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A Single Injection of the Anabolic Bone Agent, Parathyroid Hormone–Collagen Binding Domain (PTH–CBD), Results in Sustained Increases in Bone Mineral Density for up to 12 Months in Normal Female Mice

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Abstract

Parathyroid hormone (PTH) is the most effective osteoporosis treatment, but it is only effective if administered by daily injections. We fused PTH(1–33) to a collagen binding domain (PTH–CBD) to extend its activity, and have shown an anabolic bone effect with monthly dosing. We tested the duration of action of this compound with different routes of administration. Normal young C57BL/6J mice received a single intraperitoneal injection of PTH–CBD (320 µg/kg). PTH–CBD treated mice showed a 22.2 % increase in bone mineral density (BMD) at 6 months and 12.8 % increase at 12 months. When administered by subcutaneous injection, PTH–CBD again caused increases in BMD, 15.2 % at 6 months and 14.3 % at 12 months. Radiolabeled PTH–CBD was

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concentrated in bone and skin after either route of administration. We further investigated skin effects of PTH–CBD, and histological analysis revealed an apparent increase in anagen VI hair follicles. A single dose of PTH–CBD caused sustained increases in BMD by >10 % for 1 year in normal mice, regardless of the route of administration, thus showing promise as a potential osteoporosis therapy.

Keywords

Animal models; Bone density technology; DXA; Osteoporosis therapy

Introduction

Osteoporosis is a disease of reduced bone mineral density (BMD) and disrupted bone microarchitecture, resulting in increased risk of fracture. In the United States alone, estimates indicate that 10 million individuals have osteoporosis and another 34 million are at risk to develop osteoporosis. Estimated national direct expenditures for osteoporotic hip fractures were \$18 billion in 2002 [1]. According to U.S. Census Bureau, the number of individuals age 65 years and older will double from 2007 to 2030 [2], placing more individuals at risk for osteoporosis and increasing the public health impact of this disease. Most individuals with osteoporosis are treated with bisphosphonates, antiresorptive agents shown to improve BMD (3 % per year) [3] and prevent fractures (up to 40 % reduction in fracture risk) [4]. Unfortunately, the 60 % residual fracture risk in part accounts for the above statistics on morbidity and cost of osteoporotic fractures, even in a treated population.

Parathyroid hormone (PTH) is an anabolic bone agent that is more effective than bisphosphonates in the treatment of osteoporosis. PTH(1–34) increases BMD by 9 % and reduces fracture risk by 65 % [5]. PTH(1–34) is currently the only anabolic bone agent available for clinical use, marketed as teriparatide (Forteo). Anabolic agents differ from antiresorptives in that they can stimulate new bone formation, and can restore trabecular number and connectivity, resulting in increases in bone strength beyond what would be predicted on the basis of increases in BMD [6]. There are, however, some concerns. PTH(1–34) is anabolic only if it is provided by daily injection, as opposed to bisphosphonates, which can be provided orally with less frequent (weekly, monthly) dosing. PTH therapy appears to lose effectiveness over time [6], and the U.S. Food and Drug Administration's approval of PTH(1–34) is limited to 2 years of therapy [7]. Additional adverse effects include mild hypercalcemia after each injection [8] and concerns about risk of osteosarcoma, observed in rats [9] but not in humans [10].

A long-acting form of PTH would make it much easier for more patients with osteoporosis to take advantage of the most effective therapy. Ideally, such a therapy would have a much longer duration of action, providing sustained increases in bone mass with less frequent dosing. Although continuous administration of PTH by IV infusion is catabolic in bone, there are examples where continuous PTH delivery by other modes is anabolic. A fusion protein of PTH(1–34) and the Fc fragment of human IgG1 (PTH-Fc) was shown to have a significant anabolic effect in bone, although the compound also caused marked hypercalcemia [11]. A study of transgenic mice with a constitutively active mutant PTH/PTHrP receptor expressed under the *COL1A1* promoter developed increased bone mass without significant hypercalcemia [12]. Partly on the basis of this study, we constructed a fusion protein of PTH(1–33) and a collagen binding domain (CBD) derived from *Clostridium histolyticum* of ColH collagenase to target PTH delivery to the collagen-containing compartment of bone. This compound, PTH–CBD, was used to test the

hypothesis that a continuous PTH signal targeted to collagen-containing tissues could provide an anabolic effect in bone without inducing hypercalcemia.

