Effects of cyclic vs. daily treatment with human parathyroid hormone (1–34) on murine bone structure and cellular activity

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Abstract

Previously, we demonstrated that the human parathyroid hormone (1–34) fragment (hPTH(1–34)) increased bone strength in proportion to its effects on BMD and cortical bone structure in the murine femur by comparing cyclic vs. daily administration of hPTH(1–34). Both cyclic and daily regimens increased vertebral BMD similarly at 7 weeks. Here, we have examined the effects of daily and cyclic PTH regimens on bone structure and cellular activity by static and dynamic histomorphometry.

Twenty-week-old, intact female C57BL/J6 mice were treated with the following regimens (n = 7 for each group): daily injection with vehicle for 7 weeks [control]; daily injection with hPTH(1–34) (40 μg/kg/day) for 7 weeks [daily PTH]; and daily injection with hPTH(1–34) (40 μg/kg/day) and vehicle alternating weekly for 7 weeks [cyclic PTH]. At days 9 and 10, and 2 and 3 prior to euthanasia, calcein (10 mg/kg) was injected subcutaneously. At the end of study, the lumbar vertebrae 1–3 and the left femora were excised, cleaned, and processed for histomorphometry.

In the lumbar vertebrae, daily and cyclic PTH regimens significantly increased cancellous bone volume (BV/TV), trabecular number, trabecular osteoclast and osteoblast perimeters, trabecular mineral apposition rate (MAR) and bone formation rate (BFR), and periosteal MAR and BFR compared to control, with no significant difference between the two PTH-treated groups. Increased trabecular tunneling was observed in both PTH-treated groups. Both regimens tended to increase vertebral cortical bone formation parameters with the effects at the periosteum site being more marked than those at the endosteum site, resulting in a significant increase in cortical width. In the femur, the effects of cyclic PTH on BV/TV, trabecular width and number, trabecular and endocortical osteoblast and osteoclast perimeters, cortical width, and trabecular and periosteal BFR were less marked than those of daily PTH. A cyclic PTH regimen was as effective as a daily regimen in improving cancellous and cortical bone microarchitecture and cellular activity in the murine vertebra.

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Introduction

The amino terminal fragment of human parathyroid hormone (1–34) (hPTH(1–34)) is the only US FDA-approved anabolic agent for the treatment of osteoporosis [1–4]. However, its anabolic effect plateaus over time [1–3, 5–7]. To explore more efficient use of PTH, protocols with repeated cycles of on and off daily injections of hPTH(1–34) (cyclic PTH) have been developed in humans [8] and mice [9], and their efficacy on BMD and other bone variables has been compared with daily treatment. In humans, a cyclic regimen with repeated cycles of 3-month on and off daily injection of hPTH(1–34) (25 μg/day) has been shown to be as effective as daily PTH at improving vertebral bone mineral density (BMD) at 15 months in the presence of ongoing alendronate treatment [8]. In mice, we have demonstrated that a cyclic regimen with repeated cycles of 1 week on and off daily PTH injection (40 μg/kg/day) produced significant increases in BMD, cortical width, and periosteal...
carried out in a region 0.08 mm from the cranial and caudal growth plates. A one-week on and off cycle was chosen because we recently demonstrated that daily injection of hPTH(1–34) for 2 weeks markedly increased the bone resorption marker, mouse tartrate-resistant acid phosphatase (mTRACP) to a level produced by continuous PTH infusion [10]. Moreover, the effect of PTH treatment on femoral bone strength was proportional to its effects on BMD, bone markers, and bone structure [9]. As in our clinical study, both cyclic and daily PTH regimens increased vertebral BMD similarly despite the fact that less than 60% of the total daily PTH dose was given in the cyclic group [8]. However, it is well established that factors other than BMD influence bone strength and resistance to fracture [11–13]. The purpose of the present investigation was, therefore, to compare the effects of cyclic vs. daily PTH administration on cancellous and cortical bone microarchitecture and cellular activity in the mouse spine and femur.

Materials and methods

Materials

hPTH(1–34) was purchased from Bachem (Torrance, CA). Calcine, ketamine, xylene, and the reagents for processing and staining undecalcified bones, including 100% ethanol, toluidine blue, eosin, hematoxylin, silver nitrate, and sodium carbonate formaldehyde were purchased from Sigma Chemicals, Co. (St. Louis, MO). Methylmethacrylate and toluene were obtained from Fisher Scientific (Fairlawn, NJ).

