Beneficial Effects of Combined Administration of Alendronate and Alfacalcidol on Cancellous Bone Mass of the Tibia in Orchidectomized Rats: A Bone Histomorphometry Study

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(Received June 7, 2007)

Summary The purpose of the present study was to examine the effects of combined administration of alendronate (ALN) and alfacalcidol (ALF) on the cancellous and cortical bone mass of the tibia in orchidectomized rats. Fifty male Sprague-Dawley rats, 3 mo of age, were randomized by the stratified weight method into five groups: age-matched control, orchidectomy, and orchidectomy with administration of ALN (2.5 μg/kg, s.c., 5 times a week), ALF (0.05 μg/kg, p.o., 5 times a week), or ALN + ALF. The total experimental period was 12 wk. Orchidectomy reduced the cancellous bone mass of the proximal tibial metaphysis and maturation-related cortical bone gain of the tibial diaphysis as a result of increased trabecular bone resorption and decreased periosteal bone formation and also increased endocortical bone erosion and formation. ALN suppressed trabecular bone resorption and endocortical bone erosion and formation and increased periosteal bone formation, while ALF increased the number of osteoblasts and suppressed trabecular bone resorption and markedly increased periosteal and endocortical bone formation. Thus, both ALN and ALF prevented the orchidectomy-induced reduction in the cancellous bone mass and maturation-related cortical bone gain. Combined administration of ALN and ALF increased the cancellous bone mass as compared with the values observed in age-matched controls by causing more marked suppression of trabecular bone resorption. The present study showed the beneficial effects of combined administration of ALN and ALF on the cancellous bone mass of the tibia in orchidectomized rats.

Key Words orchidectomy, osteopenia, alendronate, alfacalcidol, rat

Testosterone is important for skeletal growth during the period of linear growth in males, and is also responsible for maintenance of the skeletal mass in the later stage of life (1–3). Testosterone deficiency, caused by orchidectomy (ORX), has been reported to induce high-turnover cancellous osteopenia and cortical osteopenia with decreased periosteal bone formation in rats (4, 5). We clarified, based on the findings in bone specimens obtained from 3-mo-old male Sprague-Dawley (SD) rats, that ORX induced cancellous osteopenia of the proximal tibial metaphysis as a result of increased bone resorption, and reduced maturation-related gain in the cortical bone mass of the tibial diaphysis as a result of decreased periosteal bone formation (6).

Bone growth, bone modeling, and Basic Multi-Cellular Unit (BMU)-based bone remodeling differ between the cortical and cancellous bones. Typically, bone growth and modeling drifts are predominant in cortical bone and can increase the bone mass, whereas bone remodeling occurs primarily in the cancellous bone, and can either conserve or remove bone in contact with the marrow in rats (7). Bone modeling may add bone mass but can never result in a reduction in the bone mass, whereas bone remodeling usually results in a zero or negative balance of the bone mass. Thus, it was suggested that ORX in 3-mo-old male SD rats resulted in decreased bone growth and bone modeling and increased bone remodeling.

Numerous studies have demonstrated the effects of androgen, selective androgen receptor modulators, estradiol, selective estrogen receptor modulator, prostaglandin E2, growth hormone, and parathyroid hormone, alendronate (ALN), etidronate, clodronate, vitamin K2, and soy isoflavones on the skeleton in ORX rats (5, 6, 8–24). However, very few studies have reported on the effects of vitamin D3 on the bone mass in ORX rats. Anti-resorptive agents mainly influence bone remodeling sites, while anabolic agents act on bone modeling and remodeling sites. Thus, combined administration of anti-resorptive and anabolic agents could be more effective for increasing the cancellous and cortical bone mass because of the effects on both modeling and remodeling sites in ORX rats. However, the effect of combined administration of anti-resorptive and ana-
bolic agents on the skeleton has rarely been reported in ORX rats.

ALN and active vitamin D₃, namely, alfacalcidol (ALF), are commercially available in Japan. ALN is known to inhibit osteoclast-mediated bone resorption (25), while ALF has both anabolic and anti-resorptive effects on the skeleton (26–31). The purpose of the present study was to examine the effects of combined administration of ALN and ALF on the cancellous and cortical bone mass of the tibia in 3-mo-old SD ORX rats.