We have shown previously that PTH–CBD serves as an anabolic bone agent with a longer duration of action than the PTH(1–34) without causing prolonged hypercalcemia [13]. PTH–CBD causes marked (13–15 %) increases in BMD after weekly or monthly intraperitoneal (i.p.) administration in normal young female mice. Alkaline phosphatase levels also increased with PTH–CBD treatment, indicating an anabolic mechanism of action. Treatment with PTH–CBD in mice did not show adverse effects on the microarchitecture of the bone based on micro-CT analysis; nor did it increase serum calcium. Importantly, increases in BMD after PTH–CBD therapy could still be observed 7 months after the last dose. However, the rate of decline in BMD appeared to be similar after PTH–CBD therapy and in vehicle-treated control animals. Alkaline phosphatase levels were elevated in PTH–CBD treated animals only, suggesting that there might be a sustained anabolic effect well beyond the monthly dosing interval. We therefore hypothesized that the duration of action of the drug is much longer than 1 month, and we thus proceeded with the studies described below to determine the actual duration of action of PTH–CBD.

Materials and Methods

Animals

Female C57BL/6J mice 3–5 weeks old were obtained from Jackson Laboratory (Bar Harbor, ME). Institutional animal care approval was obtained from the institution where the study was conducted (Ochsner Clinic Foundation). They were then acclimatized for 2 weeks in the animal room. They were exposed to a 12/12 h light/dark period at a temperature of 68–70 °F. The mice were given access to tap water and were provided a diet consisting of 18 % protein purchased from Harlan Company (Barton, IL, and Madison, WI).

Chemicals

PTH–CBD peptide was synthesized by recombinant DNA techniques in *Escherichia coli* [13]. Before injection, the peptide, (PTH–CBD) was dissolved in a collagen binding buffer (pH 7.5, 50 mM Tris HCl, 5 mM CaCl₂). Serum calcium levels were measured by the QuantiChrom Calcium Assay kit (DICA-500) (BioAssay Systems, Hayward, CA).

Experimental Procedure

Intraperitoneal Administration—Thirty-three animals were randomized evenly into three groups (11 animals per group): vehicle, PTH–CBD (every 3 months), or PTH–CBD (one time). After a 2-week period of acclimation, animals were anesthetized [pentobarbital (Nembutal), 50 mg/kg]; then they were weighed and basal BMD obtained. Animals in the vehicle group were injected i.p. with vehicle (collagen binding buffer). Animals in the PTH–CBD (every 3 months) group were injected i.p. with 320 µg/kg PTH–CBD [13], and animals in the PTH–CBD (one time) group received a single i.p. injection with 320 µg/kg PTH–CBD. Dual-energy X-ray absorptiometry (DXA) scans and weight assessments were obtained at 3 months, then monthly thereafter, for the first year, and again at the time of death at 15 months after the start of the study. Blood samples were collected at the time of death for serum calcium analysis. For histological purposes, skin regions from the nape of the neck to the middle of the back were obtained from the humanely killed mice. The skin was fixed in 10 % buffered formalin and processed for routine histology by hematoxylin and eosin staining.

Subcutaneous Administration—Sixteen animals were randomized evenly into two groups (vehicle and PTH–CBD, 8 animals each). After a 2-week period of acclimation,

animals were anesthetized [pentobarbital (Nembutal), 50 mg/kg]; then they were weighed and basal BMD obtained. Animals were then dosed with a single subcutaneous (s.q.) injection of 320 μ g/kg PTH–CBD or vehicle consisting of collagen binding buffer. Repeat DXA scans and weight assessments were obtained every 2 months for 1 year, and again at the time of death, which was 15 months after the start of the study.

BMD Measurement

DXA scans were read as previously described [14]. Briefly, animals were anesthetized with [pentobarbital (Nembutal) 50 mg/kg and placed in the prone position. DXA scans were obtained with a Hologic (Bradford, MA) QDR-1000 plus (high-resolution mode, scanning field 3.5×3.5 in.). Whole body scans were performed twice in succession with repositioning between scans; multiple investigators positioned the animals. After each scan was obtained, the scan was evaluated for proper vertical orientation of the spine. A spine phantom was scanned according to the manufacturer's guideline at the start and the end of the experiment to verify proper system calibration. Scans were read by selecting a one-pixel-wide vertical line as the region of interest and scanning across the selected box to determine the peak BMD value. This noninvasive technique has been shown to have a high degree of precision and reproducibility, with good correlation between multiple observers [14].

