

Compared with control (Sham/Vehicle), the Sham/CsA group had increased 1,25$(OH)_2$D levels on day 14 ($P = 0.001$) and day 28 ($P < 0.05$). Factorial analysis revealed that PTX was associated with reduced 1,25$(OH)_2$D on day 14 ($P = 0.001$) and day 28 ($P < 0.0005$). The combination group was statistically indistinguishable from the control group. No statistical interactions occurred in these factorial analyses, thus all effects seen represent the true effects of CsA and PTX.

Serum BUN

Compared with control, the Sham/CsA group had raised BUN levels on days 14 and 28 ($P < 0.0005$). Compared with control, the PTX/Vehicle group had a raised serum BUN on day 28 only ($P < 0.05$). Compared with control, BUN was also increased in the combination group on days 14 and 28 ($P < 0.0005$).

Serum Creatinine

All groups were statistically indistinguishable. However,

Table 1. Weight and serum biochemistry

Parameter	Day	Sham/Veh	Sham/CsA	PTX/Veh	PTX/CsA
Weight (g)	0	570.3 ± 5.6	567.0 ± 6.1	570.1 ± 9.8	572.1 ± 8.4
	14	563.5 ± 5.9	550.8 ± 7.3	575.3 ± 9.3	561.1 ± 7.6
	28	538.8 ± 6.9	520.7 ± 11.8	539.3 ± 8.4	537.8 ± 7.2
BGP (ng/ml)	0	49.2 ± 4.1	42.6 ± 2.2	48.0 ± 0.9	37.8 ± 5.2
	14	33.4 ± 2.0	52.8 ± 4.0[a]	35.5 ± 1.3	44.1 ± 4.2
	28	44.2 ± 3.4	64.55 ± 4.7[c]	37.8 ± 1.9	55.8 ± 3.3
$1,25(OH)_2D$ (pg/ml)	0	21.8 ± 1.6	22.1 ± 2.2	20.0 ± 2.2	26.0 ± 4.3
	14	30.2 ± 3.3	63.8 ± 8.3[b]	18.5 ± 1.6[f]	30.7 ± 4.2
	28	28.8 ± 4.3	61.4 ± 11.2[d]	10.1 ± 1.4[e]	13.9 ± 1.9
BUN (mg/dl)	0	14.5 ± 0.7	15.4 ± 0.5	17.2 ± 0.6	16.0 ± 0.6
	14	17.5 ± 0.6	25.4 ± 1.1[a]	21.4 ± 0.4	27.1 ± 1.0[a]
	28	15.1 ± 0.2	23.7 ± 1.1[a]	19.5 ± 1.0[d]	24.0 ± 1.2[a]
Creatinine (mg/dl)	0	0.4 ± 0.02	0.4 ± 0.01	0.4 ± 0.02	0.4 ± 0.03
	14	0.4 ± 0.02	0.4 ± 0.02	0.4 ± 0.00	0.4 ± 0.03
	28	0.5 ± 0.02	0.5 ± 0.02	0.5 ± 0.03	0.6 ± 0.04
Total calcium (mg/dl)	0	9.2 ± 0.1	9.1 ± 0.1	7.9 ± 0.5[c]	7.6 ± 0.2[a]
	14	9.5 ± 0.1	9.2 ± 0.1	9.3 ± 0.2	8.4 ± 0.1[a]
	28	9.0 ± 0.1	9.0 ± 0.1	8.4 ± 0.1[e]	8.1 ± 0.4[d]
Phosphate (mg/dl)	0	7.3 ± 0.3	6.5 ± 0.1	9.6 ± 0.1[a]	9.3 ± 0.3[a]
	14	6.4 ± 0.1	6.9 ± 0.1	8.8 ± 0.2[a]	9.9 ± 0.3[a]
	28	7.7 ± 0.1	7.5 ± 0.2	9.8 ± 0.4[a]	11.2 ± 0.4[a]
ALP (IU/liter)	0	72.9 ± 5.5	77.9 ± 4.0	88.1 ± 6.1	89.0 ± 7.0
	14	122.5 ± 6.8	116.7 ± 5.4	132.0 ± 8.0	152.3 ± 8.5[d]
	28	53.3 ± 1.6	53.7 ± 3.5	57.8 ± 4.2	72.4 ± 4.3[d]