Animals

The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Helen Hayes Hospital. Twenty-one, virgin female C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME) at 8 weeks of age and stabilized at the Animal Research Facility of Helen Hayes Hospital. At 20 weeks of age, animals were weighed and randomly divided into the following 3 groups: daily injection with vehicle for 7 weeks [control]; daily injection with hPTH(1–34) (40 μg/kg/day) for 7 weeks [daily PTH]; and a weekly alternating regimen of hPTH(1–34) and vehicle for 3 and 1/2 cycles [cyclic PTH] [9]. Animals were housed 3 or 4 per cage, given free access to water, and fed a standard diet (Purina Mills, St. Louis, MO) in a room maintained at 22°C with 60–75% humidity on 12-h light/dark cycles. One mouse in the control group died of unknown cause after 3 weeks of treatment. For dynamic histomorphometric analysis, animals were injected with calcine subcutaneously (10 mg/kg) at 9 and 10, and 2 and 3 days prior to euthanasia [14]. Animals were euthanized under anesthesia with a mixture of ketamine (100 mg)/xylene (3 mg/kg) at 20 to 24 h after the last PTH injection. Left femurs and lumbar vertebrae (1–3) were excised, cleaned and fixed in 10% formaldehyde for at least 48 h before processing.

Histomorphometric analysis

Excised left distal femurs and 2nd lumbar vertebrae were cut longitudinally with a low speed metallurgical saw to expose the bone marrow, dehydrated in graded ethanol, defatted in toluene, and embedded in methylmethacrylate. Five- and 10-μm-thick sagittal sections of the central regions of distal femurs and lumbar vertebral bodies were cut with a Polycut microtome (Reichert-Jung, Germany). Five-micrometer-thick sections were stained with toluidine blue for the static histomorphometric analysis. Ten-micrometer sections were left unstained for the dynamic analysis. Histomorphometry of the distal femurs was performed on the trabecular and cortical bone in an area between 0.16 mm and 1.2 mm distal to the growth plate. Histomorphometry of lumbar vertebrae was carried out in a region 0.08 mm from the cranial and caudal growth plates. Cortical width, trabecular bone area and perimeter, and trabecular osteoid, osteoblast and osteoclast perimeters and dynamic variables were measured using a digitizing image analysis system and a morphometric program, OsteoMeasure (OsteoMetrics, Inc., Atlanta, GA), at a magnification of 200× [14]. Labeled perimeter (L.Pm) was defined as (0.5 × single labeled perimeter + double labeled perimeter)/bone perimeter [%] and bone formation rate (BFR) was calculated as L.Pm × mineral apposition rate (MAR). All parameters were calculated and expressed according to standard formulae and nomenclature [15].

von Kossa staining

Five-micrometer-thick sections were stained in 5% silver nitrate for 30 min in the dark, and then reduced in 5% sodium carbonate-formaldehyde for 2 min after rinsing with 3 changes of distilled water. Before mounting on the slides, the sections were washed gently in running water for 10 min and air-dried.

Statistical analysis

All the values represent mean ± SEM. The significance of differences among groups was determined by two-way analysis of variance (ANOVA, Duncan) using SAS (Cary, NC, 9.1 version).

Results

Lumbar vertebrae

Cancellous bone

Both cyclic and daily PTH regimens significantly and almost equally increased cancellous bone volume (BV/TV) and trabecular number, and decreased trabecular separation (Figs. 1A–C). However, there was no significant effect on trabecular width with either regimen (Fig. 1D). Both cyclic and daily PTH regimens significantly and almost equally increased trabecular osteoblast and osteoclast perimeters (Fig. 1E and F). Moreover, both regimens significantly increased mineral apposition rate (MAR) and bone formation rate (BFR) to a similar degree (Figs. 1H and I).

Trabecular tunneling was observed in the lumbar vertebrae of both PTH-treated groups (Figs. 2B and C).