Biodistribution of PTH–CBD

The tissue distribution of the PTH–CBD compound was assessed by administering ^{35}S -labeled PTH–CBD via s.q. or i.p. injections. This was followed by whole mount frozen and whole body autoradiography [15]. Recombinant PTH–CBD with a phosphorylation engineered between PTH(1–33) and the CBD was purified, activated, and labeled with (gamma-35) ATP as described previously [16]. Approximately 10.8 μ g of ^{35}S -PTH–CBD (122 kcm/ μ g) was injected s.q. or i.p. (two mice each, 32–35 g, and 7 weeks old). The mice were humanely killed at 1 and 12 h after injection, then frozen in dry ice–acetone. Frozen sections (50 μ m thick) were prepared with an autocryotome, dried at -20°C , and then exposed to an image plate for 4 weeks.

Statistical Analysis

Statistical analysis was performed as follows. Where two groups were compared directly, Student's *t* test was utilized. Results were determined to be statistically significant for values $p < 0.05$. Where three or more groups were compared, analysis of variance (ANOVA) followed by tests after ANOVA as indicated were performed. Where multiple factors were compared in the same experiment (i.e., change over time with three or more groups), two-way ANOVA was performed, followed by one-way ANOVA at each time point, followed by tests after ANOVA as indicated. Survival was analyzed by Kaplan–Meier curves.

Results

Duration of Action of PTH–CBD

We tested the duration of action of PTH–CBD after i.p. or s.q. administration. Therapy by either route of administration resulted in increased BMD by DXA measurement at multiple time points compared to baseline and compared to vehicle-treated control animals (Fig. 1). Mice treated i.p. with a single dose of PTH–CBD showed a 14 % increase in BMD at 4 months (76.75 ± 1.78 vs. 67.5 ± 1.07 mg/cm², $p < 0.01$), while those treated s.q. were not different from controls at that time point (68.6 ± 1.21 vs. 64.8 ± 1.08 mg/cm², NS). Although vehicle-treated control animals showed the expected decline in BMD thereafter, those treated with PTH–CBD showed continued increases in BMD. At 6 months, mice treated with PTH–CBD by either route showed significantly higher BMD than those of

vehicle controls, with mice treated i.p. showing an 22.2 % increase (74.8 ± 1.44 vs. 61.2 ± 1.83 mg/cm², $p < 0.0001$) and those treated s.q. showing an 12.8 % increase (74.25 ± 2.97 vs. 65.8 ± 3.64 mg/cm², $p < 0.01$). These increases were sustained for up to 12 months (15.2 % for i.p., 68.9 ± 3.59 vs. 59.8 ± 1.72 mg/cm², $p < 0.05$, 14.3 % for s.q., 68.7 ± 3.22 vs. 60.1 ± 4.4 mg/cm², $p < 0.05$), but declined thereafter such that there were no statistically significant differences between groups at 15 months. Importantly, the magnitude and the trend of the increase in BMD were similar to that found in our earlier study [13].

Single versus Repeated Dosing of PTH–CBD

We compared single versus repetitive (every 3 months) dosing of PTH–CBD via the i.p. route of administration (Fig. 2). There was no apparent difference in efficacy or onset of action initially. At 10 months there appeared to be some increase in BMD observed with repeat dosing, although the only statistically significant difference between the two dosing regimens was at the 13 month time point (77.0 ± 3.35 vs. 67.4 ± 3.85 mg/cm², $p < 0.05$). Interestingly, at 15 months the BMD of mice treated with repetitive dosing declined rather sharply, which may be the result of the advanced age of the mice or which may be a manifestation of the limitations on anabolic activity observed with long-term PTH therapy [6].

Effects of PTH–CBD on Serum Calcium, Weight, and Survival

We next examined for any signs of toxic effects of the PTH–CBD compound. The results of the calcium analysis at the end of the 15-month period from the i.p.-treated animals showed no significant differences in serum calcium levels between groups (Fig. 3), although at this time point there were also no differences in BMD between groups, indicating that the treatment effects had subsided. There were no significant differences observed in the weights of the mice between groups at any time point in either study (Fig. 4). Likewise, there was no significant difference in the survival of the PTH–CBD treated animals compared to vehicle-treated animals (not shown).

Biodistribution of PTH–CBD

We next tested the biodistribution of PTH–CBD peptide by the s.q. and i.p. routes of administration. Autoradiograms of mice injected with ³⁵S-labeled PTH–CBD either i.p. or s.q. showed increased levels at the respective sites of injection after 1 h, although there was already significant redistribution to other tissues, particularly skin and bone (Fig. 5). Radioactivity was also detected in the intestinal lumen, kidney, and bladder. After 12 h, this redistribution was nearly complete, such that there appeared to be little residual radioactivity at the respective injection sites, indicating that the systemic exposure did not differ significantly between the two routes of administration.

