Table 3 Background data of women of each haplotype

Gene	Haplotype	Age (years)	Body height (cm)	Body weight (kg)	BMI	BMD (Z score)
ER ($n=261$) (*Pvu*-II: *Xba*-I)	11 ($n=5$)	69.6 (4.5)	147.0 (5.3)	47.6 (6.3)	22.0 (2.5)	0.224 (1.089)
	12 ($n=20$)	64.7 (8.1)	153.0 (6.1)	54.9 (9.5)	23.4 (3.3)	−0.470 (0.902)
	13 ($n=19$)	63.1 (10.5)	150.7 (6.4)	51.2 (5.4)	22.5 (2.2)	−0.159 (1.478)
	21 ($n=1$)	52 (–)	149 (–)	54 (–)	24.3 (–)	−0.601 (–)
	22 ($n=58$)	64.8 (9.3)	150.9 (7.2)	51.0 (7.0)	22.5 (3.5)	0.106 (1.476)
	23 ($n=80$)	63.8 (11.6)	150.7 (7.2)	50.3 (8.8)	22.1 (3.0)	−0.077 (1.413)
	32 ($n=1$)	62 (–)	158 (–)	65 (–)	26.0 (–)	0.800 (–)
	33 ($n=77$)	63.1 (10.1)	150.2 (6.2)	49.7 (8.3)	22.0 (3.0)	0.112 (1.441)
VDR ($n=318$) (*Apa*-I:*Bsm*-I)	11 ($n=7$)	61.9 (8.6)	149.1 (6.4)	45.9 (6.3)	20.7 (3.2)	−0.574 (1.170)
	12 ($n=16$)	62.8 (8.5)	152.3 (6.0)	52.9 (6.5)	23.1 (3.0)	−0.797 (1.242)
	13 ($n=21$)	64.0 (11.9)	150.0 (7.0)	48.0 (6.2)	21.3 (2.0)	0.124 (1.069)
	21 ($n=1$)	64 (–)	149 (–)	50 (–)	22.5 (–)	0.110 (–)
	22 ($n=56$)	64.3 (11.1)	150.5 (6.3)	49.8 (7.0)	21.9 (2.5)	−0.420 (1.310)
	23 ($n=91$)	63.8 (9.6)	151.3 (5.8)	51.2 (8.0)	22.3 (3.0)	0.246 (1.554)
	33 ($n=126$)	63.4 (9.8)	151.1 (7.0)	51.4 (8.7)	22.5 (3.5)	−0.850 (1.498)
PTH ($n=104$) (*Bst-Bl*:*Dra*-I)	11 ($n=64$)	63.7 (10.0)	150.7 (6.1)	50.8 (7.2)	22.4 (3.2)	0.001 (1.320)
	12 ($n=25$)	64.6 (11.5)	149.4 (5.8)	49.6 (7.2)	22.2 (2.7)	−0.509 (1.328)
	21 ($n=12$)	65.0 (11.4)	150.9 (7.3)	51.0 (7.4)	22.4 (3.1)	−0.325 (1.307)
	22 ($n=2$)	46.0 (1.4)	157.0 (5.7)	54.5 (2.1)	22.1 (0.7)	−1.600 (0.566)
	31 ($n=1$)	60 (–)	153 (–)	39 (–)	16.7 (–)	−0.700 (–)

Haplotypes are shown as the combination of two RFLP genotypes (+ + = 1, + − = 2, and − − = 3) in ER (*Pvu*-II:*Xba*-I), VDR (*Apa*-I:*Bsm*-I) or PTH (*Bst-Bl*:*Dra*-I). Data of age, body height, weight, BMI and BMD (L2-4, Z score) are expressed by the mean (SD)

lation. Here again, there was no significant association between RFLP genotypes and spondylosis severity at any levels in either population (data not shown).

Association of ER, VDR, PTH and IL-1β RFLP haplotypes with lumbar spondylosis

We further examined two RFLPs jointly in ER, VDR and PTH by haplotypic analysis. No significant difference in age, body weight, height, BMI or BMD (L2–4, Z score) was seen among the RFLP haplotypes in ER, VDR, PTH or IL-1β (Table 3, all $P>0.05$). Association studies revealed that there were significant differences in the severity of spondylosis among haplotypes of ER (Fig. 1) and VDR (Fig. 2) RFLPs, especially in the upper levels of the lumbar spine (L1/2 and L2/3). A stratified analysis by age revealed that the association of the ER haplotype was more pronounced in the younger group (≤ 63.9 years) than in the older group (>63.9 years). Contrarily, the association of the VDR haplotype was more significant in the older group (>63.6 years) than in the younger group (≤ 63.6 years). The PTH RFLP haplotype was not associated with the severity at any level in either group.

Discussion

To learn the involvement of ER, VDR, PTH and IL-1β genes in the etiology of lumbar spondylosis in post-menopausal women, this study looked at the association between the polymorphisms and the severity of spondylosis. The RFLP haplotypes of ER and VDR, although not their genotypes, were associated with the spondylosis of the upper lumbar spine. It is well known that the spondylotic change occurs more frequently in the lower levels like L4/5 or L5/S1 than in the upper levels, and that the most important environmental factor for these disorders is the accumulation of mechanical stress. Hence, it is speculated that genetic contribution may be more prominent in the upper lumbar spine where the influence of the accumulating mechanical stress is relatively small.

Interestingly, the subpopulations in which ER and VDR showed significant associations with spondylosis were different: the former in younger and the latter in older subpopulations, suggesting that these two genes distinctly contribute to lumbar spondylosis. However, the mechanisms whereby the ER and VDR genes associated with the severity pose a risk for this condition are currently unclear. Although the present study did not investigate the association of these polymorphisms with osteoarthritis of the extremities, previous reports have strongly indicated that the present findings are not specific to lumbar spondylosis, but rather commonly seen in general osteoarthritic disorders. ER polymorphism may generally affect the estrogen sensitivity in the joint cartilage, which may influence the severity in the early phase after menopause. Involvement of estrogen in osteoarthritis and spondylosis is consistent with the larger increases in women than in men in the prevalence of the disorder after 50 years of age, and actually, two estrogen

