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ORIGINAL ARTICLE

A comparative study of the bone-restorative efficacy of anabolic agents in aged ovariectomized rats

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Abstract
Introduction The study was designed to compare the bone anabolic effects of basic fibroblast growth factor (bFGF), a selective agonist for prostaglandin E receptor subtype EP4, and parathyroid hormone (PTH) in aged ovariectomized (OVX) rats with severe cancellous osteopenia.
Methods Groups of aged OVX rats were maintained untreated for 1 year postovariectomy (15 months of age) to develop severe tibial cancellous osteopenia. These animals were then treated with bFGF or the EP4 agonist (EP4) for 3 weeks. Other groups of aged OVX rats were treated with EP4 or PTH alone for 11 weeks, or sequentially with bFGF or EP4 for 3 weeks followed by PTH for 8 weeks. Cancellous and cortical bone histomorphometry were performed in the right proximal tibial metaphysis and tibial diaphysis respectively.
Results Treatment with bFGF for 3 weeks markedly increased serum osteocalcin, osteoid volume, and osteoblast and osteoid surfaces to a greater extent than EP4. Basic FGF, but not EP4 or PTH, induced formation of osteoid islands within bone marrow. EP4 stimulated cancellous bone turnover, but failed to restore lost cancellous bone in the severely osteopenic proximal tibia after 11 weeks of treatment. In contrast, EP4, much like PTH, increased cortical bone mass in the tibial diaphysis by stimulating both periosteal and endocortical bone formation. Treatment of aged OVX rats with PTH alone tended to partially reverse the severe tibial cancellous osteopenia, whereas sequential treatment with bFGF and PTH increased tibial cancellous bone mass to near the level of vehicle-treated control rats. These findings indicate that bFGF had the strongest stimulatory effect on cancellous bone formation, and was the only anabolic agent to induce formation of osteoid islands within the bone marrow of the severely osteopenic proximal tibia. Therefore, bFGF may be more effective for the reversal of severe cancellous osteopenia. PTH and EP4 increased cortical bone mass to nearly the same extent, but cancellous bone mass was greater by two-fold in PTH-treated OVX rats than in EP4-treated OVX rats.
Conclusion These findings in aged OVX rats suggest that PTH is more efficacious than EP4 for augmentation of cancellous bone in the severely osteopenic, estrogen-deplete skeleton.

Keywords Bone anabolic agents · Bone formation · Bone histomorphometry · Cancellous osteopenia · Ovariectomy · Rat

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Introduction

Parathyroid hormone (PTH) is the only bone anabolic agent approved to date for the treatment of postmenopausal osteoporosis. It has a strong stimulatory effect on bone formation in postmenopausal women and estrogen-deplete animals [1–3], but its ability to completely restore lost cancellous bone mass in the severely osteopenic skeleton

may be limited by lack of adequate numbers of bone spicules to serve as templates for new bone formation [4]. In this situation, a bone anabolic agent must induce formation of new bone spicules within bone marrow to reverse severe cancellous osteopenia, which appears to be beyond the capability of PTH. Basic fibroblast growth factor (bFGF) has been shown to have the desired osteogenic effect [5–7], which served as the basis for a therapeutic strategy involving short-term treatment with bFGF to create new bone spicules within bone marrow, followed by long-term treatment with PTH to enlarge these spicules and restore lost cancellous bone in severely osteopenic ovariectomized (OVX) rats [8]. However, systemic administration of bFGF induces significant side effects [9, 10], which makes its use in humans problematic. Therefore, development of a therapeutic alternative to bFGF with similar osteogenic effects but minimal side effects is highly desirable.

Prostaglandin E_2 (PGE_2) is another strong stimulator of bone formation, with the ability to restore lost cancellous bone mass in osteopenic OVX rats [11, 12]. Similar to bFGF, some studies in rats suggest that new bone spicules are formed within the bone marrow of the tibial diaphysis in response to PGE_2 treatment [12, 13]. Despite these impressive bone anabolic effects, PGE_2 has not been developed as an osteoporosis therapy due to concerns about adverse side effects such as diarrhea [12]. However, these side effects may be circumvented by use of a PGE_2 agonist selective for the receptor subtype EP4, which appears to promote the bone anabolic effects of PGE_2 with minimal gastrointestinal side effects [14]. The recent report of decreased cancellous bone mass and formation in EP4 receptor knockout mice is consistent with a role for this receptor in the regulation of bone formation [15]. An EP4 agonist has been shown to stimulate bone formation and prevent cancellous bone loss in immobilized male rats [14]. Treatment of intact female rats with an EP4 agonist for 3 weeks stimulated periosteal and endocortical bone formation, but this short-term treatment did not significantly increase cortical bone mass [16]. In OVX rats, an EP4 agonist has been shown to prevent cancellous bone loss [14] and to restore lost cancellous bone mass at a moderately osteopenic site [17]. Despite these promising findings, the bone restorative potential of an EP4 agonist, including the ability to induce formation of bone or osteoid islands within bone marrow, has yet to be evaluated in aged OVX rats with severe cancellous osteopenia. Furthermore, a direct comparison of the bone anabolic effects of an EP4 agonist and PTH, the "gold standard" of bone anabolic agents, has not yet been reported. Therefore, the main goal of the study is to compare the bone anabolic effects of bFGF, an EP4 agonist, and PTH in cancellous and cortical bone in aged OVX rats.

Materials and methods

Virgin female Sprague Dawley rats were obtained from Charles River (Wilmington, MA). These animals were approximately 90 days of age, and weighed an average of 240 g at the beginning of the study. All procedures with rats were approved by the Institutional Animal Care and Use Committee at the University of Florida (Gainesville, FL, USA).

Within 2 weeks of their arrival, all rats were anesthetized with an IP injection of ketamine hydrochloride and xylazine at doses of 50 and 10 mg/kg body weight respectively. Sham surgeries were performed in 22 rats, during which the ovaries were exteriorized but replaced intact. Bilateral ovariectomies were performed in 71 rats from a dorsal approach. The rats were housed in pairs at 25°C with a light/dark cycle of 13 h/11 h. Sham-operated rats were allowed unlimited access to food (Teklad LM-485 Rat Diet, Madison, WI, USA). The food consumption of OVX rats was restricted to that of sham-operated rats to minimize the increase in body weight associated with ovariectomy [18]. All rats were maintained in this manner for 1 year after surgery to allow for the development of severe cancellous osteopenia in the proximal tibial metaphysis of the OVX animals [4].