Cortical bone

Both regimens significantly increased cortical width to a similar degree (Fig. 3A), and markedly increased endocortical osteoblast and osteoclast perimeters to a similar extent (Figs. 3B and C). At both endocortical and periosteal sites, both regimens tended to increase all bone formation parameters including labeled perimeter (Figs. 3D and G), mineral apposition rate (Figs. 3E and H), and bone formation rate (Figs. 3F and I). The effects of PTH on these bone formation parameters at the periosteal surface (G–I) were more marked than those at the endocortical surface (D–F, Fig. 3). At the periosteal site, the effects of cyclic PTH on the labeled perimeter and bone formation rate were more marked than those of daily PTH (Figs. 3G and I).

Distal femur

Cancellous bone

Daily PTH increased BV/TV, trabecular number, and trabecular width by 333%, 203%, and 49% (all p<0.01 vs.
control), respectively, and decreased trabecular separation by 72% (Figs. 4A–D). Cyclic PTH also increased BV/TV, trabecular number (Tb.N), trabecular width (Tb.Wi), trabecular osteoblast perimeter (Tb.Ob.Pm), and osteoclast perimeter (Tb.Oc.Pm), trabecular labeled perimeter (Tb.L.Pm), mineral apposition rate (Tb.MAR), and bone formation rate (Tb.BFR) in the murine lumbar vertebrae. Animals were treated and bone specimens processed as described in Materials and methods. Values are mean±SEM of 6 (control) and 7 (PTH groups) replicates.

Daily PTH significantly increased femoral cancellous osteoblast and osteoclast perimeters, while the effects of cyclic PTH on those variables were not significant (Figs. 4E and F). Both regimens markedly increased all cancellous bone formation parameters including labeled perimeter, mineral apposition rate, bones, increased trabecular tunneling was observed in the femur in the PTH-treated groups (Figs. 2D–F).

Daily PTH significantly increased femoral cancellous osteoblast and osteoclast perimeters, while the effects of cyclic PTH on those variables were not significant (Figs. 4E and F). Both regimens markedly increased all cancellous bone formation parameters including labeled perimeter, mineral apposition rate,
Fig. 3. Effects of cyclic vs. daily PTH on cortical bone structure including cortical width (Ct.Wi, A), endocortical osteoblast (Ec.OB.Pm, B) and osteoclast perimeters (Ec.OC.Pm, C), endocortical labeled perimeter (Ec.L.Pm, D), mineral apposition rate (Ec.MAR, E), and bone formation rate (Ec.BFR, F), and periosteal labeled perimeter (Ps.L.Pm, G), mineral apposition rate (Ps.MAR, H), and bone formation rate (Ps.BFR, I) in the murine lumbar vertebrae. Animals were treated and bone specimens processed as described in Materials and methods. Values are mean±SEM of 6 (control) and 7 (PTH groups) replicates.

Fig. 4. Effects of cyclic vs. daily PTH on cancellous bone structure including cancellous bone volume (BV/TV, A), trabecular number (Tb.N, B), trabecular separation (Tb.Sp, C), trabecular width (Tb.Wi, D), trabecular osteoblast perimeter (Tb.OB.Pm, E) and osteoclast perimeter (Tb.OC.Pm, F), trabecular labeled perimeter (Tb.L.Pm, G), mineral apposition rate (Tb.MAR, H), and bone formation rate (Tb.BFR, I) in the murine femurs. Animals were treated and bone specimens processed as described in Materials and methods. Values are mean±SEM of 6 (control) and 7 (PTH groups) replicates.
and bone formation rate with the effects of the daily regimen being more marked than the cyclic regimen (Figs. 4G–I).

Cortical bone

Both regimens significantly increased cortical width; however, the effect of daily PTH was more marked than that of cyclic PTH (Fig. 5A). Both regimens significantly and almost equally increased endocortical osteoblast and osteoclast perimeters (Figs. 5B and C). At endocortical sites, there were trends towards increases in all bone formation parameters, but none was significant (Figs. 5D–F). By contrast, at the periosteum, both regimens significantly increased labeled perimeter, mineral apposition rate, and bone formation rate (Figs. 5G–I). The effect of cyclic PTH on periosteal-labeled perimeter was significantly less than that of daily PTH (Fig. 5G).