Weight and serum results measured as detailed under Materials and Methods. All results expressed as mean ± SEM
Veh = vehicle; BGP = osteocalcin (bone gla protein); $1,25/(OH)_2D$ = 1,25 dihydroxyvitamin D; BUN = blood urea nitrogen; ALP = alkaline phosphatase
[a] $P < 0.0005$, [b] $P \leq 0.001$, [c] $P < 0.01$, [d] $P < 0.05$ vs. sham/vehicle
[e] $P < 0.0005$, [f] $P \leq 0.001$ (main effect of PTX, factorial analysis vs. sham)

there was a statistical difference over time ($P < 0.0005$), with a uniform increase in serum creatinine by the end of the experiment.

Serum Total Calcium

Serum total calcium levels at all time points were statistically indistinguishable in the sham-operated groups (A and B). On day 0 of the experiment (18 days post-parathyroidectomy), serum total calcium, without calcium supplementation in the 12 hours prior to bleeding, was decreased in the PTX groups (C and D) (as compared with control) ($P < 0.01$). On day 14, with calcium supplementation, the PTX/Vehicle group was statistically indistinguishable from the control group (Sham/Vehicle); the combination group, however, still had a statistically significant drop in serum total calcium (as compared with control) ($P < 0.0005$). On day 28, calcium supplementation was not given during the 12 hours prior to bleeding; PTX was associated with a decrease in serum total calcium (FA, $P < 0.0005$) and the combination group had a serum total calcium that was statistically significantly lower than the control group ($P < 0.05$).

Serum Phosphate

Serum phosphate levels at all time points were statistically indistinguishable in the sham-operated groups (A and B). At all time points, the PTX/Vehicle and combination groups had increased serum phosphate levels as compared with control ($P < 0.0005$).

Serum ALP

Serum ALP was increased in all groups on day 14 and had decreased in all groups by day 28 (MANOVA time factor, $P < 0.0005$). The Sham/Vehicle, Sham/CsA, and PTX/Vehicle groups were statistically indistinguishable throughout the study; the combination group was consistently higher than the control group on days 14 and 28 ($P < 0.05$).

Serum PTH (Fig. 1)

PTH was measured on day 28 only. Compared with control, serum PTH was decreased in the PTX groups (C and D) ($P < 0.0005$). There were no statistical differences between the sham groups (A and B).

Histomorphometry (Table 2)

There were no statistical differences between the basal group and the control (Sham/Vehicle) group ($P > 0.05$).