Treatment groups

Groups of baseline control and OVX rats were sacrificed at 1 year postovariectomy when the animals were 15 months old. The treatments described below were initiated within 2 days of sacrifice of the baseline groups. For these treatments, bFGF (Chiron Corp., Emeryville, CA, USA) was dissolved in a vehicle of phosphate-buffered saline and administered SC at a dose of 1 mg/kg BW. The EP4 agonist CP-734432 was obtained from Pfizer Global Research and Development, Groton Laboratories (Groton, CT, USA), dissolved in a vehicle of 5% ethanol, and administered SC at a dose of 3 mg/kg BW. Synthetic human PTH 1–34 (Bachem, Torrance, CA, USA) was dissolved in a vehicle of acid saline and 2% heat-inactivated rat serum and administered SC at a dose of 80 μg/kg BW.

The baseline and treatment groups are listed below. Groups 1–4 were sacrificed when the rats were between 15 and 16 months of age. This first phase of the study was designed to compare the early bone anabolic response to bFGF and the EP4 agonist.

1) Baseline control (N=10)
2) Baseline OVX (N=8)
3) OVX+bFGF (N=8)

Rats in this group were injected SC with bFGF daily for 3 weeks.

4) OVX+EP4 (N=9)

Rats in this group were injected SC with the EP4 agonist daily for 3 weeks.

Groups 5–10 were sacrificed after 11 weeks of treatment, when the rats were nearly 18 months of age. This second phase of the study was designed to determine whether sequential treatment initially with bFGF or EP4 followed by PTH would be more effective for restoring lost cancellous bone mass than treatment with PTH alone. In addition, the bone anabolic effects of long-term treatment with the EP4 agonist were compared to those of PTH. The experimental design did not include a group of OVX rats treated for 11 weeks with bFGF, due to concerns about the ability of the animals to maintain good health in view of the adverse side effects of the growth factor.

5) Control+Vehicle (N=12)

Half of the rats in this group were injected SC with 5% ethanol daily for 11 weeks. The remaining rats were injected SC with acid saline 5 days/week for 11 weeks.

6) OVX+Vehicle (N=8)

Rats in this group were treated as described above for control rats in Group 5.

7) OVX+bFGF+PTH (N=9)

Rats in this group were injected SC with bFGF daily for 3 weeks. Treatment with bFGF was then withdrawn, followed by SC injections with PTH 5 days/week for 8 weeks.

8) OVX+EP4+PTH (N=10)

Rats in this group were injected SC with the EP4 agonist daily for 3 weeks. Treatment with EP4 was then withdrawn, followed by SC injections with PTH 5 days/week for 8 weeks.

9) OVX+EP4 (N=10)

Rats in this group were injected SC with the EP4 agonist daily for 11 weeks.

10) OVX+PTH (N=9)

Rats in this group were injected SC with PTH 5 days/week for 11 weeks.

All rats were injected with demeclocycline (Sigma Chemical Co., St. Louis, MO) at a dose of 15 mg/kg BW on the 17th (SC) and 16th (IP) days before sacrifice and with the same dose of calcein (Sigma) on the 7th (SC) and 6th (IP) days before sacrifice to label actively forming bone surfaces. Euthanasia was achieved by exsanguination from the abdominal aorta under ketamine/xylazine anesthesia. Hematocrit was measured at the time of necropsy with a micro-hematocrit reader (Clay Adams, Parsippany, NJ, USA). The right tibia from each animal was stripped of musculature, cut in half cross-sectionally, and placed in 10% phosphate-buffered formalin for 24 h for tissue fixation. Serum samples were stored at −80°C for future analyses.

Serum biochemistry

Serum calcium and phosphorus were measured by the o-cresolphthalein compliance method and the ammonium molybdate method respectively, with a Hitachi 911 Chemistry Analyzer (Roche, Indianapolis, IN, USA). Serum osteocalcin was assayed as a systemic marker for bone formation with a rat osteocalcin ELISA kit (Nordic Biosciences, Denmark).

Cancellous bone histomorphometry

The right proximal tibiae were dehydrated in ethanol, embedded undecalcified in modified methyl methacrylate [19], and sectioned longitudinally with Jung 2065 and 2165 microtomes (Leica Corp., Rockleigh, NJ) at thicknesses of 4 and 8 μm. The thinner sections were stained according to the von Kossa method with a tetrachrome counterstain (Polysciences, Warrington, PA, USA) whereas the 8 μm-thick sections remained unstained for collection of fluorochrome-based data.

The region of interest for data collection consisted of cancellous bone tissue within the proximal tibial metaphysis in an area beginning 0.75 mm distal to the growth plate/metaphyseal junction, which excludes the primary spongiosa. Histomorphometric measurements were performed with the Bioquant Bone Morphometry System (R&M Biometrics Corp., Nashville, TN, USA) and the Osteomeasure/Trabecular Analysis System (Osteometrics, Inc., Atlanta, GA, USA). Cancellous bone volume was measured in stained sections at a magnification of 20×. Osteoid volume, as a percentage of bone tissue area, and osteoblast, osteoid, and osteoclast surfaces, as percentages of total cancellous perimeter, were measured in the same sections at 200×. Fluorochrome-based indices of bone formation, including mineralizing surface (% cancellous bone perimeter with double fluorochrome labels) and mineral apposition rate, were measured at 200× in the thicker, unstained sections. Values for mineral apposition rate were not corrected for obliquity of the plane of section in cancellous bone [20]. Bone formation rate (surface referent) was calculated by multiplying mineralizing surface by mineral apposition rate [20].

Cortical bone histomorphometry

The distal half of the right tibia was dehydrated and defatted in 100% ethanol and acetone, then embedded undecalcified in a styrene monomer that polymerizes into a polyester resin (Tap Plastics, San Jose, CA, USA). The tibial diaphysis 1–2 mm proximal to the tibiofibular junction was sawed into cross sections of ~100 μm thickness with an Isomet low-speed saw (Buehler, Lake

Bluff, IL, USA). These cross sections were then ground to a thickness of 50 μm for histomorphometric measurements with the Osteomeasure System. Cortical bone tissue area and bone marrow area were measured in one cross section per animal at a magnification of 20×. Cortical bone area was calculated by subtracting marrow area from cortical bone tissue area. The distance from the periosteal to the endocortical surfaces was also measured for calculation of cortical width. In addition, periosteal and endocortical mineralizing surface, mineral apposition rate, and bone formation rate were determined as described above for cancellous bone, with the exception that mineralizing surface was calculated as % double-labeled perimeter + 1/2% single-labeled perimeter.