Effects of PTH–CBD on the Skin

Because the biodistribution studies showed significant uptake of PTH–CBD in the skin, we examined for any evidence of biologic effects in this tissue. At gross examination, there were no obvious differences in the skin or the coat of mice between PTH–CBD-treated groups and vehicle-treated control groups. However, histological examination of the skin samples obtained from i.p.-administered animals (experimental procedure 1) showed that there was an apparent increase in the number of hair follicles, including those in the subcutaneous fat region (anagen VI follicles), in the PTH–CBD treated group (Fig. 6). Importantly, anagen VI follicles are in the growth phase of the hair cycle, suggesting that PTH–CBD might have positive effects on hair growth.

Discussion

We synthesized a bone-targeting PTH analog, PTH-CBD, to extend the duration of action of PTH injections while maintaining the anabolic effect. PTH-CBD is a hybrid protein containing a PTH domain that activates bone formation and a CBD that targets and retains PTH delivery to the bone [13]. Our previous study suggested that this peptide may have a duration of action beyond 7 months. We found that BMD increased by 22.2 % (compared to vehicle controls) after 6 months in animals that received a single i.p. dose of PTH-CBD and 12.8 % after 6 months in animals that received a single s.q. dose. Micro-CT analysis from bones collected at the time of death was attempted, but the readings obtained did not pass validation tests, presumably because of the advanced age of the animals. The response to PTH-CBD was delayed, with significant increases in BMD not observed until 4–6 months after initiation of therapy. This delay may be intrinsic to the action of PTH-CBD, or early anabolic effects may have been obscured by the rapid increase in BMD normally observed in young mice. Of note, when tested in chemotherapy-induced osteoporosis, PTH-CBD shows a much more rapid effect on BMD, evident in as little as 4 weeks after therapy [17].

PTH-CBD administered by either i.p. or s.q. routes of administration resulted in increases in BMD, although the BMD response was earlier and greater after i.p. administration. Distribution studies indicated that PTH-CBD is absorbed completely after either route of administration, indicating systemic exposure that was not significantly different, although the distribution to bone might still be different between the two routes of administration. Unfortunately, the whole body mount technique does not allow quantitative comparison of relative tissue uptake. Additional studies will examine if increasing the dose or frequency of PTH-CBD administration by the s.q. route might compensate for this apparent reduction in efficacy.

There was no additional efficacy observed with 3-month dosing of PTH-CBD versus single injection; however, after 9 months, the BMD appeared to decline in the group receiving the single PTH-CBD injection, while the BMD increase was sustained for a longer period of time with redosing every 3 months. The dosing of PTH-CBD every 3 months would be expected, in retrospect, to have resulted in increased drug accumulation in the bone with each dose. This dose stacking led to an effective increase in drug exposure to the target tissue. Despite this, there was no apparent further increase in observed efficacy, suggesting that at least in mice, a 320- μ g/kg dose may be sufficient for a maximal response on BMD, at least by the i.p. route of administration. There was marked decrease in BMD observed at 15 months in the mice treated with PTH-CBD every 3 months; this may be the result of the advanced age of the mice, or it may be the result of increases in bone removal that have been described with long-term PTH therapy [6].

Our findings that BMD are highest at 3 and 4 months of age in normal mice are in agreement with the findings of other investigators [18]. In mice, sexual maturation occurs at approximately 5–6 weeks of life, corresponding to adolescence in humans [19], and peak bone mass is attained between 3 and 4 months of age—a period of time that from a developmental perspective is approximately adulthood in humans.

While PTH(1–34) is a very effective treatment for osteoporosis [20], increased incidence of osteosarcoma and hypercalcemia are reported adverse effects of PTH(1–34) therapy [9]. In our studies, the mice were evaluated for signs of osteosarcomas or other tumors by physical examination, whole body DXA scans, and necropsy at time of death; there was no evidence of tumor formation. Although there were no observed changes in serum calcium at the end of the study, blood collections were not attempted during the course of the study to minimize animal mortality. We have, in a separate study in rats, found that PTH-CBD does

not increase serum calcium either acutely or chronically [21]. PTH–CBD therapy did not affect the weight of the animals, and their mortality did not increase (data not shown).