MATERIALS AND METHODS

Treatment of animals. Fifty male SD rats, 3 mo of age, were purchased from Hilltop Lab. Animals, Inc. (Scottsdale, PA, USA). The animals were housed under local vivarium conditions (temperature 23.8°C and 12-h on/off light cycle), and were fed a pelleted standard chow diet containing 1.36% calcium and 2,400 IU/kg of vitamin D (Rodent Diet 8604, Harlan Teklad, Madison, WI, USA), with free access to water. Following a 1-wk adaptation period in the new environment, the rats were randomized by the stratified weight method into five groups of 10 rats each according to the treatment protocols. All the rats were labeled with ketamine injected intraperitoneally at 80 mg/kg, and then administered by gavage deep into the mouth at the dose of 1.5 μg/kg body weight five times a week. ALF (Teijin Pharma, Tokyo, Japan) was dissolved in 0.1 mL of PBS containing 0.25% ethanol and 0.1% Tween 20, and then administered by gavage deep into the mouth at the dose of 0.05 μg/kg body weight five times a week. These doses of ALN and ALF were considered to be effective in rats, in accordance with previously published data (26–32). The body weight of the rats was monitored weekly, and the total experimental period was 12 wk. The study was carried out at Winthrop-University Hospital, and the animals were maintained according to the National Institutes of Health (NIH) Guidelines for Care and Use of Laboratory Animals. All the animal experimental protocols were approved by the Laboratory Animal Care Committee of Winthrop-University Hospital.

Preparation of specimens. All the rats were labeled with 10 mg/kg of calcine (Sigma Chemical, St. Louis, MO, USA) injected intramuscularly 10 d and 3 d before they were sacrificed. The animals were anesthetized with ketamine injected intraperitoneally at 80 mg/kg, together with xylazine at 12 mg/kg, and sacrificed by exsanguination. The right tibia was collected from every animal. The tibiae were used for bone histomorphometric analysis; the bones were fixed overnight in 40% cold ethanol, and then cut into three parts using an Isomet saw (Buehler, Lake Bluff, IL, USA). The proximal tibial metaphyses and tibial diaphyses were stained with Villanueva Osteochrome Bone Stain (PolyScience, Warrington, PA, USA) for 5 d. The specimens were then dehydrated sequentially in ascending concentrations of ethanol (70, 95, and 100%) and xylene and then embedded in methyl methacrylate (EM Science, Gibbstown, NJ, USA) at 4°C, in accordance with the method of Erben (33). Cross-sections of the tibial diaphysis just proximal to the tibio-fibular junction were cut at 40-μm thickness using a diamond wire Histo-Saw machine (Delaware Diamond Knives, Wilmington, DE, USA), and the thickness of each cross-sectional specimen was determined with an Inspectors’ Dial Bench Gauge (L.S. Starrett, Athol, MA, USA). Frontal sections of the proximal tibial metaphysis were cut at 5-μm thickness using a microtome (Leica RM2155; Leica Inc., Nussloch, Germany), transferred onto chromium-gelatin-coated slides, dried overnight under pressure at 42°C, and coverslipped with Eukitt mounting medium (Calibrated Instruments, Hawthorne, NY, USA) for static and dynamic histomorphometric analyses.

Bone histomorphometric analysis of the tibia. A digitizing morphometric system was used to measure the bone histomorphometric parameters. The system consisted of an epifluorescence microscope (Nikon E-400, OsteoMetrics, Atlanta, GA, USA), an Osteomeasure High Resolution Color Subsystem (OsteoMetrics) coupled to an IBM computer, and a morphometry program (OsteoMetrics). The measured parameters for cancellous bone included the total tissue volume (TV), bone volume (BV), bone surface (BS), eroded surface (ES), single- and double-labeled surfaces (sLS and dLS, respectively), number of osteoblasts (N.Ob) and osteoclasts (N.Oc), and interlabel width. These data were used to calculate the percent cancellous bone volume (BV/TV), trabecular number (Tb N), trabecular thickness (Tb Th), trabecular separation (Tb Sp), ES/BS, mineralizing surface (MS)/BS [(sLS/2+dLS)/BS], mineral apposition rate (MAR), bone formation rate (BFR)/BS, N.Ob/BS, and N.Oc/BS, in accordance with the standard nomenclature proposed by Parfitt et al. (34). In the present study, the region of cancellous bone measured was 1.5–4.5 mm distal to the lower margin of the growth plate in the proximal tibial metaphysis, which consists of secondary spongiosa. The following parameters of cortical bone were measured: the total tissue area (Tt Ar) and cortical bone area (Ct Ar), as well as the periosteal and endocortical BS (perimeter), sLS, dLS, and interlabel width, and endocortical ES. These data were used to calculate the marrow area (Ma Ar), percent Ct Ar, and percent Ma Ar, as well as the periosteal and endocortical MS/BS [(sLS/2+dLS)/BS], MAR, and BFR/BS, and endocortical ES/BS.