Statistical analysis

Data are presented as the mean ± SD for each group. The nonparametric Kruskal–Wallis test followed by a post-hoc test (*t* statistic adjusted for the number of groups and comparisons) was used to determine statistically significant differences among groups [21]. Data for groups 1–4 (baseline and 3 weeks of treatment) and 5–10 (11 weeks of treatment) were analyzed separately. *P* values less than 0.05 were considered to be significant.

Results

The mean body weight for baseline OVX rats (384.9± 44.3 g) was very similar to that of baseline control rats (391.0±43.1 g). OVX rats treated for 3 weeks with bFGF weighed 2.3% less than baseline OVX rats, but this difference was not statistically significant. Treatment of OVX rats for 3 weeks with the EP4 agonist did not affect body weight (394.2±38.7 g). After 11 weeks of treatment, mean body weights for OVX rats treated with PTH alone, or sequentially with bFGF + PTH or EP4 + PTH, did not differ from that of vehicle-treated OVX rats. The mean body weight for OVX rats treated for 11 weeks with EP4 alone was 7.6% less than that of vehicle-treated OVX rats, but this difference was not statistically significant. The EP4-treated OVX rats did not exhibit signs of gastrointestinal side effects such as diarrhea.

Treatment of OVX rats for 3 weeks with bFGF significantly decreased blood hematocrit levels, to 17.3± 3.0%. All other groups had mean hematocrit values of 38–43%. All groups of rats were found to be normocalcemic and normophosphatemic. However, the lowest mean serum phosphorus values were detected in OVX rats treated for 3 weeks with bFGF (4.6±0.8 mg/dl), although this value was not significantly different from that of baseline OVX rats (4.9±0.7 mg/dl).

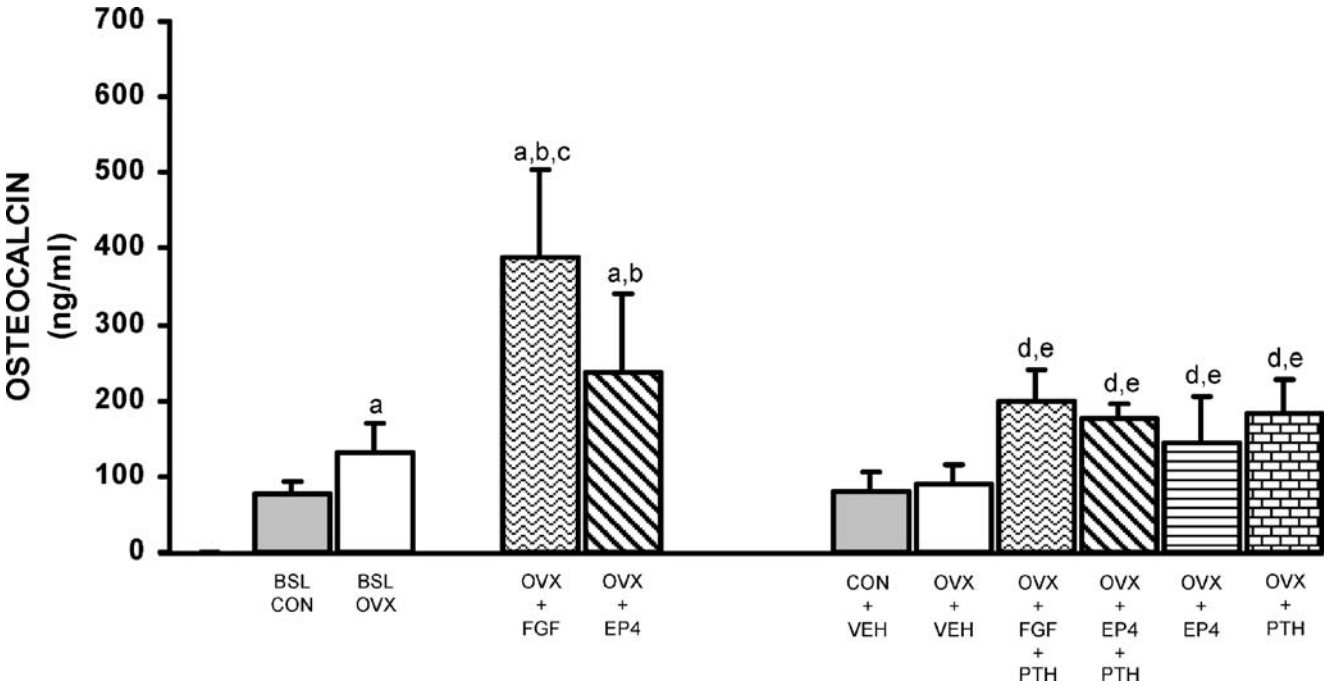

Fig. 1 Mean values (± SD) for serum osteocalcin in 10 groups of rats. Baseline control (BSL CON) and baseline OVX (BSL OVX) rats were sacrificed at 15 months of age (1 year postovariectomy). The adjacent groups of OVX rats were treated for 3 weeks with bFGF or an EP4 agonist alone (OVX+FGF and OVX+EP4). Rats from the 6 groups to the right were treated with vehicle alone for 11 weeks (CON+VEH and OVX+VEH), 3 weeks with FGF or EP4 followed by 8 weeks with PTH (OVX+FGF+PTH and OVX+EP4+PTH), and 11 weeks with EP4 or PTH alone (OVX+EP4 and OVX+PTH). [a] Significantly different from the BSL CON group. [b] Significantly different from the BSL OVX group. [c] Significantly different from the OVX + EP4 group at 3 weeks of treatment. [d] Significantly different from the CON + VEH group. [e] Significantly different from the OVX + VEH group

Serum osteocalcin (Fig. 1) was significantly increased in baseline OVX rats compared with baseline control rats. OVX rats treated for 3 weeks with bFGF exhibited a 3–4 fold increase in serum osteocalcin compared to the baseline OVX group. This systemic index of bone formation was increased by nearly 2-fold in OVX rats treated for 3 weeks with the EP4 agonist, but this increment was significantly less than that of bFGF-treated OVX rats. Treatment of OVX rats for 11 weeks with the bone anabolic agents, either singly or sequentially, induced an approximately 2-fold increase in serum osteocalcin compared to the vehicle-treated control and OVX rats.