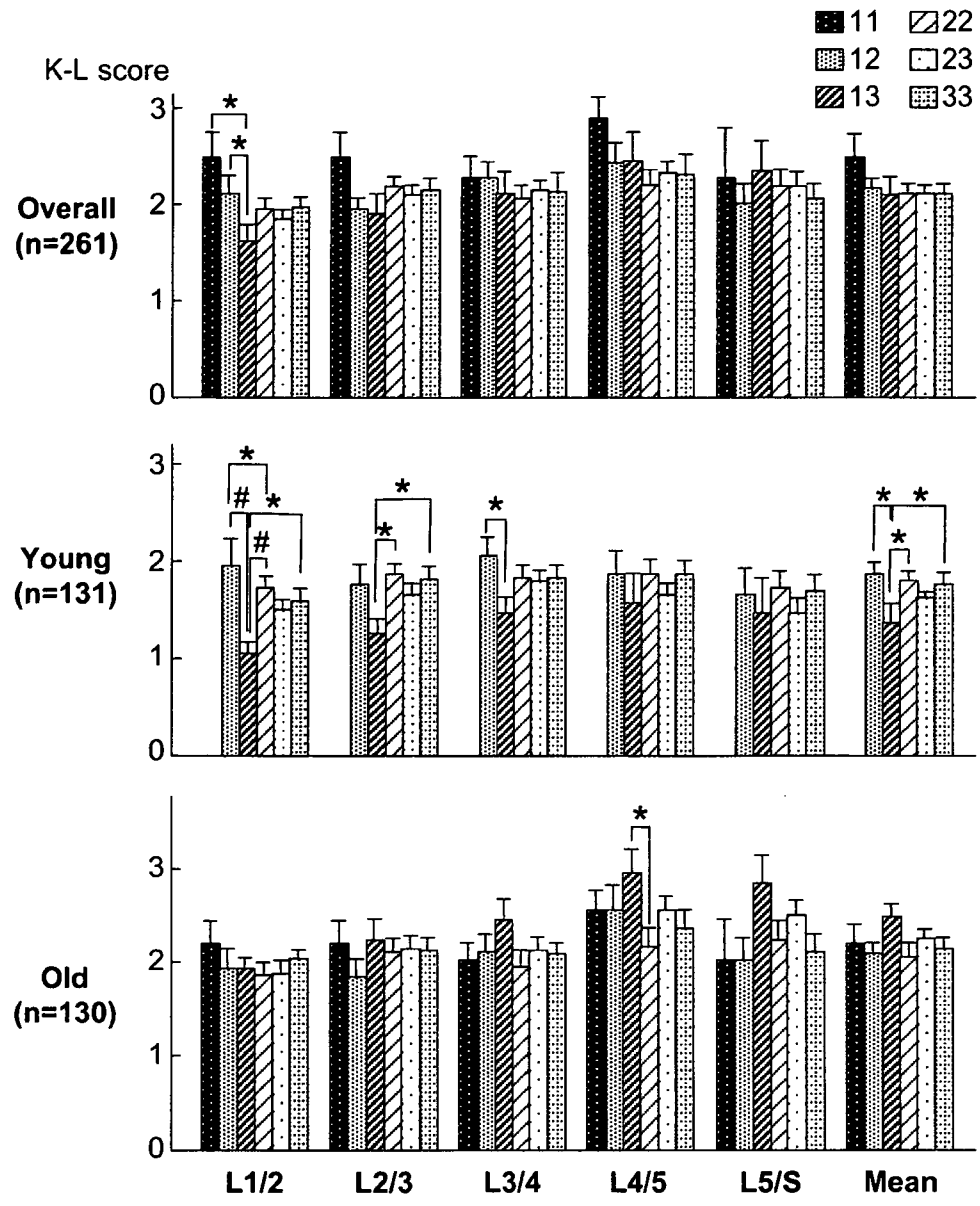

Fig. 1 Association between ER RFLP haplotypes and the severity of spondylosis at disk levels (L1/2-L5/S1) in the overall ($n=261$), younger ($n=131$) and older ($n=130$) populations of post-menopausal women. The allele that could be digested by *Pvu*-II and *Xba*-I endonucleases was expressed by (+) while that which could not by (−), and the genotype was shown by the combination: + + = 1, + − = 2, and − − = 3. The haplotypes were further expressed by the combination of genotypes (11, 12, 13, 21, 22, 23, 31, 32, 33). The severity of spondylosis at each disk level was graded according to the Kellgren–Lawrence score (grade 0–4) on a lateral radiograph of the lumbar spine. Patients were divided into two subpopulations younger and older than the average age: 63.6 years. Data are expressed as means (*bars*) ± SEMs (*error bars*). $*P<0.05$, $\#P<0.01$; significant difference

receptors (ERα and ERβ) have been identified in normal and osteoarthritic cartilage [26, 35], indicating that the cartilage can respond to estrogen. Furthermore, hormone replacement therapy for menopause seems to be associated with a decrease in the prevalence of symptoms and radiological alterations related to osteoarthritis [25]. Estrogen can influence chondrocyte function on multiple levels by interacting with cellular growth factors, adhesion molecules and cytokines. Nevertheless, findings regarding the correlation between estrogen and osteoarthritis are inconsistent and inconclusive and range from estrogen protecting against osteoarthritis to cartilage damage mediated by high levels of estrogen and higher binding to estrogen receptors [9]. Contrary to estrogen, vitamin D may play an important role in spondylosis at older ages when the contribution of estrogen loss is weaker. Expression of vitamin D receptor has also been detected in osteoarthritic cartilage and human articular chondrocytes; however, its function on chondrocytes is unclear [31]. Indeed, the present and previous studies could implicate the VDR gene itself to be functionally involved in the etiology of osteoarthritis [16, 33, 36].

Alternatively, since there are a number of reports indicating that ER and VDR genotypes are implicated in determining bone density [2, 7, 12, 19, 20, 22], the

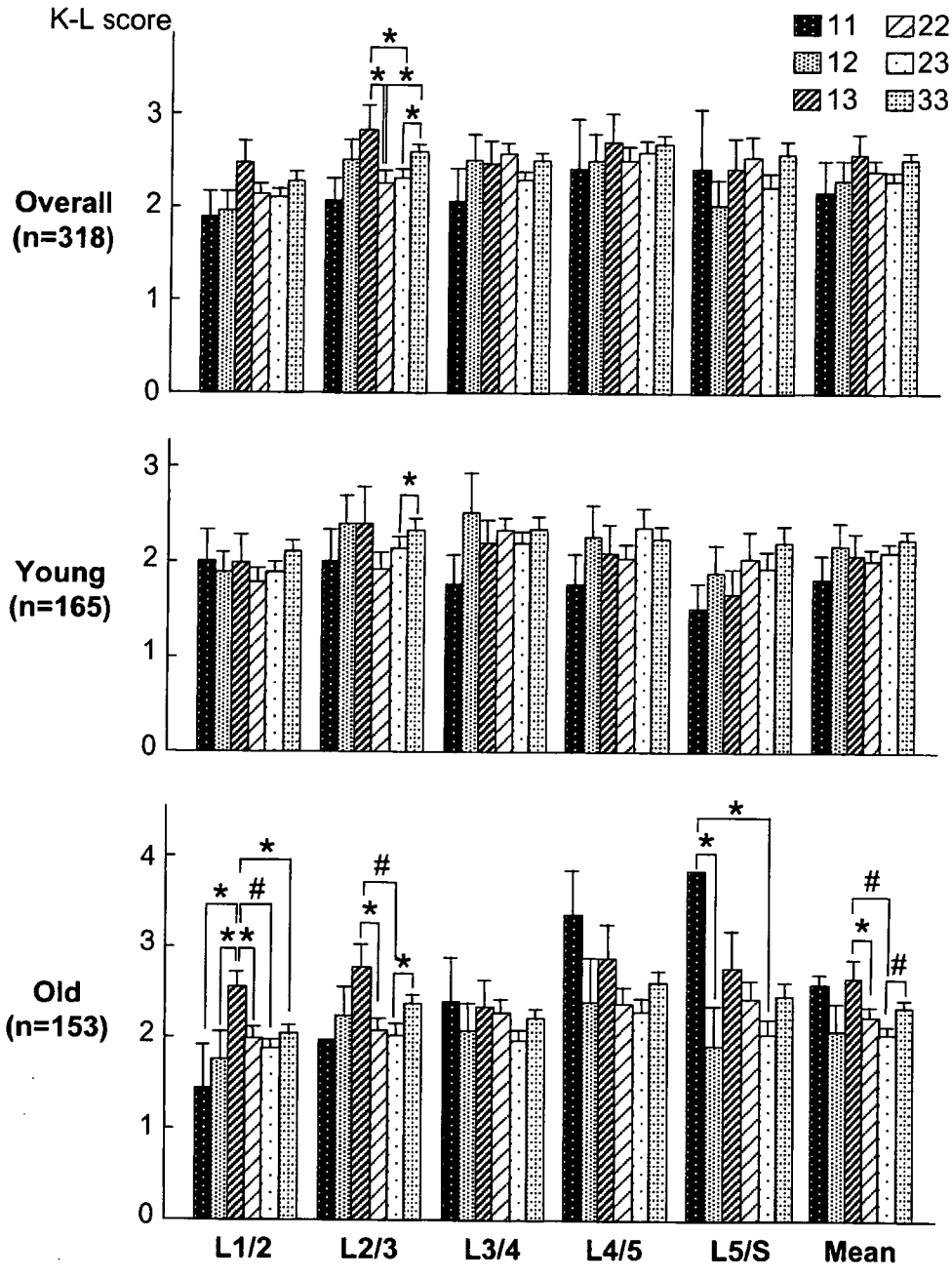

Fig. 2 Association between VDR RFLP haplotypes and the severity of spondylosis at disk levels (L1/2-L5/S1) in the overall ($n=318$), younger ($n=165$) and older ($n=153$) populations of postmenopausal women. The haplotypes were expressed by the combination of RFLP genotypes by *Apa*-I and *Bsm*-I endonuclease digestions as described in Fig. 1. The severity of spondylosis at each disk level was graded according to the Kellgren–Lawrence score (grade 0–4) on a lateral radiograph of the lumbar spine. Patients were divided into two subpopulations younger and older than the average age: 63.9 years. Data are expressed as means (*bars*) ± SEMs (*error bars*). $*P<0.05$, $\#P<0.01$; significant difference

significant association between the RFLPs and spondylosis might be secondary to the effects on bone density. This would be in line with the suggestion that decreased bone density could lead to less damage of articular cartilage because of differential impact loading [24]. In the present study, however, the fact that there was no difference in bone density among the RFLP genotypes and haplotypes (Tables 2, 3) indicates that the findings on spondylosis are independent of bone density in the lumbar spine.

Considering that neither of the ER and VDR polymorphic sites is located in exons but instead in introns, these nucleotide variants do not directly lead to amino acid substitutions. The most likely mechanism may be that these intronic substitutions affect the splicing mechanism; the one base substitution may yield an alternate transcript with abnormal function or affect the expression level of proteins. The next task ahead of us will be to investigate the relevance of these polymorphisms to the production or function of ER and VDR.

Regarding the clinical utility of this study, the practical application of this genetic information of individuals to specific diagnostic or therapeutic modifications is a conceptual goal. It is true that the ER and VDR gene polymorphisms might possibly be useful diagnostic markers to predict the progression of lumbar spondylosis; however, this study demonstrated no more than the possible involvement of ER and VDR signalings in this disorder, and is incomplete and premature for clinical use. For example, the cut-off between 'young' and 'old' is the average age of the population just happened to be enrolled in the present study, but not a definite age that is applicable to the general population. Our stratified analysis to decide the cut-off age by dividing the population into five generations: < 56, 56–60, 61–65, 66–70, and > 70, failed to show the clear association of ER or VDR polymorphism with the lumbar spondylosis (data not shown). This may be at least partly due to the insufficiency of the population in each subgroup, which results in significant difference ($P < 0.05$) of the genotypic frequencies from those expected in Hardy–Weinberg equilibrium in the subpopulations. To decide the cut-off age for the exact diagnostic criteria by the ER and VDR polymorphisms, it will be essential to increase the population studied, so that more precise stratified analyses can be done. In addition, although the objective of this study is limited to post-menopausal women, the conclusion will be more enhanced with the inclusion of other ages like premenopausal women. Regarding therapeutic modifications, estrogen and vitamin D themselves or regulators of their expressions or functions might possibly lead to conservative treatment to prevent the progression. In reality, however, such modification is an era away from the current generation. The legal, social, and ethical implications of this information are also overwhelming.