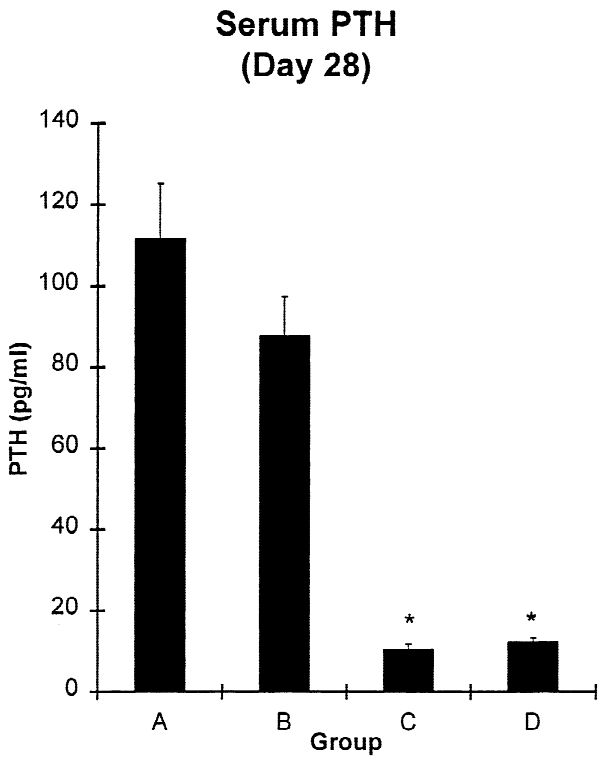

Fig. 1. Serum parathyroid hormone (PTH) measured on day 28 as detailed under Materials and Methods. **(A)** Sham/Vehicle Group **(B)** Sham/CsA Group, **(C)** PTX/Vehicle Group, **(D)** PTX/CsA Group. All values are mean ± SEM. $^{*}P \leq 0.0005$ vs. Sham/Vehicle, Sham/CsA.

First, the structural parameters were analyzed. CsA was associated with a reduced bone mass (%Tb.Ar) (FA, $P < 0.0005$), decreased trabecular number (FA, $P = 0.001$), and increased trabecular separation (FA, $P < 0.01$). Compared with control, the PTX/Vehicle group had an increased bone mass (%Tb.Ar) ($P < 0.01$) and an increased trabecular width ($P < 0.01$). In addition, PTX was associated with an increased trabecular number (FA, $P < 0.05$) and decreased trabecular separation (FA, $P < 0.05$). The combination group showed no statistical differences when compared with the Sham/Vehicle control group.

With regard to the kinetic histomorphometric indices, the Sham/CsA group, as compared with controls, had an increased percent mineralizing surface ($P < 0.0005$), mineral apposition rate ($P < 0.05$), bone formation rate/tissue volume ($P = 0.001$), and bone formation rate/bone volume ($P < 0.0005$). Compared with control, the PTX/Vehicle group had a decreased percent mineralizing surface ($P < 0.0005$), mineral apposition rate ($P < 0.0005$), bone formation rate/tissue volume ($P < 0.0005$), and bone formation rate/bone volume ($P < 0.01$). When compared with the controls, the combination group showed a decrease in mineral apposition rate ($P < 0.01$) and bone formation rate/tissue volume ($P < 0.01$). All other comparisons between the combination group and the control groups were not statistically significant. No statistical interaction occurred in the factorial analysis of the bone histomorphometry, and thus all effects seen represent the true main effects of CsA and PTX.

Discussion

When considered independently, these studies show that PTX and CsA have dramatic and contrasting effects on the circulating biochemical and histomorphometric parameters of bone mineral metabolism in the rat.

This study is consistent with both our previous work and the work of others [15, 28, 32, 38] showing that in the rat, CsA alone produces a high turnover osteopenia with a decreased bone mass, increased osteoblast activity and recruitment, and increased serum BGP and $1,25(OH)_2D$. In this experiment, histomorphometric kinetic parameter of resorption was not affected by CsA (% E.Pm not statistically significant), although overall CsA was associated with a decreased Tb.Ar, Tb.N, and increased Tb.Sp which translates into less bone, therefore indicating an increased net resorptive process, as impaired bone formation leading to less bone is unlikely given the increased osteoblastic activity.

The BGP values probably reflect an increase in bone remodeling and not only formation [40], i.e., both osteoblastic and osteoclastic activity that is not translated into a net increase in bone volume (mass), as resorption exceeded formation. The raised serum BGP may also reflect the increased $1,25(OH)_2D$ levels [41, 42]. Another explanation of the raised BGP is the presence of impaired renal function. Serum BUN was increased above the reference range (15–21 mg/dl) [43]. However, this latter explanation is unlikely as serum creatinine, a more accurate reflection of renal function, did not differ between the groups.