Cancellous bone histomorphometry

Baseline OVX rats were characterized by a marked decrease in cancellous bone volume compared with baseline control rats (Fig. 2). This cancellous osteopenia in aged OVX rats was associated with increased indices of bone formation such as osteoblast and osteoid surfaces, and

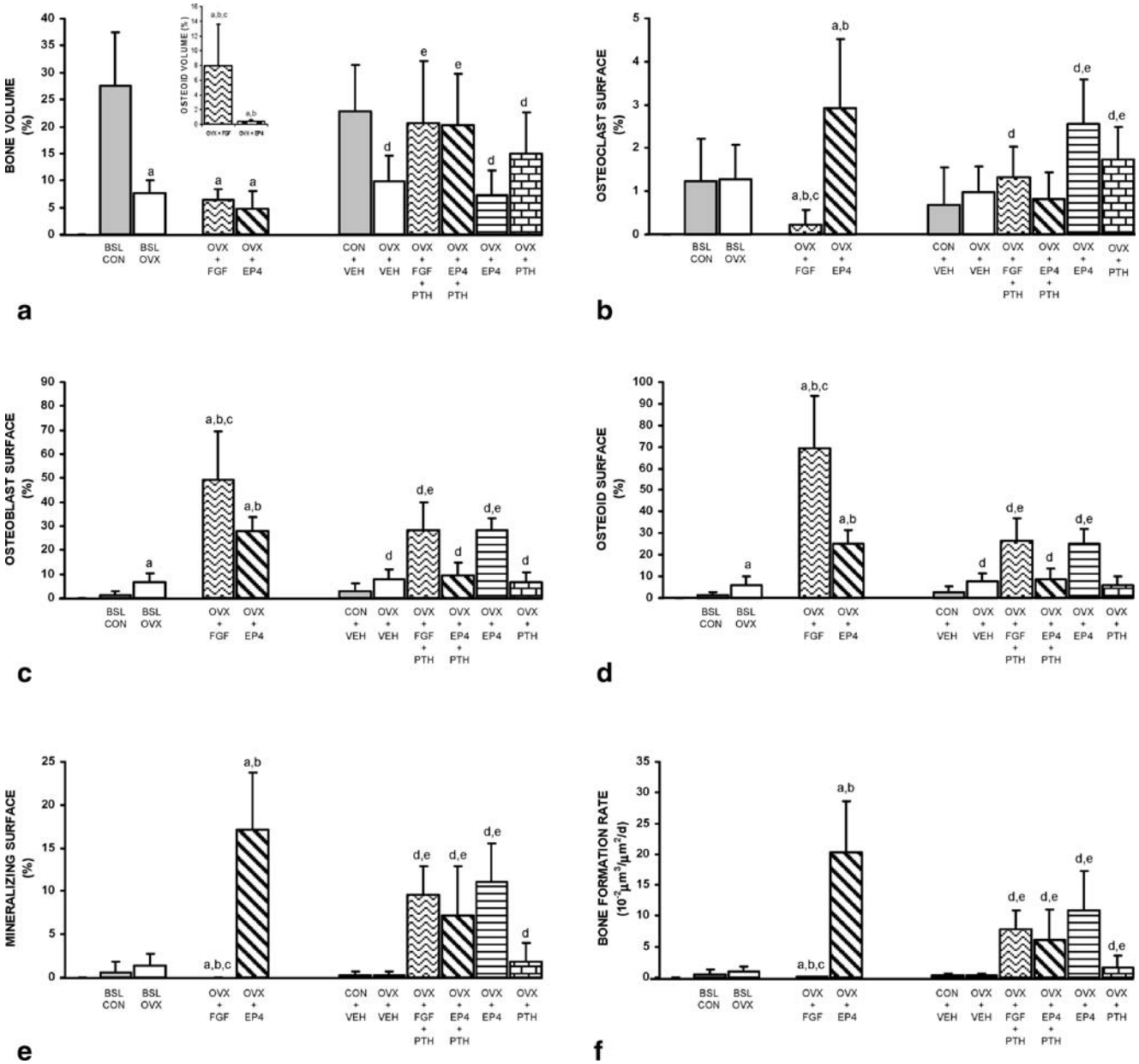

Fig. 2 Mean values (± SD) for cancellous bone volume (**a**), osteoclast surface (**b**), osteoblast surface (**c**), osteoid surface (**d**), mineralizing surface (**e**), and surface-referent bone formation rate (**f**) in the proximal tibial metaphysis of 10 groups of rats. The *inset* within **a** depicts mean values for osteoid volume in OVX rats treated for 3 weeks with bFGF alone and EP4 alone. Osteoid volume was negligible (<0.1%) in the other 8 groups of rats. See legend for Fig. 1 for details. [a] Significantly different from the BSL CON group. [b] Significantly different from the BSL OVX group. [c] Significantly different from the OVX + EP4 group at 3 weeks of treatment. [d] Significantly different from the CON + VEH group. [e] Significantly different from the OVX + VEH group

strong trends for increased mineralizing surface and bone formation rate, but osteoclast surface was nearly identical in baseline control and OVX rats. Treatment with bFGF or EP4 alone for 3 weeks did not increase cancellous bone mass in aged OVX rats. However, osteoid volume was markedly increased in OVX rats treated with bFGF alone, and slightly increased in OVX rats treated for 3 weeks with the EP4 agonist (Fig. 2a). In contrast, osteoid volume was minimal in baseline control and OVX rats (<0.1%). Osteoid islands without any apparent connections to pre-existing bone spicules were observed in OVX rats treated with bFGF alone (Fig. 3), but not in EP4-treated OVX rats.

Regarding cancellous bone turnover (Fig. 2), treatment with bFGF alone induced 7.5- and 11-fold increases in osteoblast and osteoid surfaces respectively, compared to baseline OVX rats. Treatment of OVX rats for 3 weeks with the EP4 agonist induced 4-fold increases in osteoblast and osteoid surfaces. The increment in these variables was significantly greater in bFGF-treated OVX rats compared with EP4-treated OVX rats. Fluorochrome-based indices of bone formation were negligible in OVX rats treated with bFGF alone, due to the impairment of bone mineralization by the growth factor [6–8]. In contrast, bone formation rate was substantially increased in OVX rats treated with the EP4 agonist, due to significant increases in mineralizing surface (Fig. 2e) and mineral apposition rate (1.2±0.2 μm/d vs 0.5±0.3 μm/d) compared to baseline OVX rats. OVX rats treated with bFGF alone had very low mean values for osteoclast surface, whereas this index of bone resorption was increased by at least a factor of 2 in EP4-treated OVX rats compared to baseline OVX rats (Fig. 2b).