Using the information gained, we demonstrated possible associations of ER and VDR genes with lumbar spondylosis. These genes might have a relatively small attributable risk fraction for this disease; however, identification of the disease-susceptibility and disease-severity gene could lead to a better understanding of the molecular pathogenesis of the condition, which is essential to develop a significant diagnosis and treatment. Further studies on the functional relevance of these gene abnormalities could provide new biological insights into the etiology of diseases with osteoarthritis including spondylosis.

Acknowledgements This work was supported by a Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology (#15209049).

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Exp. Anim. 55(4), 333–342, 2006

Therapeutic Effect of Risedronate on Cancellous and Cortical Bone in Ovariectomized Osteopenic Rats: A Comparison with the Effects of Alfacalcidol

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Abstract: *The purpose of the present study was to compare the therapeutic effects of risedronate (RIS) and alfacalcidol (ALF) on cancellous and cortical bone in ovariectomized osteopenic rats. Forty-two female Sprague-Dawley rats, 7 months of age, were randomized by the stratified weight method into six groups: the sham-operated control (Sham) group, and five ovariectomized groups: treated with vehicle, RIS (0.1, 1.0, or 2.5 mg/kg, p.o., daily), and ALF (0.5 μg/kg, p.o., daily). Treatment was started 6 weeks after surgery and continued for 6 weeks. Evaluation at 12 weeks after surgery revealed that ovariectomy (OVX) decreased the cancellous bone volume/total tissue volume (BV/TV) of the proximal tibial metaphysis as a result of an increase of the bone formation rate/bone surface (BFR/BS), BFR/BV, and eroded surface (ES/BS), while having no effect on the cortical area (Ct Ar) of the tibial diaphysis. OVX also decreased the maximum load of the femoral distal metaphysis, while having no effect on any mechanical property parameters of the femoral diaphysis. RIS (at all the doses) increased the BV/TV relative to the value in the OVX-Vehicle group, but the value was not restored to that observed in the Sham group. The effects of RIS (1.0 mg/kg and 2.5 mg/kg) were similar, and greater than those of RIS (0.1 mg/kg). ALF also increased the BV/TV relative to the OVX-Vehicle group, but the value was not restored to that observed in the Sham group, similar to the results of RIS (1.0 mg/kg and 2.5 mg/kg) treatment. The alterations of the structural parameters induced by RIS (at the doses) were attributable to suppression of the increase of ES/BS, BFR/BS, and BFR/BV. The alterations of the structural parameters induced by ALF were attributable to suppression of the increase of ES/BS and attenuation of the increase of BFR/BV, while the BFR/BS was maintained. ALF also increased the Ct Ar to beyond the value observed in the Sham group. RIS (at all the doses) had no effect on the mechanical properties of the femoral distal metaphysis, whereas ALF prevented the loss of the maximum load of the femoral distal metaphysis. Thus, the results of the present study show differential effects of RIS and ALF on cancellous and cortical bone in ovariectomized osteopenic rats.*

Key words: *alfacalcidol, osteopenia, ovariectomy, rat, risedronate,*

(Received 24 November 2005 / Accepted 3 February 2006)

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Introduction

Osteoporosis is recognized as a major public health problem. Because estrogen deficiency associated with menopause causes marked bone loss, osteoporosis primarily affects postmenopausal women. The bisphosphonate, risedronate (RIS) and the active vitamin D_3, alfacalcidol (ALF), have been widely used for postmenopausal osteoporosis in Japan. Several large randomized controlled trials (RCTs) have demonstrated that RIS reduces the incidence of vertebral and hip fractures in postmenopausal osteoporotic women [7, 8, 12]. However, because no large RCTs have been conducted to determine the anti-fracture efficacy of ALF in postmenopausal osteoporotic women, its efficacy in the treatment of postmenopausal osteoporosis remains to be established.

Several preclinical studies have reported on the effects of RIS and ALF against cancellous osteopenia using a rat model of postmenopausal osteoporosis (the preventive effects of RIS and ALF on osteopenia) [6, 15, 20]. RIS suppresses bone resorption and prevents cancellous bone loss in ovariectomized rats [6, 15]. On the other hand, ALF suppresses bone resorption, but maintains or even stimulates bone formation, thereby increasing the bone mineral density (BMD) and improving the mechanical properties of the bone [20]. However, very few studies have reported on the therapeutic effects of RIS or ALF on the bone mass and mechanical properties in ovariectomized osteopenic rats (the therapeutic effects of RIS and ALF on established osteopenia). The purpose of the present study was to compare the therapeutic effects of RIS and ALF on cancellous and cortical bone in ovariectomized osteopenic rats.

Materials and Methods

Treatment of animals

Forty-two female Sprague-Dawley rats, 7 months of age, were purchased from Charles River Japan (Kanagawa, Japan). They were fed a pelleted standard chow diet containing 1.25% calcium and 0.9% phosphorus (CRF-1: Oriental Kobo, Co., Ltd., Tokyo, Japan). The animals were housed under local vivarium conditions (temperature 23.3°C, humidity 55%, and 12 h on/off light cycle), with free access to water. After allowing one week for adaptation to the new environment, the rats were randomized by the stratified weight method into the following six groups: sham-operation + vehicle (Sham, n=5) group, bilateral ovariectomy (OVX, n=5) + vehicle group, OVX + RIS (0.1 mg/kg [n=8], 1.0 mg/kg [n=8], and 2.5 mg/kg [n=8]) groups, and OVX + ALF (0.5 μg/kg, n=8) group. The treatment with vehicle, RIS, or ALF was started 6 weeks after surgery and continued for 6 weeks. Bilateral OVX was performed under general anesthesia induced by intraperitoneal injection of 25–30 mg/kg pentobarbital sodium. Tablet forms of RIS (Actonel, Aventis Pharma, Tokyo, Japan) or ALF (One-alfa, Teijin Pharma, Tokyo, Japan) were pulverized, dissolved in 0.1 ml of sterile saline, and administered orally to the animals daily by gavage deep into the mouth. The doses of RIS and ALF were determined based on the results of previous studies [6, 13, 15, 20]; the daily dose of ALF (0.5 μg/kg) was 5 times higher than the effective daily dose (0.1 μg/kg) for preventing the loss of the proximal, middle, and distal femoral BMD in OVX rats [20], while the daily doses of RIS, 0.1 mg/kg, 1.0 mg/kg, and 2.5 mg/kg were within the range of doses previously tested in OVX or hind-limb immobilized rats [6, 13, 15]. The skeletal efficacy of ALF in ovariectomized rats has clearly been established [20]. Because the present study focused on investigating the effect of RIS on established osteopenia after OVX and comparing the skeletal efficacy between RIS and ALF, the highly effective dose of ALF and the low, middle, and high dose of RIS were selected. Only vehicle (0.1 ml of sterile saline) was also administered orally to the animals daily by gavage in the Sham-vehicle and OVX-vehicle groups. The body weight of the rats was monitored weekly, and the total duration of the experiment was 12 weeks. The present study was carried out at the laboratory of Hamri Co., Ltd. (Ibaraki, Japan). The animals were maintained according to the National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals, and the animal experiment protocols were approved by the Laboratory Animal Care Committee of Hamri Co., Ltd. (Ibaraki, Japan).

Preparation of specimens

Urine samples from all the rats were collected over a 24 h period using metabolic cages 6, 9, and 12 weeks after the start of the experiment, and the specimens

were stored at –20°C. All the rats were labeled with 25 mg/kg of tetracycline (Sigma Chemical, St. Louis, MO, USA) injected intramuscularly and 8 mg/kg of calcein (Sigma Chemical, St. Louis, MO, USA) injected subcutaneously, 9 days and 3 days, respectively, before sacrifice. The animals were sacrificed 12 weeks after the surgery by exsanguination under anesthesia induced by intraperitoneal injection of 25–30 mg/kg of pentobarbital sodium. Upon sacrifice, serum specimens were collected from all the rats, and the right femur and right tibia were isolated.