PTX was confirmed by markedly reduced PTH levels in the parathyroidectomized rats, and by the alteration in serum total Ca and phosphate. PTX resulted in an increase in bone mass (%Tb.Ar). However, it also decreased osteoblast activity and recruitment, and serum $1,25(OH)_2D$ levels. Serum levels of BGP were unaffected by PTX. The increase in bone mass is possibly related to the decrease in activation frequency with the known effect of PTH increasing remodeling negated in hypoparathyroidism. These effects on bone mineral metabolism are in keeping with known PTH physiology and the clinical situation of hypoparathyroidism and increased bone density [44, 45].

The effects of CsA and PTX on bone mineral metabolism were mostly opposite to one another, and the combination group D (PTX/CsA) showed little deviation from the control group (Sham/Vehicle). The combination group had no statistical change, from control, in structural histomorphometric parameters (%Tb.Ar, Tb.Wi, Tb.N, and Tb.Sp), histomorphometric resorption parameter (%E.Pm), and some of the formation indices (%MS and BFR/BV). However, there was a reduction in MAR and BFR/TV representing reduced osteoblast activity and recruitment, reflecting the effects of PTX. Though serum BGP and $1,25(OH)_2D$ levels were indistinguishable with the control, there was a reduced serum PTH level, reflecting the effect of PTX.

Table 2. Effect of PTX and CsA on bone histomorphometry

Parameter	Basal	Sham/Veh	Sham/CsA	PTX/Veh	PTX/CsA
Structural parameters					
Percent trabecular area (%Tb.Ar%)	13.9 ± 0.9	13.6 ± 1.3	10.3 ± 0.6[e]	19.9 ± 1.0[c]	13.1 ± 0.9
Trabecular width (μml)	59.8 ± 2.0	54.8 ± 2.7	54.6 ± 1.9	68.5 ± 2.9[c]	60.1 ± 3.1
Trabecular number (#/mm)	2.4 ± 0.2	2.4 ± 0.2	1.9 ± 0.1[f]	2.9 ± 0.2[i]	2.2 ± 0.2
Trabecular separation (μm)	384.2 ± 27.6	391.1 ± 45.5	511.5 ± 41.6[g]	280.5 ± 16.8[i]	426 ± 55.4
Formation kinetic parameters					
Percent mineralizing surface	29.1 ± 1.6	32.8 ± 2.4	50.8 ± 2.5[a]	11.1 ± 1.2[a]	24.9 ± 2.7
Mineral apposition rate (μm/day)	0.7 ± 0.08	0.8 ± 0.05	1.1 ± 0.07[d]	0.4 ± 0.05[a]	0.6 ± 0.03[c]
Bone formation rate/tissue Volume (BFR/TV %/year)	31.9 ± 5.9	40.0 ± 4.0	61.0 ± 4.7[b]	8.3 ± 2.0[a]	17.7 ± 1.9[c]
Bone formation rate/bone Volume (BFR/BV %year)	227.3 ± 36.7	339.3 ± 59.1	631.4 ± 58.1[a]	40.4 ± 8.3[c]	142.5 ± 19.1
Resorption kinetic parameters					
Percent eroded perimeter (%)	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.05	0.6 ± 0.1

Tibial histomorphometry measured at day 28, as detailed under Materials and Methods. All results are expressed as mean ± SEM. Veh = Vehicle

[a] $P < 0.0005$, [b] $P \leq 0.001$, [c] $P < 0.01$, [d] $P < 0.05$ vs. Sham/Vehicle

[e] $P < 0.0005$, [f] $P \leq 0.001$, [g] $P < 0.01$ (main effect of CsA, factorial analysis vs. vehicle).