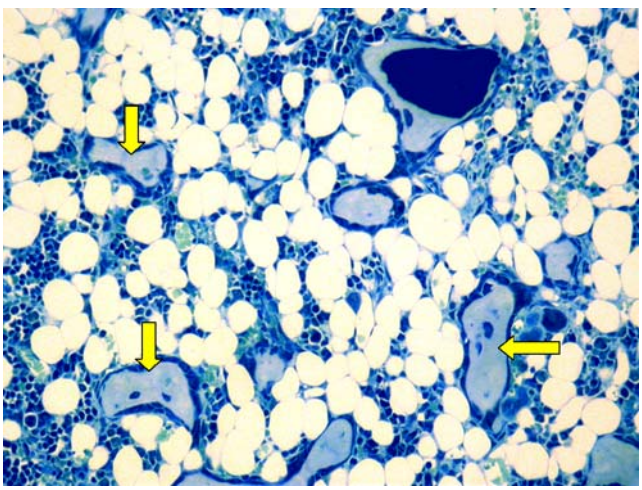

Fig. 3 Cancellous bone tissue in the proximal tibial metaphysis of an aged OVX rat treated for 3 weeks with bFGF. Note the osteoid islands (*arrows*) without any apparent connections to black-stained bone spicules. These osteoid islands were not observed in aged OVX rats treated with the EP4 agonist. (Von Kossa/tetrachrome stain; original magnification ×100)

After 11 weeks of vehicle treatment, OVX rats remained markedly osteopenic, with high cancellous bone turnover compared with sham-operated control rats. Sequential treatment with bFGF + PTH and EP4 + PTH restored cancellous bone mass to near the level of sham-operated control rats. The mean cancellous bone volume for OVX rats treated with PTH alone was approximately 50% greater than that of vehicle-treated OVX rats, but this difference was not statistically significant. Treatment of OVX rats for 11 weeks with EP4 alone failed to increase cancellous bone mass compared to vehicle treatment of OVX rats. Furthermore, the mean cancellous bone volume for OVX rats treated with EP4 alone was half that of OVX rats treated with PTH alone. Osteoid volume was negligible (<0.1%) in all groups. Differences in cancellous bone mass among some of the treatment groups are seen in Fig. 4.

Osteoblast and osteoid surfaces (Fig. 2) were increased by at least a factor of 3 in OVX rats treated with bFGF + PTH and EP4 alone, compared with vehicle-treated OVX rats. These indices of bone formation were not significantly increased in OVX rats treated with EP4 + PTH and PTH alone. Bone formation rate (surface-referent) was markedly increased by at least an order of magnitude in OVX rats treated with bFGF + PTH, EP4 + PTH, and EP4 alone. Bone formation rate was also significantly increased in OVX rats treated with PTH alone, but to a lesser extent than the other treatment groups. The observed increase in bone formation rate was due to an increase in mineralizing surface. Mineral apposition rate ranged from 0.7–0.9 μm/day with no significant differences among the groups.

OVX rats treated with bFGF + PTH exhibited a nonsignificant trend for increased osteoclast surface compared with vehicle-treated OVX rats. This index of bone resorption was significantly increased by approximately a factor of 2 in OVX rats treated with EP4 alone and PTH alone.

Cortical bone histomorphometry

Baseline OVX rats had decreased cortical bone area and width compared with baseline control rats (Fig. 5). Treatment for 3 weeks with the EP4 agonist significantly increased these indices of cortical bone structure as well as cortical bone tissue area, to at least the level of baseline control rats, whereas treatment with bFGF was not as effective in this regard. Baseline control and OVX rats had similar mean values for periosteal and endocortical bone formation rates. These indices of cortical bone formation were not significantly increased in bFGF-treated OVX rats compared with vehicle-treated OVX rats. In contrast, EP4 treatment for 3 weeks markedly increased both periosteal and endocortical bone formation rates in aged OVX rats. The observed increases in these variables were due to EP4-