The serum samples were stored at –20°C. The urine and serum samples were used for the measurements of biochemical markers as described below. The femurs were stored at –20°C and then used for biomechanical testing as described below. The tibiae were processed for bone histomorphometric analyses. The bones were fixed in cold 40% ethanol overnight, and then cut into three parts using an Isomet saw (Buehler, Lake Bluff, IL, USA). The proximal tibial metaphysis and tibial diaphysis with the fibular junction were stained with Villanueva Osteochrome Bone Stain (Polyscience, Warrington, PA, USA) for 5 days. The specimens were 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 according to the method of Erben [5]. 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 8 μm or 4 μm thickness using a microtome (Leica RM2155; Leica Inc., Nussloch, Germany). The 8 μm sections were then transferred onto chromalum-gelatin-coated slides and dried overnight under a press at 42°C. All the sections were coverslipped with Eukitt (Calibrated Instruments, Hawthorne, NY, USA) for the static and dynamic histomorphometric analyses. For tartrate-resistant acid phosphatase (TRAP) histochemistry, the 8-μm sections of the proximal tibial metaphysis were deplasticized with three changes of 2-methoxyethylacetate for 30 min each, two changes of acetone for 5 min each, and sequential changes of ethanol (95%, 70%, and 40%), followed by two changes of deionized water for 5 min each for rehydration. The deplasticized and rehydrated sections (8 μm thickness) were placed in 0.1 M acetate buffer at pH 5.0 for 5 min, and the TRAP reaction was performed using a leukocyte acid phosphatase kit (Sigma Chemical, St. Louis, MO, USA). Sections stained for TRAP were counterstained with Mayer's hematoxylin (1 min) and then air-dried and mounted with a plastic UV mounting medium (Polysciences Inc., Warrington, PA, USA). For Goldner Trichrom staining to count the osteoblast surface, adjacent 4-μm sections of the proximal tibia metaphysis were deplasticized and rehydrated, followed by the procedure of Goldner Trichrom stain and mounting with Eukitt (Calibrated Instruments, Hawthorne, NY, USA).

Urine and serum biochemical analyses

The levels of urinary deoxypyridinoline (DPD) as a bone resorption marker were measured by enzyme-immunoassay (EIA) using a Pyrilinks-D kit (Metra Biosystems Inc., CA, USA). The serum calcium and phosphorus levels were measured by the o-CPC and ammonium molybdate colorimetric methods, respectively, using an autoanalyzer (Dada Behring Model RXL, Bakersfield, CA, USA). The levels of serum osteocalcin (OC) as a bone formation marker were measured by immunoradiometric assay (IRMA) using a Rat Osteocalcin IRMA kit (Immutopics, Inc., CA, USA).

Biomechanical testing

The mechanical properties of the diaphysis of the femur were evaluated by the three-point bending test. Load was applied midway between two supports placed 15 mm apart on the bone. The femur was positioned so that the loading point was at the center of the femoral diaphysis and bending occurred about the medial-lateral axis. The specimens were tested in a saline bath at 37°C. Each specimen was submerged in the saline bath for about 3 min before testing, to allow temperature equilibration. Load-displacement curves were recorded at a crosshead speed of 20 mm/min using a materials-testing machine (MZ500D; Maruto, Co., Ltd., Tokyo, Japan). The parameters analyzed were the maximum load, stiffness, and breaking energy.

Immediately after the three-point bending test of the right femoral diaphysis, the distal metaphysis was isolated over a length of 10 mm from the joint surface of

the femoral condyle. The mechanical properties of this segment were then measured by the compression test. Compressive load was applied by the rectangular parallelepiped crosshead (length 2 cm, width 2 cm, and height 1 cm) on the specimens from the lateral to the medial aspect. The specimens were tested in a saline bath at 37°C. Each specimen was submerged in the saline bath for about 3 min before testing, to allow temperature equilibration. Load-displacement curves were recorded at a crosshead speed of 10 mm/min and compression depth of 2.5 mm, using a materials-testing machine (MZ500D; Maruto, Co., Ltd., Tokyo, Japan). The parameters analyzed were the maximum load, stiffness, and breaking energy.

Bone histomorphometry of the tibia

A digitizing morphometry system was used to measure the bone histomorphometric parameters of the tibial specimens. The system consisted of an epifluorescence microscope (Nikon E-400, OsteoMetrics, Atlanta, GA, USA), an Osteomeasure High Resolution Color Subsystem (OsteoMetrics, Atlanta, GA, USA), and a digitizing pad (Numonics 2206; Numonics Corp., Montomerville, PA, USA) coupled to an IBM computer, and a morphometry program (OsteoMetrics, Atlanta, GA, USA). 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), and osteoblast surface (ObS). 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, MS/BS [(sLS/2+dLS)/BS], mineral apposition rate (MAR), bone formation rate (BFR)/BS, BFR/BV, and ObS/BS, in accordance with the standard nomenclature described by Parfitt *et al.* [16]. In the present study, the region of cancellous bone measured was 1–4 mm distal to the lower margin of the growth plate in the proximal tibia, which consists of secondary spongiosa. Cells showing positive staining for TRAP were counted in the region extending from the distal end of the growth plate to 0.2 mm from the growth plate, and the number of osteoclasts (N.Oc) and the osteoclast surface (OcS) per BS were calculated. The measured parameters for cortical bone were the total tissue area (Tt Ar), cortical bone area (Ct Ar), endocortical ES, periosteal and endocortical BS, sLS, dLS, and the interlabel width. These data were used to calculate the marrow area (Ma Ar), endocortical ES/BS, and periosteal and endocortical MS/BS [(sLS/2+dLS)/BS], MAR, and BFR/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 Fisher's protected least significant difference (PLSD) 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

Changes in body weight (Table 1)

The body weight at surgery did not differ significantly among the six groups. OVX was associated with an increase in the body weight of the animals. Neither RIS nor ALF affected the body weight of the ovariectomized animals.

Biochemical markers (Table 2)

OVX increased the urinary DPD and serum OC levels. RIS (at all the doses) decreased the serum phosphorus levels with the greatest decrease by RIS (2.5 mg/kg), while serum calcium levels were only decreased by RIS (2.5 mg/kg). RIS (1.0 mg/kg and 2.5 mg/kg) prevented the elevation of both the serum OC and urinary DPD levels, however, RIS (0.1 mg/kg) only attenuated the increase of the urinary DPD levels. A greater decrease of the serum OC levels was observed in the RIS (2.5 mg/kg) group than in the RIS (1.0 mg/kg) group. On the other hand, ALF mildly prevented the elevation of both the markers, without inducing any significant hypercalcemia.

Bone histomorphometric analysis of the cancellous bone of the proximal tibial metaphysis (Fig. 1 and Table 3)

The cancellous BV/TV, Tb N, and Tb Th were decreased, and the Tb Sp was increased, 12 weeks after OVX, as a result of increased bone resorption (ES/BS, N.Oc/BS, OcS/BS) and bone formation (ObS/BS, MS/BBS, BFR/BS, BFR/BV). RIS (at all the doses) increased the BV/TV, Tb N, and decreased the Tb Sp

Table 1. Changes in body weight

	Body weight (g)		
	Baseline (at surgery)	6 weeks (start of administration)	12 weeks
Sham	366 ± 32	381 ± 48	351 ± 38
OVX			
Vehicle	364 ± 29	446 ± 23[a]	404 ± 9[a]
RIS (0.1 mg/kg)	368 ± 27	440 ± 33[a]	411 ± 28[a]
RIS (1.0 mg/kg)	364 ± 27	431 ± 26[a]	403 ± 35[a]
RIS (2.5 mg/kg)	374 ± 40	440 ± 33[a]	404 ± 33[a]
ALF (0.5 μg/kg)	371 ± 30	436 ± 24[a]	388 ± 30[a]

Data are expressed as mean ± SD. ANOVA with Fisher's PLSD test was used to compare the data among the groups. [a]: significant vs Sham.

Table 2. Biochemical markers

	Deoxypyridinoline (nmol/mmol Cr)			Osteocalcin (ng/ml)	Calcium (mg/dl)	Phosphorus (mg/dl)
	6 weeks (start of administration)	9 weeks	12 weeks			
Sham	19.6 ± 7.4	17.0 ± 3.7	14.8 ± 5.1	21.8 ± 5.3	9.7 ± 0.2	6.4 ± 0.3
OVX						
Vehicle	44.3 ± 10.9[a]	58.3 ± 18.4[a]	54.5 ± 20.6[a]	33.3 ± 5.8[a]	9.9 ± 0.3	6.7 ± 0.3
RIS (0.1 mg/kg)	49.7 ± 18.3[a]	42.6 ± 8.5[ab]	29.3 ± 5.6[ab]	30.8 ± 7.3[a]	9.6 ± 0.5	5.8 ± 0.4[ab]
RIS (1.0 mg/kg)	50.3 ± 14.7[a]	22.3 ± 5.9[bc]	16.4 ± 4.4[bc]	23.8 ± 8.1[b]	9.5 ± 0.4	5.6 ± 0.5[ab]
RIS (2.5 mg/kg)	48.7 ± 14.8[a]	25.7 ± 9.9[bc]	13.9 ± 3.8[bc]	13.9 ± 3.0[bcd]	9.4 ± 0.2[b]	5.1 ± 0.2[abcd]
ALF (0.5 μg/kg)	49.8 ± 13.2[a]	38.7 ± 16.9[abde]	24.8 ± 10.7[be]	23.7 ± 12.1[be]	10.1 ± 0.6[f]	6.9 ± 0.6[ace]

Data are expressed as mean ± SD. ANOVA with Fisher's PLSD test was used to compare the data among the groups. [a]: significant vs Sham, [b]: significant vs Vehicle. [c]: significant vs RIS (0.1 mg/kg), [d]: significant vs RIS (1.0 mg/kg), [e]: significant vs RIS (2.5 mg/kg), [f]: significant vs RIS (all doses).

relative to the values observed in the OVX-Vehicle group, but the values were not restored to those observed in the Sham group. The effects of RIS (1.0 mg/kg and 2.5 mg/kg) were greater than those of RIS (0.1 mg/kg). The OVX-induced decrease of the Tb Th was entirely prevented by RIS (at all the doses), with the value of this parameter being restored to the value observed in the Sham group. ALF also increased the BV/TV and Tb N and decreased the Tb Sp relative to the values observed in the OVX-Vehicle group, but the values were not restored to those observed in the Sham group. Furthermore, ALF also increased the Tb Th and the increase of this parameter following ALF treatment was more marked than that following RIS treatment (at all the doses). Thus, the alterations of the structural parameters induced by RIS (at all the doses) were attributable to suppression of the increase of bone resorption (ES/BS) and formation (BFR/BS, BFR/BV), and the alterations of the structural parameters induced by ALF were attributable to suppression of the increase of bone resorption (ES/BS), while bone formation (ObS/BS, MS/BS, BFR/BS) was maintained. The effect of ALF on cancellous BV/TV was similar to that of RIS (1.0 mg/kg and 2.5 mg/kg).