[i] $P < 0.05$ (main effect of PTX, factorial analysis vs. sham)

This study showed that $1,25(OH)_2D$ was increased by CsA and decreased by PTX. We have previously shown the increase in 1α-hydroxylase activity and the significant elevations in serum $1,25(OH)_2D$ levels [16, 32, 33]. It is also known that PTH stimulates 1α-hydroxylase activity [46]. It is therefore possible that PTX decreased 1α-hydroxylase activity producing the decrease in $1,25(OH)_2D$ seen in this study. Thus, it seems that the effect of combined CsA and PTX on 1α-hydroxylase activity produces a level of activity similar to the control which may imply that despite a near absence of PTH, CsA can still promote $1,25(OH)_2D$ production, or alternatively, that the lack of PTH "may blunt" CsA's stimulating action on 1α-hydroxylase.

Serum total Ca, without supplementation in the 12 hours prior to bleeding, was decreased in the PTX groups, confirming the effect of the PTX. In addition, serum phosphate was elevated in both the PTX/Vehicle and the combination groups. Since increased PTH induces phosphaturia [47], it is possible that the increase in serum phosphate is due to PTX-induced increase in renal phosphate reabsorption, as seen in the clinical situation. The increase in serum phosphate may also be due to decreased renal clearance as a consequence of CsA, but this is unlikely given that the CsA/Sham group did not manifest this.

We have no conclusive explanation for the increase in serum ALP on day 14, nor the fact that the combination group had a higher serum ALP than the other groups. However, a study by Loertscher et al. [6] demonstrated a persistent elevation of ALP in renal transplant recipients after CsA treatment. This ALP was thought to be of osseus origin, thus reflecting increased bone formation. Hypoparathyroidism, however, is not usually associated with alterations in ALP [48]. It is also possible that the alterations in serum ALP are due to contributions from sources other than bone, because bone-specific ALP was not measured. In addition, it is known that ALP does not always mirror BGP as seen in Paget's disease of bone where ALP is disproportionately higher than BGP [49].

This study shows that CsA and PTX have generally opposite effects on bone mineral metabolism, producing, respectively, either high turnover osteopenia or increased bone density, thus negating the individual effects of each other and resulting in a histomorphometric picture that is statistically indistinguishable with the control group.

One explanation of this effect is that CsA and PTX exert their effect via separate mechanisms, negating each other, resulting in a net effect that is similar to control. Another explanation is that to demonstrate the profound accelerated bone loss (30–40% of cancellous bone) produced by CsA in normal rats, normal circulating levels of PTH must be present. This is in accord with experiments where no increase in circulating PTH (i.e., normal levels) have been found with CsA administration [28, 50, 51], yet major bone loss occurs. In the clinical situation with transplant recipients, there is conflicting data as either PTH levels are normal, relatively increased, or even low compared with controls

[11, 24–26], and rapid and severe bone loss with fractures occur. A permissive role or a synergistic sensitizing effect in the face of normal PTH and CsA administration may be possible with IL-6 being a mediator of bone resorption as both CsA and PTH increase IL-6 locally [19, 20, 23]. Thus, with PTX, the lack of PTH lessens the role of IL-6 and prevents or modifies resorption of bone.

One of the ways to substantiate the role of PTH would be to administer PTH and CsA to the PTX rat and observe whether changes are produced that are equivalent to CsA treatment of nonparathyroidectomized rats. However it would be difficult to assess the ideal dose of PTH to administer as a physiological replacement, and the dose of PTH would need to be given intermittently to mimic the circadian rhythm of parathyroid hormone secretion [52, 53].

In conclusion, recent studies have suggested that PTH, as part of secondary hyperparathyroidism, may be involved in posttransplantation bone disease, which is usually a high turnover, rapid remodeling disease in humans. This secondary hyperparathyroidism may in turn be due to the cocktail of immunosuppressants used to prevent organ rejection. This study has shown that there is an interaction between CsA and circulating PTH levels resulting in a modified histomorphometric picture with PTX, as compared with CsA alone. This may indicate that controlling posttransplantation levels of PTH (if elevated) may decrease the rapid remodeling high turnover state and lessen the risks and effects of posttransplantation bone disease. Further studies are needed to elucidate the mechanisms of the PTH-CsA interaction.

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