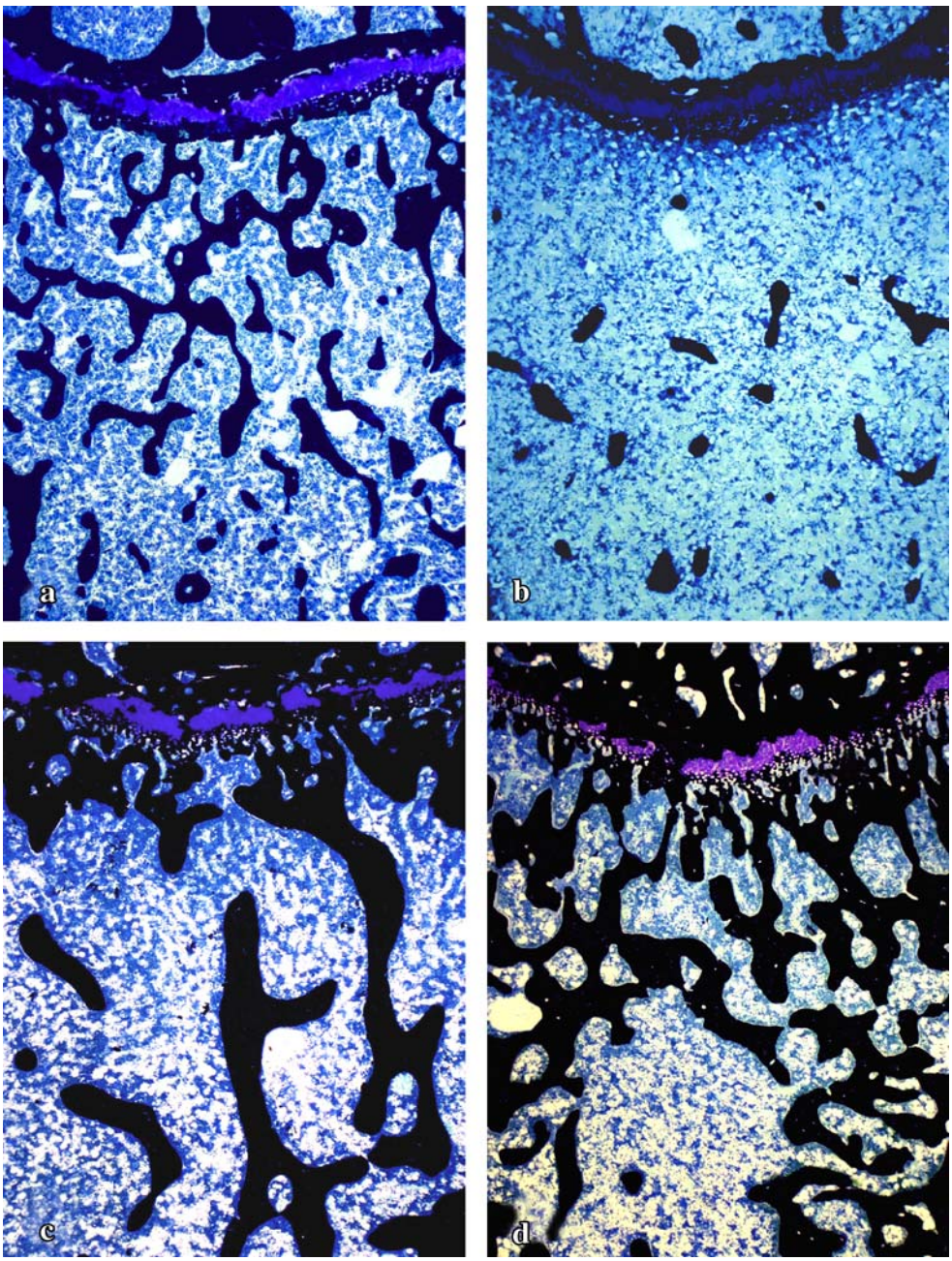

Fig. 4 Proximal tibial metaphyses from (**a**) a baseline control rat, (**b**) a baseline OVX rat, (**c**) an OVX rat treated with PTH alone, and **d**) an OVX rat treated sequentially with bFGF and PTH. Note the reduced mass of black-stained cancellous bone indicative of severe cancellous osteopenia in the baseline OVX rat. Treatment with PTH alone tended to partially restore lost cancellous bone, and sequential treatment with bFGF and PTH completely reversed cancellous osteopenia in this aged OVX rat. (Von Kossa/tetrachrome stain; original magnification ×40)

induced increases in both mineralizing surface and mineral apposition rate (Table 1).

After 11 weeks of vehicle treatment, OVX rats exhibited trends for decreased cortical bone area and width, as well as increased marrow area compared with vehicle-treated control rats. Treatment of aged OVX rats with EP4 and PTH alone, as well as sequential treatment with FGF + PTH and EP4 + PTH, significantly increased cortical bone area and width and decreased marrow area, compared with both vehicle-treated control and OVX rats. This augmentation of cortical bone mass was associated with a stimulation of both periosteal and endocortical bone formation rates in all OVX groups treated singly or sequentially with anabolic agents, due primarily to significant increases in mineralizing surface (Table 1). The marked increases in cortical bone mass in the tibial diaphysis of OVX rats treated with the anabolic agents are seen in Fig. 6.

Discussion

This study detected significant differences in the bone anabolic effects of bFGF, an EP4 receptor agonist, and PTH in aged OVX rats. Based on marked increases in serum osteocalcin, osteoblast surface, and osteoid variables, bFGF had the strongest stimulatory effect on cancellous bone