Discussion

We have recently reported that a cyclical regimen, in which PTH is given daily for three months followed by 3 months without PTH and the cycle is then repeated, improved vertebral BMD to the same extent as daily PTH in osteoporotic women [8]. These data lend support to the concept that PTH can be effectively used in a cyclic fashion. Since it is now well established that BMD does not assess all aspects of bone strength [11–13], we explored the effects of cyclical PTH treatment on histomorphometric variables related to bone strength in a murine model. Here we report that, similar to our previous densitometric studies in this model [9], there were no significant differences between the effects of cyclic and daily PTH regimens in most variables of trabecular and cortical bone structure and bone cell activity in the lumbar vertebrae, while the effects of cyclic PTH, although statistically significant for some variables, were proportionally less than those of daily PTH on cancellous and cortical bone structure in the femur.

Two key observations of this study were the effects of PTH on trabecular architecture and cortical bone formation at the periosteal surfaces. As for the former, we observed that both cyclic and daily regimens markedly increased trabecular tunneling in both lumbar vertebrae and femur. We believe that this is the first study to demonstrate PTH-stimulated trabecular tunneling in mice as a likely mechanism for the structural changes in cancellous bone that result from the anabolic action of PTH. PTH-induced trabecular tunneling has previously been demonstrated in other species including monkeys [16,17] and dogs [18], but not in rodents. There are a few reports in rat long bones (tibiae) where PTH significantly increased trabecular thickness with a slight but non-significant increase in trabecular number and without detectable tunneling with a 7-day administration of a daily 1-h infusion of PTH [19] or a 4-week treatment of daily
subcutaneous injections with PTH (6 days a week) [20,21]. In the present study, both PTH regimens increased trabecular number without any increase in trabecular thickness in the vertebræ, while they increased both trabecular number and width in the femur. This difference may be in part explained by differences in baseline trabecular thickness at the two sites. Comparison of the values of trabecular thickness (Figs. 1D and 4D) showed that the mean vertebral trabecular width ranges from 49 μm (control) to 54 μm (daily PTH), whereas the mean femoral trabecular width ranges from 33 μm (control) to 49 μm (daily PTH). Therefore, we speculate that there may be an upper limit for trabecular thickness of around 50 μm in mice. Thus, when PTH begins to increase vertebral trabecular thickness, intra trabecular resorption is immediately activated and the trabecula is divided, resulting in increased trabecular number with unchanged thickness. On the other hand, femoral trabeculae (around 30–35 μm) can still increase their width by 50% before reaching 50 μm. Thus, PTH increases both trabecular thickness and number in the femur. One potential stimulus to trabecular tunneling may be osteocyte hypoxia. There is growing evidence that hypoxia plays an important role in cartilage differentiation and endochondral bone development [22,23], osteocyte function [24,25], and osteoclast formation and bone resorption [26,27]. Schipani et al. have demonstrated that the growth plate of mice at 15.5 days of embryonic gestation (E15.5) is hypoxic and this hypoxia occurs in its interior rather than at its periphery [23]. If this can be applied to the trabeculae of cancellous bones of mature mice, we hypothesize that each trabecula may have a gradient of oxygen tension from its outer surface to the center. As trabecular thickness increases under the influence of PTH, osteocytes in the center may become hypoxic and initiate tunneling resorption. Arnett et al. have shown that hypoxia induced progressive increases in osteoclast number and resorption activity in vitro [26]. The concept that intra trabecular resorption serves as a means to maintain adequate nutrient supply to osteocytes has previously been proposed and discussed at length [28,29].

The current study also demonstrated a significant positive effect of PTH on periosteal bone formation in normal adult mice. Both PTH regimens stimulated cortical bone formation more markedly at the periosteal surface than the endocortical surface at both skeletal sites. This is consistent with our previous findings by pQCT that both cyclic and daily PTH regimens markedly increased periosteal circumference and were without effect on endocortical circumference in the femur [9]. The importance of periosteal bone formation in improving bone strength has been appreciated [30,31]. There are some studies (e.g. [32–34]) that have suggested that PTH stimulates periosteal bone formation in animals; however, we believe that this is the first study to demonstrate such an effect with a cyclical regimen. PTH-induced periosteal bone formation in humans remains to be definitively established, while some [35], but not all [36] studies have provided evidence for an increase in bone size. Chen et al. have also shown that an excess of endogenous PTH stimulated periosteal circumference, which might partly compensate for the decrease in BMD and bone strength [37]. Animal studies are beginning to identify some of the genes that may be important in modulating the differential responses to both intermittent and continuous PTH on cancellous, endocortical, and periosteal surfaces [38–42].