Statistical analysis. All the data were expressed as means and standard deviation (SD). Multiple comparisons of data among the groups were performed by analysis of variance (ANOVA) with the Turkey-Kramer test. All statistical analyses were performed using the Stat View J-5.0 program on a Macintosh computer. A significance level of p<0.05 was used for all the comparisons.

RESULTS

Body weight

The initial body weights of the CON, ORX,
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ORX, ORX/ALN, ORX/ALF, and ORX/ALN+ALF groups were 384±6, 386±10, 389±10, 383±6, and 389±10 g, respectively, with no significant differences in the initial body weight among the groups. The respective final body weights were 504±14, 492±16, 503±20, 496±20, and 497±23 g, respectively, with no significant differences in the final body weight among the groups.

Effects of ORX

Previously, we clarified that ORX in 3-mo-old SD rats induced cancellous osteopenia in the proximal tibial metaphysis and reduced maturation-related gain in the cortical bone mass of the tibial diaphysis (6). In the present study, taking into consideration our previous data on the maturation-related changes in bone size and bone mass, ORX induced loss of cancellous BV/TV of the proximal tibial metaphysis (Fig. 1) and decreased maturation-related gain in the percent Ct Ar, and reduction in the percent Ma Ar of the tibial diaphysis (Fig. 2). The decrease in cancellous BV/TV, which was associated with a reduction in the Tb N and increase in the Tb Sp, was attributable to increased bone resorption (N.Oc/BS) (Fig. 1 and Table 1). Alterations in the percent Ct Ar and percent Ma Ar were attributable to decreased periosteal bone formation (MAR, BFR/BS) and increased endocortical bone erosion and formation.
Table 1. Bone histomorphometric analysis of cancellous bone of the proximal tibial metaphysis—Formative and resorptive parameters.

|          | N.Ob/BS (1/mm) | MS/BS (%) | MAR (μm/d) | BFR/BS (μm²/μm²/d) | N.Oc/BS (1/mm) | ES/BS (%) |
|----------|----------------|-----------|------------|--------------------|----------------|-----------|
| CON      | 2.7 ± 1.1      | 24.9 ± 5.9| 1.31 ± 0.15| 33.0 ± 9.6         | 2.6 ± 1.1      | 5.9 ± 2.3 |
| ORX      | 2.1 ± 1.1      | 24.9 ± 7.0| 1.38 ± 0.24| 34.3 ± 9.8         | 5.5 ± 1.5      | 12.2 ± 2.8 |
| ORX + ALN| 1.0 ± 0.3     | 13.8 ± 5.5| 1.07 ± 0.31| 15.5 ± 7.0         | 2.2 ± 1.2      | 4.5 ± 1.4 |
| ORX + ALF| 3.4 ± 1.3      | 12.5 ± 5.0| 1.43 ± 0.35| 29.1 ± 10.2        | 2.8 ± 1.2      | 9.8 ± 4.4 |
| ORX + ALN + ALF| 0.7 ± 0.6 | 11.9 ± 3.2| 1.23 ± 0.29| 15.0 ± 6.2         | 0.9 ± 0.8      | 2.9 ± 2.2 |

Data are expressed as mean ± SD. ANOVA with Tukey-Kramer test was used to compare the data among the groups.

* Significant vs. CON; † significant vs. ORX; ‡ significant vs. ALN; § significant vs. ALF.