Bone histomorphometric analysis of the cortical bone of the tibial diaphysis (Fig. 2 and Table 4)

OVX did not affect the Tt At or Ct Ar, despite stimulated periosteal bone formation (MS/BS, BFR/BS), but increased the Ma Ar as a result of increased endocortical bone resorption (ES/BS) and, subsequently, increased endocortical bone formation (BFR/BS). RIS (at all the doses) prevented the increase of the Ma Ar, restoring it to the value observed in the Sham group. On the other

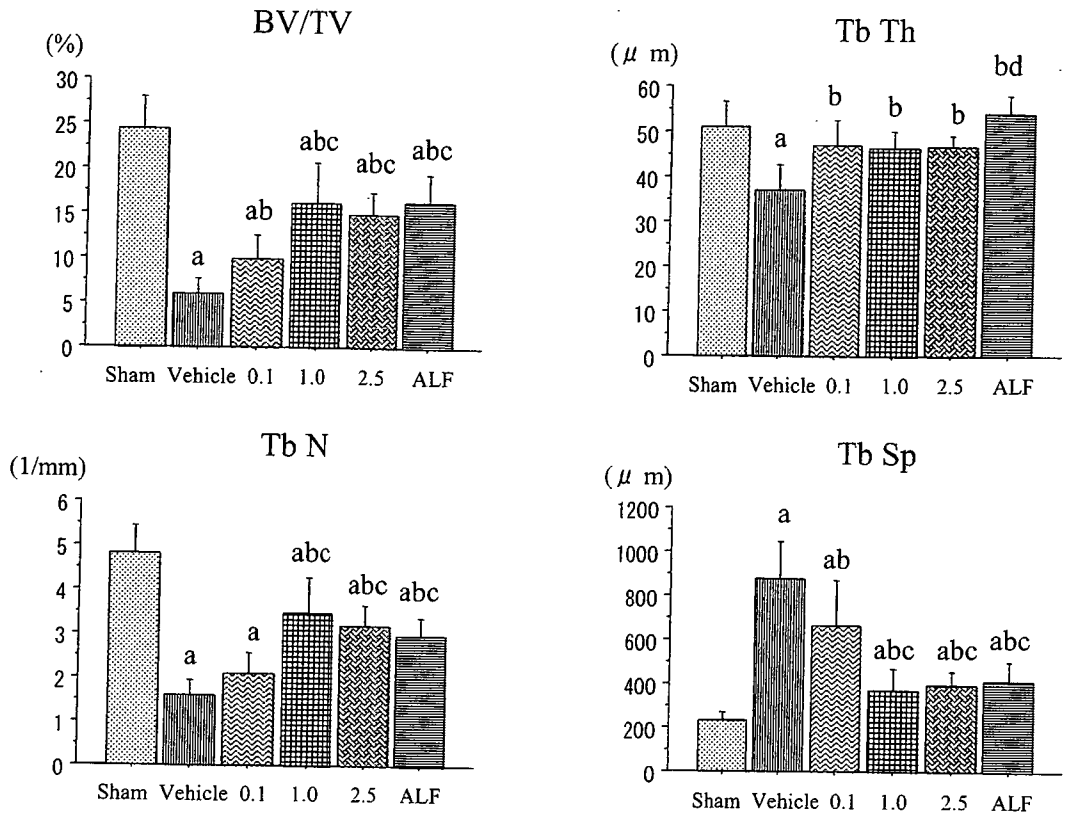

Fig. 1. Bone histomorphometric analysis of the cancellous bone of the proximal tibial metaphysis. —Structural parameters—. Data are expressed as mean ± SD. ANOVA with Fisher's PLSD test was used to compare the data among the groups. Sham: Sham-operated control, Vehicle: OVX+Vehicle, 0.1: OVX+RIS (0.1 mg/kg), 1.0: OVX+RIS (1.0 mg/kg), 2.5: OVX+RIS (2.5 mg/kg), ALF: OVX+ALF. a: significant vs Sham, b: significant vs Vehicle, c: significant vs RIS (0.1 mg/kg), d: significant vs RIS (all doses). BV/TV: bone volume/total tissue volume, Tb N: trabecular number, Tb Th: trabecular thickness, Tb Sp: trabecular separation.

Table 3. Histomorphometric analysis of the cancellous bone of the proximal tibial metaphysis —Formative and resorptive variables—

	ES/BS (%)	N.Oc/BS (#/mm)	OcS/BS (%)	ObS/BS (%)	MS/BS (%)	MAR (μm/day)	BFR/BS (μm³/μm²/day)	BFR/BV (%/year)
Sham	7.2 ± 3.4	1.11 ± 0.20	4.2 ± 0.6	13.4 ± 0.5	10.6 ± 4.5	0.96 ± 0.17	9.9 ± 4.3	119 ± 50
OVX								
Vehicle	12.6 ± 4.2[a]	4.40 ± 1.00[a]	15.4 ± 2.5[a]	19.6 ± 2.5[a]	19.2 ± 3.6[a]	0.88 ± 0.12	17.2 ± 5.7[a]	281 ± 63[a]
RIS (0.1 mg/kg)	8.0 ± 2.0[b]	1.82 ± 0.35[ab]	6.6 ± 1.8[ab]	15.3 ± 2.9[b]	15.7 ± 4.2[a]	0.77 ± 0.19	12.1 ± 4.4[b]	156 ± 53[b]
RIS (1.0 mg/kg)	7.5 ± 2.4[b]	1.70 ± 0.39[ab]	5.9 ± 1.3[ab]	12.0 ± 2.7[bc]	13.5 ± 3.4[b]	0.67 ± 0.19[ab]	8.9 ± 2.8[b]	117 ± 33[b]
RIS (2.5 mg/kg)	6.2 ± 1.5[b]	1.76 ± 0.51[ab]	6.3 ± 1.7[ab]	13.0 ± 2.4[b]	10.5 ± 3.8[bc]	0.56 ± 0.07[abc]	5.9 ± 2.4[bc]	76 ± 28[bc]
ALF (0.5 μg/kg)	7.6 ± 3.1[b]	2.14 ± 0.45[ab]	7.9 ± 1.8[ab]	18.8 ± 2.1[af]	22.4 ± 4.1[af]	0.86 ± 0.26[de]	19.1 ± 5.6[af]	213 ± 53[abf]

Data are expressed as mean ± SD. ANOVA with Fisher's PLSD test was used to compare the data among the groups. [a]: significant vs Sham, [b]: significant vs Vehicle, [c]: significant vs RIS (0.1 mg/kg), [d]: significant vs RIS (1.0 mg/kg), [e]: significant vs RIS (2.5 mg/kg), [f]: significant vs RIS (all doses). ES: eroded surface, BS: bone surface, N.Oc: number of osteoclast, ObS: osteoblast surface, MS: mineralizing surface, MAR: mineral apposition rate, BFR: bone formation rate, BV: bone volume.