The mechanism of action of PTH–CBD in bone is currently unknown. There may be preferential activation of bone formation rather than bone removal, as PTH–CBD appears to have limited uptake to the bone marrow, where the osteoclast precursor cells are mobilized from in a PTH-dependent manner. T cells are required for the catabolic effect of continuous PTH therapy [22], and these cells may have reduced exposure to PTH–CBD. PTH–CBD may be released from the collagen matrix in a pulsatile fashion during remodeling events, mimicking daily injections of PTH(1–34). PTH–CBD may initiate an anabolic bone response that is self-sustaining. Further study is required to elucidate the actual mechanism of action of PTH–CBD.

Regarding the effects of PTH–CBD in the skin, although there were no obvious changes in the coat of the animals, there were indications in the histological analysis that the number of anagen VI hair follicles was increased with PTH–CBD treatment. In a separate study that used a more rigorous protocol, we examined skin effects of PTH–CBD in a mouse model of chemotherapy-induced alopecia and found that PTH–CBD treatment promotes hair growth and results in an increased number of anagen VI hair follicles [23]. These findings are consistent with the qualitative observations in this study and likely the result of positive effects of PTH–CBD on WNT signaling in skin, causing increased production of β -catenin [24], a known promoter of the hair cycle [25].

Our novel hybrid peptide of PTH and a CBD caused long-term (up to 12 months) increases in BMD in normal female mice after a single dose. These increases are observed with either i.p. or s.q. administration, although i.p. administration results in greater and more rapid BMD changes. There was no observed hypercalcemia or tumor formation associated with these BMD changes. With these encouraging results in normal mice, we are now pursuing studies of efficacy in models of postmenopausal osteoporosis.

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Fig. 1.

Lumbar spine BMD after intraperitoneal (i.p.) or subcutaneous (s.q.) administration of parathyroid hormone–collagen binding domain (PTH–CBD). Seven-week-old female C57BL/6J mice received a single i.p. or s.q. injections of either vehicle or PTH–CBD (320 μ g/kg) as indicated. Spinal BMD was measured by DXA. Results are expressed as mean $\pm$ standard deviation. * p < 0.01 versus vehicle i.p. at the indicated time point. # p < 0.01 versus vehicle s.q. at the indicated time point

Fig. 2.

Lumbar spine BMD after intraperitoneal (i.p.) administration of parathyroid hormone–collagen binding domain (PTH–CBD), comparing single versus repeated dosing. Seven-week-old female C57BL/6J mice received i.p. injections of PTH–CBD (320 µg/kg), either a single injection or every 3 months, as indicated. Spinal BMD was measured by DXA. Results are expressed as mean ± standard deviation. * $p < 0.01$ versus PTH–CBD i.p. (one time) at the indicated time point

Fig. 3.

Serum calcium after intraperitoneal (i.p.) administration of parathyroid hormone–collagen binding domain (PTH–CBD). Seven-week-old female C57BL/6J mice received i.p. injections of vehicle, PTH–CBD (every 3 months) (320 μ g/kg), or PTH–CBD (one time) (320 μ g/kg). Blood samples were obtained at the time the animal was humanely killed (15 months) and serum calcium was measured with the QuantiChrom™ Calcium Assay Kit (Bioassay Systems, Hayward, CA). Results are expressed as mg/dL, mean $\pm$ standard deviation. NS by ANOVA

Fig. 4. Animal weights after intraperitoneal (i.p.) or subcutaneous (s.q.) dosing of parathyroid hormone–collagen binding domain (PTH–CBD). Seven-week-old female C57BL/6J mice received either single or every-3-months i.p. injections of the PTH–CBD (320 μ g/kg), single s.q. injection of PTH–CBD peptide (320 μ g/kg), or vehicle control. Results are expressed in grams mean $\pm$ standard deviation

Fig. 5. Tissue distribution of ^{35}S -labeled parathyroid hormone–collagen binding domain (PTH–CBD). Whole body autoradiograms obtained 1 or 12 h after administration of ^{35}S -labeled PTH–CBD, either by intraperitoneal (i.p.) or subcutaneous (s.q.) administration, as indicated by the *large labeled arrows*. Slices are oriented with the caudal end at the *top* and the dorsum to the *left*. *Solid arrows* show uptake into skin; *dashed arrows* show uptake into spine

Fig. 6.

Histological analysis of the skin after administration of a single dose of parathyroid hormone–collagen binding domain (PTH–CBD) (320 μ g/kg) by intraperitoneal (i.p.) injections. Skin regions from the nape of the neck to the middle of the back were obtained from the dead mice. The skin was fixed in 10 % buffered formalin and processed for routine histology by hematoxylin and eosin staining