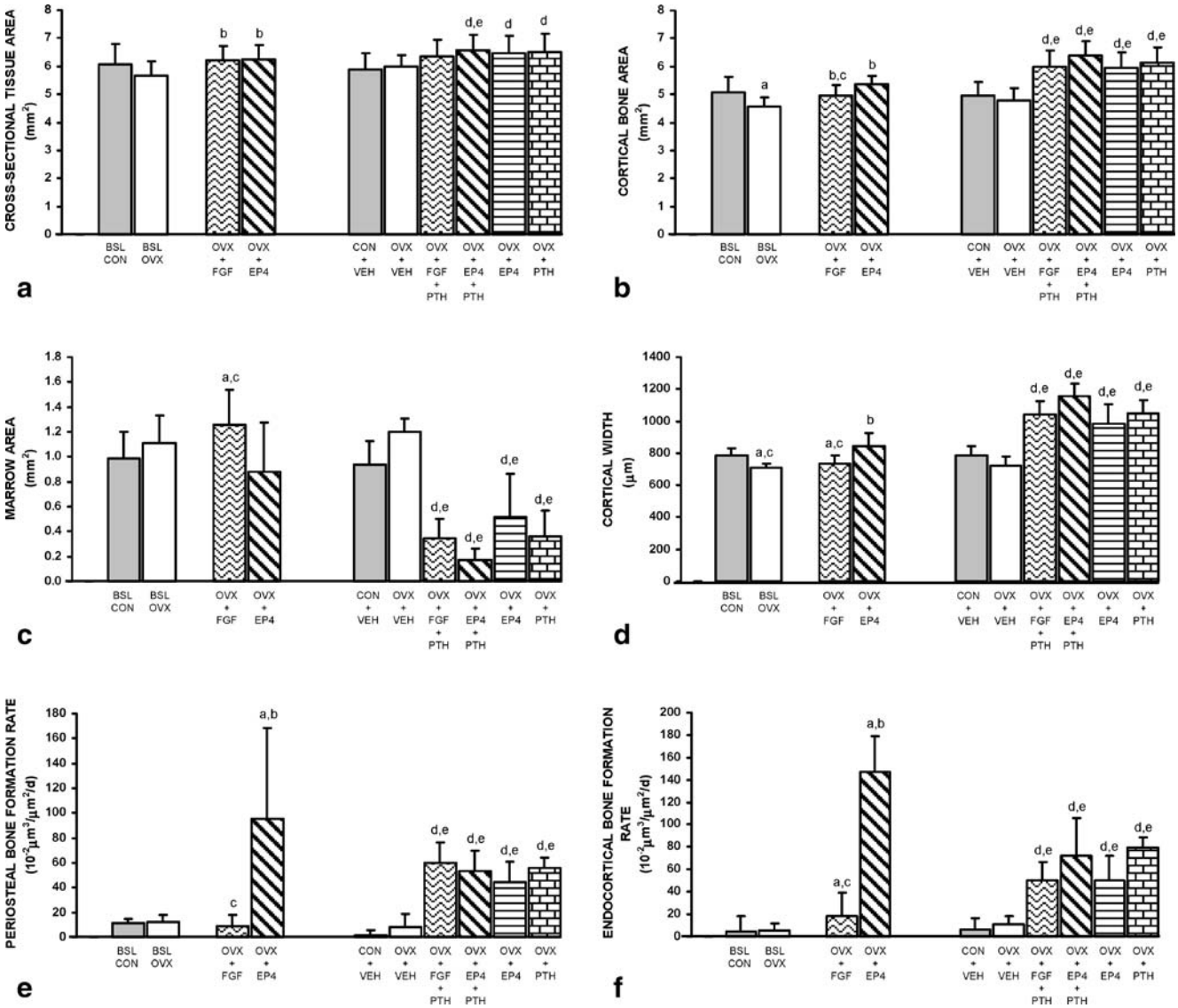

Fig. 5 Mean values (± SD) for cortical bone tissue area (**a**), cortical bone area (**b**), marrow area (**c**), cortical width (**d**), periosteal bone formation rate (**e**), and endocortical bone formation rate (**f**) in the tibial diaphyses of 10 groups of rats. See legend for Fig. 1 for details. [a] Significantly different from the BSL CON group. [b] Significantly different from the BSL OVX group. [c] Significantly different from the OVX + EP4 group at 3 weeks of treatment. [d] Significantly different from the CON + VEH group. [e] Significantly different from the OVX + VEH group

formation. Although long-term treatment with the EP4 agonist stimulated cancellous bone formation to a greater extent than PTH, OVX rats treated with the hormone had greater cancellous bone mass than those treated with the EP4 agonist. This finding is probably a consequence of an accompanying strong stimulation of bone resorption by the EP4 agonist, which was in balance with the stimulation of bone formation so that a net increase in cancellous bone mass failed to occur. In contrast, treatment of aged OVX rats with PTH tended to induce a positive bone balance with the increment in bone formation exceeding the increment in bone resorption, which resulted in a strong trend for partial restoration of lost cancellous bone. In cortical bone, however, the anabolic effects of the EP4 agonist were very similar to those of PTH, in that both therapeutic agents strongly stimulated periosteal and endocortical bone formation, and markedly increased cortical bone mass. These skeletal effects of the EP4 agonist appear to be similar to those of growth hormone, which was also found to augment cortical but not cancellous bone mass in rats [22, 23] and humans [24].

The observed anabolic effects of the EP4 agonist on cortical bone are consistent with the recent findings of Ke et al. [17], but, in contrast to the current study, these investigators reported complete restoration of lost cancellous bone mass in EP4-treated OVX rats with established cancellous osteopenia. The sample site for cancellous bone analyses in the former study was the lumbar vertebral body,

Table 1 Effects of anabolic treatments on periosteal and endocortical mineralizing surface and mineral apposition rate in the tibial diaphysis

	Treatment									
	Baseline		3 weeks		11 weeks					
	BSL CON	BSL OVX	OVX+ FGF	OVX+ EP4	CON+ VEH	OVX+ VEH	OVX+FGF+ PTH	OVX+EP4+ PTH	OVX+ EP4	OVX+ PTH
Periosteal surface										
Mineralizing surface (%)	14.4±9.1	13.9±9.8	15.6±14.3	68.3±23.9[a,b,c]	10.6±16.0	15.4+12.6	77.0±9.0[d,e]	72.4±14.5[d,e]	59.5±17.5[d,e]	69.7±5.6[d,e]
Mineral apposition rate (μm/d)	0.55±0.09	0.56±0.06	0.47±0.09	1.24±0.63[a,b,c]	0.84±0.28	0.64±0.13	0.77±0.14	0.71±0.12	0.72±0.11	0.81±0.14
Endocortical surface										
Mineralizing surface (%)	13.7±8.4	10.7±5.2	20.7±15.8	86.7±10.0[a,b,c]	12.7±7.5	16.6±6.7	57.3±12.4[d,e]	75.7±21.8[d,e]	58.4±23.2[d,e]	74.3±8.4[d,e]
Mineral apposition rate (μm/d)	1.29±0.0	0.74±0.21	1.15±0.24	1.68±0.23[b,c]	0.84±0.28	0.64±0.29	0.86±0.20	0.92±0.25[e]	0.84±0.14	1.07±0.08[e]