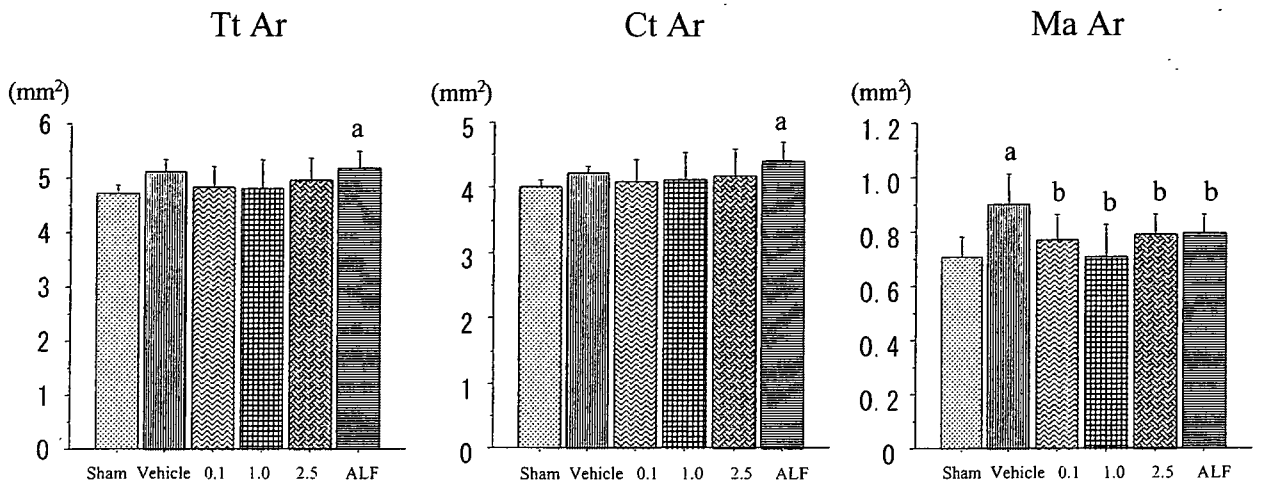

Fig. 2. Bone histomorphometric analysis of the cortical bone of the tibial diaphysis. —Structural parameters—. Data are expressed as mean ± SD. ANOVA with Fisher's PLSD test was used to compare the data among the groups. Sham: Sham-operated control, Vehicle: OVX+Vehicle, 0.1: OVX+RIS (0.1 mg/kg), 1.0: OVX+RIS (1.0 mg/kg), 2.5: OVX+RIS (2.5 mg/kg), ALF: OVX+ALF. a: significant vs Sham, b: significant vs Vehicle. Tt Ar: total tissue area, Ct Ar: cortical area, Ma Ar: marrow area.

Table 4. Histomorphometric analysis of the cortical bone of the tibial diaphysis —Formative and resorptive variables—

	Periosteal			Endocortical			
	MS/BS (%)	MAR (μm/day)	BFR/BS (μm³/μm²/day)	ES/BS (%)	MS/BS (%)	MAR (μm/day)	BFR/BS (μm³/μm²/day)
Sham	39.6 ± 10.7	1.49 ± 0.22	59.7 ± 19.7	27.7 ± 3.5	5.5 ± 1.2	0.00 ± 0.00	0.0 ± 0.0
OVX							
Vehicle	66.4 ± 10.2[a]	1.51 ± 0.24	99.8 ± 17.3[a]	40.0 ± 5.0[a]	12.9 ± 5.9[a]	0.63 ± 0.63[a]	9.8 ± 12.3[a]
RIS (0.1 mg/kg)	60.9 ± 13.3[a]	1.26 ± 0.25	77.2 ± 22.7	33.7 ± 5.4	13.3 ± 4.7[a]	0.15 ± 0.28[b]	2.5 ± 4.7
RIS (1.0 mg/kg)	59.1 ± 10.6[a]	1.19 ± 0.27[ab]	70.8 ± 23.2	33.9 ± 7.0	8.1 ± 3.8[c]	0.16 ± 0.30[b]	1.9 ± 3.6
RIS (2.5 mg/kg)	57.1 ± 23.2	1.17 ± 0.26[b]	69.2 ± 36.4	26.6 ± 10.0[b]	6.0 ± 4.6[bc]	0.13 ± 0.35[b]	1.2 ± 3.4[b]
ALF (0.5μg/kg)	53.8 ± 20.0	1.69 ± 0.22[f]	92.9 ± 43.8	25.6 ± 11.0[bd]	18.3 ± 6.5[ae]	1.29 ± 0.23[abe]	24.1 ± 10.6[abe]

Data are expressed as mean ± SD. ANOVA with Fisher's PLSD test was used to compare the data among the groups. [a]: significant vs Sham, [b]: significant vs Vehicle, [c]: significant vs RIS (0.1 mg/kg), [d]: significant vs RIS (1.0 mg/kg), [e]: significant vs RIS (2.5 mg/kg), [f]: significant vs RIS (all doses). MS: mineralizing surface, BS: bone surface, MAR: mineral apposition rate, BFR: bone formation rate, ES: eroded surface.

hand, only RIS (2.5 mg/kg) suppressed endocortical bone resorption (ES/BS) and formation (MS/BS, MAR, BFR/BS). ALF increased the Tt Ar and Ct Ar to beyond the values observed in the Sham group, and prevented the increase of the Ma Ar after OVX. Furthermore, it also suppressed endocortical bone resorption (ES/BS), while even stimulating endocortical bone formation (MS/BS, BFR/BS) to beyond the values observed in the OVX-Vehicle group.

Biomechanical test of the femur (Table 5)

OVX decreased the maximum load of the femoral distal metaphysis, without any effect on the mechanical properties of the femoral diaphysis. RIS (at all the doses) had no effects on the mechanical properties of the femoral distal metaphysis or diaphysis, whereas ALF prevented the loss of the maximum load and increased the breaking energy of the femoral distal metaphysis, without any effect on the mechanical properties of the femoral diaphysis.

Table 5. Mechanical properties of the femur

	Distal metaphysis			Diaphysis		
	Maximum load (N)	Stiffness (N/cm)	Breaking energy ($x10^{-5}N \cdot m$)	Maximum load (N)	Stiffness (N/cm)	Breaking energy ($x10^{-5}N \cdot m$)
Sham	362 ± 67	526 ± 142	45885 ± 11076	120 ± 6	167 ± 24	4956 ± 917
OVX						
Vehicle	279 ± 28[a]	388 ± 106	41079 ± 3598	131 ± 19	169 ± 44	6175 ± 1746
RIS (0.1 mg/kg)	313 ± 27	436 ± 72	44135 ± 10637	132 ± 9	174 ± 21	6262 ± 937
RIS (1.0 mg/kg)	312 ± 35	464 ± 52	49013 ± 5927	132 ± 14	179 ± 35	6591 ± 1575
RIS (2.5 mg/kg)	335 ± 39	502 ± 132	47561 ± 6154	132 ± 18	170 ± 42	6976 ± 2839
ALF (0.5 μg/kg)	404 ± 76[bc]	551 ± 177[b]	58193 ± 10422[ac]	131 ± 9	170 ± 16	6904 ± 1397

Data are expressed as mean ± SD. ANOVA with Fisher's PLSD test was used to compare the data among the groups. [a]: significant vs Sham, [b]: significant vs Vehicle, [c]: significant vs RIS (all doses).

Discussion

The present study showed the differential effects of RIS and ALF on cancellous and cortical bone in ovariectomized osteopenic rats. The strengths of this study are the detailed bone histomorphometric analyses of cancellous and cortical bone, the measurement of biochemical markers of bone turnover, and the measurement of the mechanical properties of the femoral distal metaphysis. The weaknesses are the ineffectiveness of both treatments in restoring the cancellous BV/TV to the osteopenic skeleton after OVX, despite decreased multiple parameters related to bone remodeling. The two treatments increased the cancellous BV/TV compared with OVX-Vehicle-controls, but could not restore to the values seen in Sham-controls. Because the two treatments were impotent, switching to potent anabolic agents such as parathyroid hormone (PTH) might completely restore the cancellous BV/TV in ovariectomized osteopenic rats.

The effects of OVX on cancellous and cortical bone have already been established, and our results can be comparative with those of a number of previous studies. In particular, we confirmed that OVX resulted in cancellous osteopenia by 6 weeks after surgery in 6-month-old rats, without inducing cortical osteopenia because of the absence of Haversian-based remodeling in rat cortical bone [11]. According to this report, our study animals would also have developed cancellous osteopenia by 6 weeks after the OVX.

Bisphosphonates inhibit osteoclast-mediated bone resorption, and loss of osteoclast function and apoptosis is a consequence of loss of function of one or more important signaling proteins. A nitrogen-containing bisphosphonate like RIS is not metabolized but can inhibit enzymes of the mevalonate pathway, thereby preventing the biosynthesis of isoprenoid compounds that are essential for the post-translational modification of small GTPases [18]. RIS has a potent anti-resorptive effect on the bone.

RIS improved not only the connectivity of trabecular bone, but also its thickness by suppressing bone turnover in ovariectomized osteopenic rats. The effects of RIS (1.0 mg/kg) and RIS (2.5 mg/kg), which were similar, were more marked than those of RIS (0.1 mg/kg). Therefore, 1.0 mg/kg was considered to be the minimum effective dose of RIS in the present study. However, RIS did not completely restore the cancellous BV/TV to the value observed in the Sham group, reflecting its limitation in increasing cancellous bone mass. It is possible that RIS reduced the amount of remodeling space and then increased the cancellous bone mass. The decreases of the serum calcium and phosphorus levels after RIS treatment, with the greatest decreases by RIS (2.5 mg/kg), suggest that RIS treatment resulted in accumulation of minerals in the bone. The effect of RIS on bone metabolism was similar in ovariectomized rats with and without established osteopenia [6, 15].

The active and hormonal form of vitamin D, 1α, 25-dihydroxyvitamin D_3, plays a central role in bone and mineral homeostasis through binding to the vitamin D receptor in calcium-related target organs, including the intestine, bone, kidney, and parathyroid gland [1, 17].