CON: age-matched control, ORX: orchidectomy, ALN: administration of alendronate, ALF: administration of alfacalcidol, ALN + ALF: administration of alendronate and alfacalcidol.

N.Ob: number of osteoblasts, BS: bone surface, MS: mineralizing surface, MAR: mineral apposition rate, BFR: bone formation rate, N.Oc: number of osteoclasts, ES: eroded surface, NS: not significant.

Table 2. Bone histomorphometric analysis of cortical bone of the tibial diaphysis—Formative and resorptive parameters.

|          | MS/BS (%) | MAR (μm/d) | BFR/BS (μm²/μm²/d) | ES/BS (%) |
|----------|-----------|------------|--------------------|-----------|
| CON      | 34.4 ± 13.2| 1.15 ± 0.09| 39.5 ± 14.8        | 2.2 ± 1.6 |
| ORX      | 21.9 ± 10.5| 0.96 ± 0.08| 21.4 ± 10.5        | 0.6 ± 0.6 |
| ORX + ALN| 40.0 ± 12.7| 1.00 ± 0.11| 40.2 ± 14.2        | 1.9 ± 1.9 |
| ORX + ALF| 54.1 ± 10.4| 1.22 ± 0.12| 66.1 ± 15.5        | 1.1 ± 0.03 |
| ORX + ALN + ALF| 55.1 ± 7.5| 1.35 ± 0.18| 74.2 ± 14.9        | 2.2 ± 1.0 |

Data are expressed as mean ± SD. ANOVA with Fisher’s PLSD test was used to compare the data among the groups.

* Significant vs. CON; † significant vs. ORX; ‡ significant vs. ALN; § significant vs. ALF.

CON: age-matched control, ORX: orchidectomy, ALN: administration of alendronate, ALF: administration of alfacalcidol, ALN + ALF: administration of alendronate and alfacalcidol.

MS: mineralizing surface, BS: bone surface, MAR: mineral apposition rate, BFR: bone formation rate, ES: eroded surface, NS: not significant.

Effects of combined administration of ALN and ALF in ORX rats

Combined administration of ALN and ALF increased the cancellous BV/TV (Fig. 1), as compared with the values in age-matched controls by causing more marked suppression, as compared with each drug administered alone, of trabecular bone resorption (N.Oc/BS) (Tables 1 and 2). In particular, ALN and ALF had synergistic effects on the cancellous bone mass. However, there was no benefit of combined administration of ALN and ALF for the cortical bone mass, as compared with each drug administration alone.

**DISCUSSION**

To our knowledge, the combined effects of anti-resorptive and anabolic agents on the skeleton have not specially been assessed in ORX rats. The present study was conducted to clarify the efficacy of combined administration of ALN and ALF on the cancellous and cortical bone mass in ORX rats. ALN suppressed trabecular bone resorption and endocortical bone erosion and...
formation and increased periosteal bone formation, while ALF increased the N.Ob/BS and suppressed trabecular bone resorption, and markedly increased periosteal and endocortical bone formation. As a result, both ALN and ALF prevented the ORX-induced reductions in cancellous bone mass and maturation-related cortical bone gain. Combined administration of ALN and ALF increased the cancellous bone mass as compared with the values in age-matched controls by more markedly suppressing trabecular bone resorption. Thus, we confirmed the beneficial effects of combined administration of ALN and ALF on the cancellous bone mass in ORX rats.

ALN is known to inhibit osteoclast-mediated bone resorption (25). There are a few previous reports on the effects of ALN on the skeleton in ORX rats; ALN reduced the bone turnover, prevented loss of bone density, and preserved or increased the mechanical strength of the femur (18, 19). Similarly, in the present study, ALN suppressed bone remodeling on the trabecular bone surface, resulting in prevention of ORX-induced cancellous osteopenia. However, ALN not only suppressed bone remodeling at the endocortical surface, but also prevented the ORX-induced reduction in periosteal bone formation, resulting in prevention of the decreases in the maturation-related gain in percent Ct Ar and reduction in the percent Ma Ar. Thus, ALN (2.5 µg/kg, s.c., 5 times a week) effectively prevented the ORX-induced reductions in cancellous bone mass and maturation-related cortical bone gain in the 3-mo-old SD rats.