Data are mean ± SD

[a] Significantly different from BSL CON group

[b] Significantly different from BSL OVX group

[c] Significantly different from OVX+FGF at 3 weeks of treatment

[d] Significantly different from CON+VEH group

[e] Significantly different from OVX+VEH group

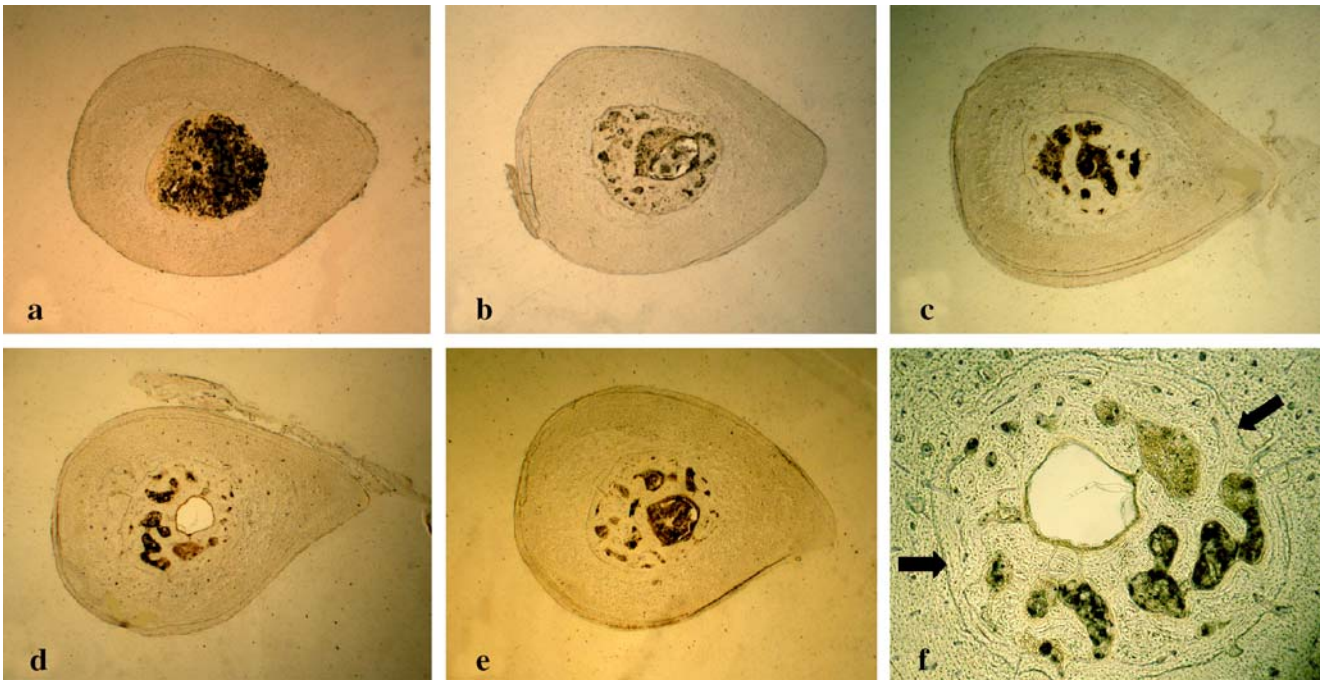

Fig. 6 Cross sections of the tibial diaphysis from aged OVX rats treated for 11 weeks with vehicle (**a**), sequentially with FGF and PTH (**b**), sequentially with EP4 and PTH (**c**), EP4 alone (**d**), and PTH alone (**e**). Note the increased bone mass within the marrow cavity of all OVX rats treated with these bone anabolic regimens. At higher magnification (**f**), the new bone deposited within the original endocortical surface (*arrows*) can be seen in an OVX rat treated with EP4 alone. (original magnification *A-E*: ×10; *F*: ×40)

which is moderately osteopenic in aged OVX rats, whereas the sample site in the current study was the severely osteopenic proximal tibial metaphysis. This difference in the magnitude of cancellous osteopenia at the beginning of EP4 treatment may explain the divergent results. However, Sibonga et al. [25] reported that PGE_2 reversed severe cancellous osteopenia in the proximal tibia of senescent rats when treatment was initiated 20 months after ovariectomy. This finding suggests that the EP4 receptor agonist does not completely mimic the effects of PGE_2 on bone. There is evidence that the bone catabolic effects of PGE_2 are largely mediated through the EP4 receptor [26, 27]. On the other hand, bone strength is greatly reduced in EP2 knockout mice [28], and bone fracture healing is enhanced by an EP2 selective agonist [29]. Therefore, an optimal bone anabolic response may require activation of several PGE_2 receptor subtypes.

When comparing the efficacy of the bone anabolic agents, short-term treatment (3 weeks) with bFGF appears to have a stronger stimulatory effect on cancellous bone formation than the EP4 agonist, as indicated by significantly greater increases in osteoblast surface and serum osteocalcin. OVX rats treated with the EP4 agonist for 3 weeks exhibited a greater increase in cancellous mineralizing surface and bone formation rate than bFGF-treated OVX rats, but this finding is probably a consequence of the inhibitory effect of bFGF on bone mineralization [6–8], which makes fluorochrome-based data unreliable in animals treated with the growth factor. The EP4 agonist, but not bFGF, was found to also stimulate bone resorption, as indicated by increased osteoclast surface. However, the low osteoclast surface in bFGF-treated OVX rats may not be a direct effect of the growth factor but rather secondary to the accumulation of osteoid, since osteoclasts are rarely found adjacent to unmineralized bone surfaces. In any case, bFGF induced formation of osteoid islands within the bone marrow and osteoid bridges between pre-existing bone spicules, which was not observed in EP4-treated OVX rats. Therefore, bFGF appears to be more promising for the treatment of severe cancellous osteopenia. Nevertheless, the EP4 agonist markedly stimulated both periosteal and endocortical bone formation, and augmented cortical bone mass, which are highly desirable skeletal effects. Furthermore, OVX rats treated with the EP4 agonist did not exhibit the gastrointestinal side effects (weight loss and diarrhea) associated with PGE_2, which supports the EP4 agonist for consideration as an osteoporosis therapy.

As in some previous studies [4, 8], PTH treatment of aged OVX rats with severe cancellous osteopenia failed to completely restore lost cancellous bone, probably due to lack of adequate numbers of bone spicules to serve as templates for new bone formation. Somewhat surprisingly, some indices of cancellous bone formation—such as osteoblast surface, mineralizing surface, and bone formation rate—were only marginally increased in OVX rats treated for 11 weeks with PTH alone, but the observed increase in

serum osteocalcin indicates that bone formation was elevated in these animals. The PTH-induced stimulation of cancellous bone formation is known to decline with time in OVX rats during long-term treatment with the hormone [30], which may explain the lower-than-expected increases in histomorphometric indices of cancellous bone formation after 11 weeks of treatment with PTH alone.

Previous treatment of severely osteopenic OVX rats with bFGF to induce formation of new bone spicules before initiation of PTH treatment was expected to augment cancellous bone mass to a greater extent than treatment with PTH alone. Although OVX rats treated sequentially with bFGF and PTH exhibited a trend for greater cancellous bone mass than OVX rats treated with PTH alone, this trend was not statistically significant. In contrast to bFGF, the EP4 agonist did not induce formation of bone/osteoid spicules within bone marrow, yet the cancellous bone volumes for aged OVX rats treated sequentially with EP4 + PTH and bFGF + PTH were nearly identical. This finding is difficult to explain, especially in view of the failure of treatment with EP4 alone to augment cancellous bone mass. Perhaps the increased bone-forming surfaces in EP4-treated OVX rats at the beginning of PTH treatment served as a foundation for a greater initial stimulatory effect of the hormone on bone formation, which resulted in the strong trend for OVX rats treated with EP4 + PTH to have greater cancellous bone mass than those treated with PTH alone.

In summary, the current study detected some significant differences in the skeletal effects of bone anabolic agents in aged OVX rats. Basic FGF, but not the EP4 agonist or PTH, induced formation of osteoid islands within the bone marrow of the severely osteopenic proximal tibia, and had the strongest stimulatory effect on cancellous bone formation. A strong trend for increased cancellous bone mass was observed in aged OVX rats treated with PTH alone. In contrast, the EP4 agonist stimulated cancellous bone turnover, but did not augment cancellous bone mass. However, much like PTH, the EP4 agonist strongly stimulated both periosteal and endocortical bone formation, which resulted in a marked increase in cortical bone mass. Based on these results, bFGF appears to be the most promising bone anabolic agent for the reversal of severe cancellous osteopenia, but its adverse side effects must be minimized before this growth factor can be advanced as an osteoporosis therapy. Furthermore, PTH appears to be more efficacious than the EP4 agonist for augmentation of cancellous bone in the severely osteopenic, estrogen-deplete skeleton.

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