One α, $25(OH)_2D_3$ stimulates calcium absorption from the intestine, regulates bone resorption as well as formation, and enhances calcium reabsorption in the distal renal tubules, while it represses parathyroid hormone gene transcription in the parathyroid glands [1, 17]. One α-hydroxyvitamin D_3 (ALF), which is the prodrug of 1α, 25-dihydroxyvitamin D_3, has been widely used in the treatment of a variety of metabolic bone diseases, such as rickets/osteomalacia, renal osteodystrophy, and osteoporosis [1, 17]. A clinical study showed that ALF reduced bone turnover and prevented vertebral fractures in postmenopausal women with osteoporosis [14]. However, ALF shows a relatively low effectiveness and the risk of developing hypercalciuria/hypercalcemia and urinary stones, resulting in a relatively narrow therapeutic window [4]. An experimental study, which was conducted to investigate the preventive effect of ALF on osteopenia in ovariectomized rats, clearly showed that ALF suppressed bone resorption, but maintained or even stimulated bone formation [20].

ALF improved not only the connectivity of trabecular bone, but also its thickness by suppressing bone turnover but maintaining bone formation in ovariectomized osteopenic rats. These alterations in bone formation and resorption in ovariectomized osteopenic rats were similar to those observed in a previous study that examined the preventive effect of ALF on the cancellous bone loss in ovariectomized rats [20]. The effect of ALF on the cancellous BV/TV was similar to that of RIS (1.0 mg/kg and 2.5 mg/kg). However, the suppression of bone turnover by ALF was milder than that by RIS (at all the doses). Thus, ALF had a milder antiresorptive effect than RIS on cancellous bone in ovariectomized osteopenic rats, and also appeared to have the potential to maintain bone formation, differing in this respect from RIS. The increase in the Tb Th induced by ALF was more marked than that induced by RIS (at all the doses), probably due to maintained bone formation. However, the cancellous BV/TV was not restored to the level observed in the Sham group, reflecting the limitation of ALF at our dose setting in increasing the cancellous bone mass.

OVX decreased the maximum load of the femoral distal metaphysis, associated with a decrease in the cancellous BV/TV. Despite the similar effects of RIS and ALF on the cancellous BV/TV, RIS (at all the doses) had no effects on the mechanical properties of the femoral distal metaphysis, whereas ALF prevented the loss of the maximum load and increased the breaking energy of the femoral distal metaphysis. These results may partly be attributable to the more pronounced effect of ALF than RIS on the Tb Th. The Tb Th may be an important factor in determining the bone strength, as observed in rats treated with vitamin K_2 [9, 10]. Thus, the efficacy of ALF in improving the mechanical properties of the bone in ovariectomized osteopenic rats observed in the present study might be attributable, at least in part, to the marked increase of the Tb Th induced by the drug.

Clinically, it is apparent that RIS is more effective than ALF in increasing the lumbar BMD and reducing the incidence of vertebral fractures in postmenopausal osteoporotic women [3]. Nevertheless, in the present study, ALF induced an increase of the cancellous BV/TV similar to that of RIS. This discrepancy between the clinical and experimental results may be due to the differential responses of cancellous bone to ALF between ovariectomized osteopenic rats and postmenopausal osteoporotic women (humans). In fact, the potent preventive effect of alfacalcidol on cancellous bone loss after OVX in rats has been confirmed in the previous studies [20]; thus, ALF might exert greater beneficial effects on rat bones than on human bones.

Increased endocortical bone resorption and periosteal bone formation seem to be similar in ovariectomized osteopenic rats and postmenopausal osteoporotic women [19]. Thus, the pharmacological effects of the drugs on the endocortical bone in ovariectomized osteopenic rats can be exactly translatable into clinically beneficial effects on the endocortical bone in postmenopausal osteoporotic women. ALF, but not RIS, increased the Ct Ar, mainly by decreasing endocortical bone resorption and increasing endocortical bone formation. However, perhaps because this effect was modest, the mechanical properties of the femoral diaphysis were not improved. Also, RIS did not decrease endocortical bone resorption in the present study, probably because osteoclasts on the endocortical surface may be less responsive to bisphosphonates than those on the trabecular surface [2].

In conclusion, the present study demonstrated that RIS and ALF increased the cancellous bone mass by suppressing bone turnover in ovariectomized osteopenic

rats. The effects of both ALF and RIS (at effective doses) were similar on the cancellous bone mass. ALF was associated with maintained or even stimulated bone formation and a marked increase of the trabecular thickness. Also, only ALF increased the cortical bone mass, and prevented the loss of the maximum load of the metaphysis of the femur. Thus, the present study showed the differential effects of RIS and ALF on cancellous and cortical bone in ovariectomized osteopenic rats.

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Exp. Anim. 55(4), 349–355, 2006

Preventive Effects of Risedronate and Calcitriol on Cancellous Osteopenia in Rats Treated with High-Dose Glucocorticoid

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Abstract: *We compared the effects of risedronate (Ris) and calcitriol (Cal) on cancellous osteopenia in rats treated with high-dose glucocorticoid (GC). Forty female Sprague-Dawley rats, 4 months of age, were randomized by the stratified weight method into four groups of 10 rats each according to the following treatment schedule: intact control, and GC administration with vehicle, Ris, or Cal. The GC (methylprednisolone sodium succinate, 5.0 mg/kg, s.c.), Ris (10 μg/kg, s.c.), and Cal (0.1 μg/kg, p.o.) were administered 3 times a week. At the end of the 4-week treatment period, bone histomorphometric analysis was performed for cancellous bone of the proximal tibial metaphysis. The GC administration decreased cancellous bone volume (BV/total tissue volume [TV]), trabecular number (Tb N), and trabecular thickness (Tb Th), as a result of increased bone resorption and decreased bone formation. Ris treatment markedly increased cancellous BV/TV and Tb N above the control level as a result of suppressed bone turnover. On the other hand, Cal treatment attenuated the GC-induced decrease in cancellous BV/TV and Tb Th as a result of suppressed bone resorption and maintained bone formation. This study showed the differential effects of Ris and Cal on cancellous osteopenia in rats treated with high-dose GC.*

Key words: *calcitriol, cancellous osteopenia, glucocorticoid, rat, risedronate*

Introduction

Glucocorticoid (GC) therapy has been associated with an increased risk of osteoporosis, and consequently, an increased incidence of fractures. The loss of bone mineral density (BMD) is more evident in cancellous bone than in cortical bone [23]. Several meta-analyses/systematic-reviews have reported the effects of vitamin D,

(Received 15 November 2005 / Accepted 3 March 2006)

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calcitonin, and bisphosphonates on the BMD of the lumbar spine and hip in patients treated with GCs; vitamin D (both active and native vitamin D) and calcitonin stabilize lumbar spine BMD, while bisphosphonates stabilize both lumbar spine and hip BMD, with, nonetheless, a less certain effect on hip BMD [2, 8, 9, 21]. In particular, bisphosphonates have been reported to be the most effective in stabilizing lumbar BMD in patients treated with GCs [1]. In Japan, it is generally agreed that bisphosphonates should be the first line drugs in the treatment of GC-induced osteoporosis, while active vitamin D is the second line treatment [14]. However, the countermeasures against the rapid and vigorous loss of BMD by high-dose GC therapy [24], i.e., pulse steroid therapy are less certain, and the mechanism for the efficacy of bisphosphonates and active vitamin D in stabilizing lumbar BMD in patients treated with GCs in terms of the effect of these drugs on cancellous microarchitecture remains to be established. The purpose of the present study was to use an animal model to compare the effects of risedronate (Ris) and calcitriol (Cal) on high-dose GC-induced cancellous osteopenia by means of bone histomorphometry.

Materials and Methods

Treatment of animals

Forty female Sprague-Dawley rats, 4 months of age, were purchased from Hilltop Lab. Animals, Inc. (Scottdale, 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 vitamin D (Rodent Diet 8604, Harlan Teklad, Madison, WI, USA), with free access to water. After allowing one-week's adaptation to the new environment, the rats were randomized by the stratified weight method into four groups of 10 rats each according to the following treatment schedule: intact control (CON), and GC administration with vehicle, Ris, or Cal as preventive treatment. Methylprednisolone sodium succinate (Pharmacia & Upjohn Company, Kalamazoo, MI, USA) was administered as the GC, at a dose of 5.0 mg/kg body weight, three times a week by subcutaneous injection. Ris (Aventis Pharma, Tokyo, Japan) was dissolved in 0.1 ml of sterile saline, and then administered by subcutaneous injection at a dose of 10 μg/kg body weight three times a week. Cal (Chugai, 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 a dose of 0.1 μg/kg body weight three times a week. The Ris and Cal doses were considered to be effective, in accordance with previously published data [10, 11, 13]. The body weight of the rats was monitored weekly and the experimental period was 4 weeks. 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 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 calcein (Sigma Chemical, St. Louis, MO, USA) injected intramuscularly 10 days and 3 days 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. A serum specimen, the left femur and right tibia were collected from every animal.

The serum samples were stored at –20°C until use for the measurements of serum calcium and phosphorus levels with an automated instrument (Dada Behring Model RXL, Bakersfield, CA, USA). The femurs were stored at –20°C until use for BMD measurement as described below. The tibial length was measured with dial calipers and the bones were then used for bone histomorphometric analysis; they 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 were stained with Villanueva Osteochrome Bone Stain (Polyscience, Warrington, PA, USA) for 5 days. 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 [3]. 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.