Because ALN has been reported to suppress bone formation on the periosteal surface in intact rats (35), and ALF stimulates periosteal bone formation in ovariectomized rats (28–31), the efficacy of the combined administration of ALN and ALF on periosteal bone formation and cortical bone mass had been expected in ORX rats. However, periosteal bone formation was unexpectedly stimulated following administration of ALN in the present study. It has also been reported that the RANKL inhibitor increases cortical volumetric bone mineral content and density, cortical thickness in terms of periosteal circumference, and cross-sectional moment of inertia at the proximal tibia and distal radius in intact monkeys (36). On the other hand, an iliac biopsy study has reported that long-term ALN treatment increases the mean wall thickness in postmenopausal women with osteoporosis (37), possibly suggesting an anabolic effect of ALN on the cancellous bone, although we did not detect any anabolic effect of ALN on the cancellous bone in the present short-term experimental study. The mechanism for anabolic effects of bisphosphonates remains unknown. However, one possibility for increased periosteal bone formation following administration of ALN might be the anabolic actions of parathyroid hormone (PTH), because ALN is known to decrease the serum calcium level and increase the serum PTH level (38). Another possibility might be the action of ALN on the osteoblast, because in vitro studies have demonstrated that bisphosphonates inhibit osteocyte and osteoblast apoptosis (39), and may stimulate osteoblast proliferation and differentiation (40–42), leading to increased bone formation. Thus, the anabolic effect of ALN administration on the cortical bone might partly be the direct action of ALN or the indirect action of intrinsic PTH on the osteoblasts on the periosteal surface in ORX rats. However, further studies are needed to clarify the mechanism for increased periosteal bone formation after ALN treatment in ORX rats.

ALF has been reported to suppress trabecular and endocortical bone resorption while maintaining or even increasing trabecular and endocortical bone formation, and also to increase periosteal bone formation in ovariectomized rats (27–31), suggesting the anti-resorptive and anabolic effects of ALF on the bone. Thus, ALF supercouples bone resorption and formation in ovariectomized rats. However, very few studies have reported on the effects of ALF on the skeleton in ORX rats. ALF mildly suppressed bone remodeling as indicated by the suppressed trabecular bone resorption and increased bone modeling as indicated by the increased N.Ob/BS on the trabecular surface and increased periosteal BFR/BS. ALF might also supercouple bone resorption and formation in ORX rats. ALF (0.05 µg/kg, p.o., 5 times a week) effectively prevented the ORX-induced reductions in the cancellous bone mass and maturation-related cortical bone gain in 3-mo-old SD rats.

The more pronounced effect of combined administration of ALN and ALF on the cancellous bone mass in the lumbar spine as compared with single administration of either agent alone has been reported to be attributable to a reduction in the bone turnover marker levels in ovariectomized rats (43). This is the first report to show the effects of combined administration of ALN and ALF on the bone mass in ORX rats. Combined administration of the two agents increased the cancellous bone mass as compared with the values observed in age-matched controls in ORX rats by more markedly suppressing trabecular bone resorption. Combined administration of the two agents exerted more pronounced effects, as compared to either agent alone, on the bone remodeling site, the cancellous bone. We speculate that osteoclasts in the cancellous bone might be more responsive to combined administration of ALN and ALF than those in the cortical bone.

There an obvious limitation to be addressed in the present study: a growing rat model was used. Thus, maturation-related gains in bone mass could not be ignored in ORX rats. Furthermore, the effect of ALN and/or ALF on cortical porosity, which could be observed in aged ORX rats (5, 20), was not assessed. Therefore, the results of the present study can not be translated into human in terms of men with primary osteoporosis. Thus, further studies are needed to confirm the beneficial effects of combined administration of ALN and ALF on the bone mass in ORX aged rats.

In conclusion, the present study showed that both ALN and ALF were effective in preventing the ORX-induced reductions in the cancellous bone mass and maturation-related cortical bone gain in 3-mo-old SD rats. However, combined administration of the two
agents effectively increased the cancellous bone mass as compared with the values in age-matched controls. Thus, we confirmed the beneficial effects of combined administration of ALN and ALF on the cancellous bone mass of the tibia in ORX rats.

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