We have recently reported [9] that both cyclic and daily PTH treatment increased BMD in the lumbar spine and femur. The increments in femoral BMD were accompanied by an increase in femoral bone strength. The increase in vertebral BMD did not, however, reflect an improvement in vertebral strength. We argued that the lack of improvement in vertebral strength was most likely due to technical difficulties in assessing strength at this site in addition to a small sample size, rather than a real discrepancy between BMD and strength. The current findings tend to support this argument in that significant and identical improvements were seen in vertebral cancellous and cortical bone microarchitecture with both regimens. Our finding that a cyclic PTH regimen was as effective as the daily regimen in the vertebral but not femoral bones raises the question of why there is an apparent site-specific difference in response. As we speculated in our previous study [9], it may be in part due to the differences in enrichment of cancellous vs. compact bones or site-specific differences in the distribution and activity of osteoblasts and osteoclasts in response to PTH exposure and withdrawal [43,44]. It has previously been suggested that the anabolic action of PTH is limited if the cancellous bone volume is very low at the beginning of treatment in rats [45,46] and mice [14]. In the current study, the control value for cancellous bone volume (BV/TV) of the femur was only one-quarter of that of the lumbar vertebrae (7% vs. 28%). Therefore, it may be that the anabolic action afforded in the cyclic regimen was insufficient to mount an effective response in the femur whereas it was sufficient in the spine. Vertebral Ps.L.Pm was significantly increased in the cyclic PTH group (Fig. 3G), but this did not appear to have an effect on cortical width (Fig. 3A). One possible explanation for this is that there was greater viability in Ps.L.Pm in the cyclical group, and another would be that this difference appeared later in the treatment period and that there was insufficient time for it to be translated into a structural difference.

Furthermore, except for femoral cancellous bone, the effects of PTH on osteoblast perimeter (Ob.Pm) tended to be greater than those on labeled perimeter (L.Pm). We suspect that label escape contributed to an underestimation of the lack of mineralization at the perimeter, particularly in the PTH-treated groups in which osteoclast perimeter was significantly increased.

Both regimens increased the indices of formation and resorption to a similar extent by 7 weeks, suggesting that the increase in bone mass may have reached a maximum by that time. We suspect that this phenomenon may also occur in humans, and that shorter treatment periods may be as beneficial as longer ones for this reason.

Finally, the aim of the current study was to provide further support for the effectiveness of cyclical PTH regimens in humans, such as in our recent clinical study [8]. However, we should note that there are substantial differences between our murine model and our clinical study. For example, the women were estrogen-deficient, whereas the mice were estrogen-replete and the dose of PTH was much higher than in our clinical study [8,9]. Moreover, in the clinical study, patients were treated with
daily or cyclic PTH in addition to the ongoing alendronate treatment [8]. Thus, further investigation using ovariectomized mice, lower doses of PTH, and different cyclical protocols, in combination with anti-resorptive agents would be desirable. We are currently exploring such regimens. In addition, it would be helpful if we could examine the measured variables at earlier time points such as weeks 1, 2, and 3. It would especially be of interest to determine what happens to bone structure and strength at 2 weeks in the cyclic PTH group when the BMD response was identical to the daily group with half the amount of PTH administered [7,9]. Results of bone markers, histomorphometric analyses, and strength tests at earlier time points may provide us with more information on the dynamics of the anabolic and catabolic actions of PTH and shed light on the cellular mechanisms underlying the site-specific responses.

In conclusion, we have demonstrated that cyclic PTH administration stimulated periosteal bone formation and improved vertebral cancellous and cortical bone microarchitecture as effectively as daily PTH administration in mice. Cyclic PTH administration also resulted in significant improvements in a number of structural and cellular variables at the distal femur, although the response at that site was less pronounced than that seen with the daily regimen. Continued exploration of cyclic PTH delivery, either alone or in combination with anti-resorptive agents, seems warranted.

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