Femoral BMD

The BMD of the whole left femur was determined by dual energy X-ray absorptiometry (DXA) using a Hologic QDR-2000 plus (Hologic Inc., Bedford, MA, USA). The instrument was adapted for an ultra-resolution mode, with line spacing of 0.0254 cm, resolution of 0.0127 cm, and collimation of 0.9 cm diameter. The bone was placed in a Petri dish, and to simulate soft-tissue density, tap water was poured around the bone to a depth of 1 cm. The bone mineral content and bone area were measured, and the BMD of that area was calculated by dividing bone mineral content by bone area. The coefficient of variation of these measurements at our laboratory was less than 1.0% [20].

Bone histomorphometric analysis of the tibia

A digitizing morphometric system was used to measure bone histomorphometric parameters. The system consisted of an epifluorescence microscope (Nikon E-400, OsteoMetrics, Atlanta, GA, USA), an Osteomeasure High Resolution Color Subsystem (OsteoMetrics, Atlanta, GA, USA) coupled to an IBM computer, and a morphometry program (OsteoMetrics, Atlanta, GA, USA). The measured parameters for cancellous bone included total tissue volume (TV), bone volume (BV), bone surface (BS), eroded surface (ES), single- and double-labeled surfaces (sLS and dLS, respectively), and interlabel width. These data were used to calculate 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, and BFR/BV, in accordance with the standard nomenclature proposed by Parfitt *et al.* [19]. In the present study, the region of cancellous bone measured was 1–4 mm distal to the lower margin of the growth plate in the proximal tibial metaphysis, which consists of secondary spongiosa. In addition to measurement of the above parameters, interlabel width beneath the growth plate was used to calculate longitudinal growth rate (LGR).

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 Fisher's protected least significant difference (PLSD) 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, tibial length, femoral BMD, and serum calcium and phosphorus levels (Table 1)

The initial body weight did not differ significantly among the groups.

The GC administration decreased body weight, tibial length, and serum calcium levels, but had no effect on femoral BMD.

Ris prevented the GC-induced decrease in tibial

Table 1. Body weight, tibial length, femoral BMD, and serum calcium and phosphorus levels

	Initial body weight (g)	Final body weight (g)	Tibial length (mm)	Femoral BMD (mg/cm^2)	Serum	
					Calcium (mg/dl)	Phosphorus (mg/dl)
CON	243 ± 6	293 ± 10	39.0 ± 0.5	223 ± 7	9.7 ± 0.2	8.0 ± 0.6
GC						
Vehicle	237 ± 10	272 ± 16[a]	38.2 ± 0.6[a]	219 ± 7	9.4 ± 0.1[a]	7.3 ± 0.6[a]
Ris	234 ± 9	271 ± 16[a]	38.9 ± 0.4[b]	225 ± 8	9.2 ± 0.2[ab]	7.3 ± 0.6[a]
Cal	243 ± 10	285 ± 13[bc]	38.7 ± 0.6[b]	226 ± 5	9.4 ± 0.3[ac]	7.1 ± 0.8[a]

Data are expressed as mean ± SD. ANOVA with Fisher's PLSD test was used to compare the data among the groups. CON: intact control; GC: glucocorticoid; Ris: risedronate; Cal: calcitriol. [a], significant vs CON; [b], significant vs GC; [c], significant vs Ris. BMD: bone mineral density.

length, but showed no effect on femoral BMD; Ris also accelerated the GC-induced decrease in serum calcium levels. On the other hand, Cal prevented the GC-induced decrease in body weight and tibial length, but showed no effect on serum calcium levels, or femoral BMD.

Bone histomorphometric analysis of cancellous bone of the proximal tibial metaphysis (Figs. 1 and 2)

The GC administration decreased cancellous BV/TV, Tb N, and Tb Th, and increased Tb Sp. This cancellous osteopenia was associated with decreased bone formation (MS/BS, MAR, BFR/BS, BFR/BV), and increased ES/BS. The GC administration also decreased LGR.

Ris increased cancellous BV/TV and Tb N above the values observed in the CON group, and decreased Tb Sp to below the value observed in the CON group, as a result of markedly decreased bone turnover, indicated by decreases in ES/BS, BFR/BS and BFR/BV, while Cal attenuated the GC-induced decrease in cancellous BV/TV and Tb Th by decreasing ES/BS and maintaining MS/BS. Ris prevented the GC-induced decrease in LGR, whereas Cal had no effect on this parameter.

Discussion

GC induces cancellous osteopenia in rats. It has been demonstrated that GC induces the loss of cancellous BV/TV, Tb N, and Tb Th, decreases longitudinal

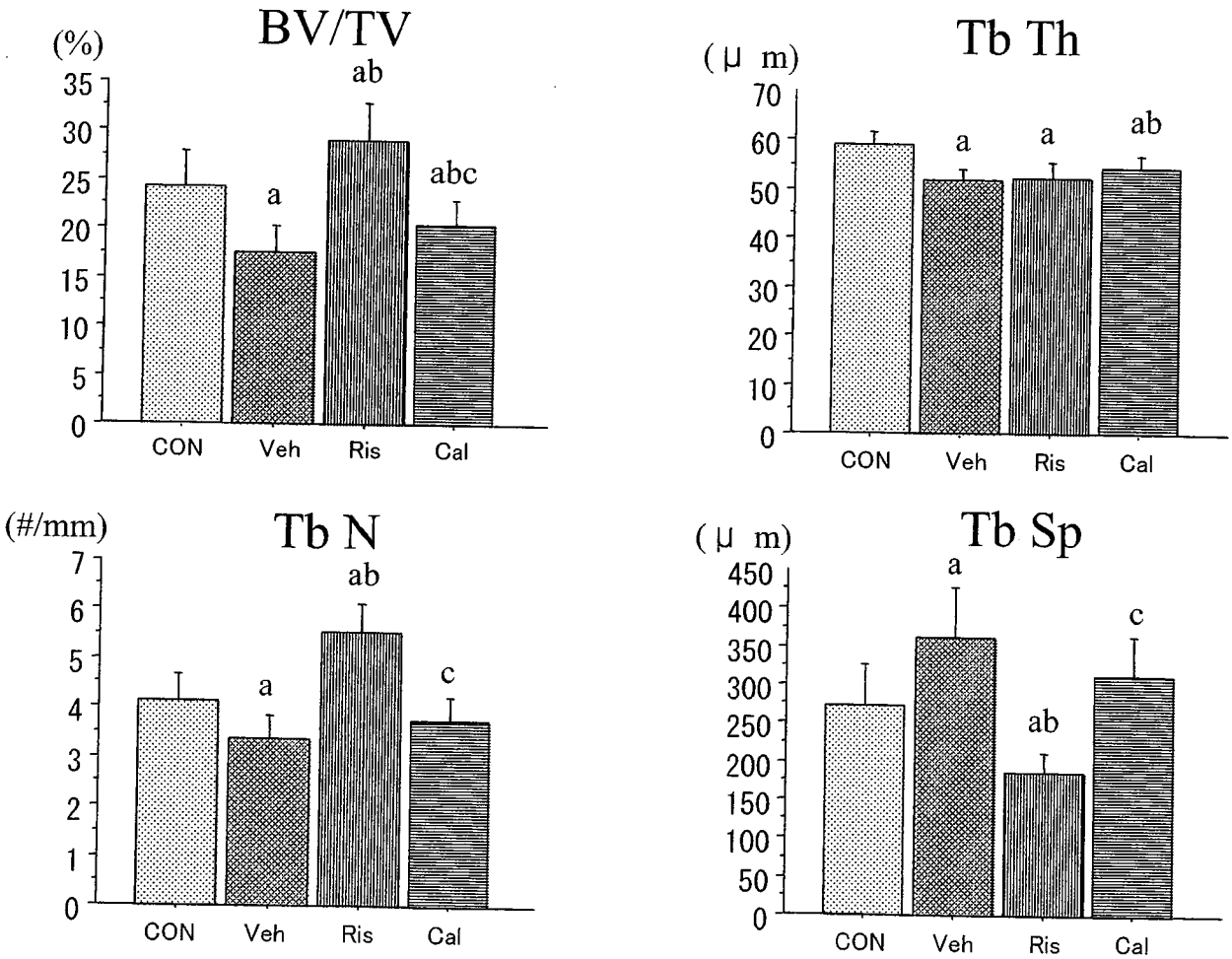

Fig. 1. Bone histomorphometric analysis of cancellous bone of the proximal tibial metaphysis. –Structural variables– Data are expressed as mean ± SD. ANOVA with Fisher's PLSD test was used to compare the data among the groups. CON: intact controls; Veh: GC(glucocorticoid)+Vehicle; Ris: GC+Risedronate; Cal: GC+Calcitriol. a, significant vs CON; b, significant vs Veh; c, significant vs Ris. BV/TV: bone volume/total tissue volume; Tb N: trabecular number; Tb Th: trabecular thickness; Tb Sp: trabecular separation.

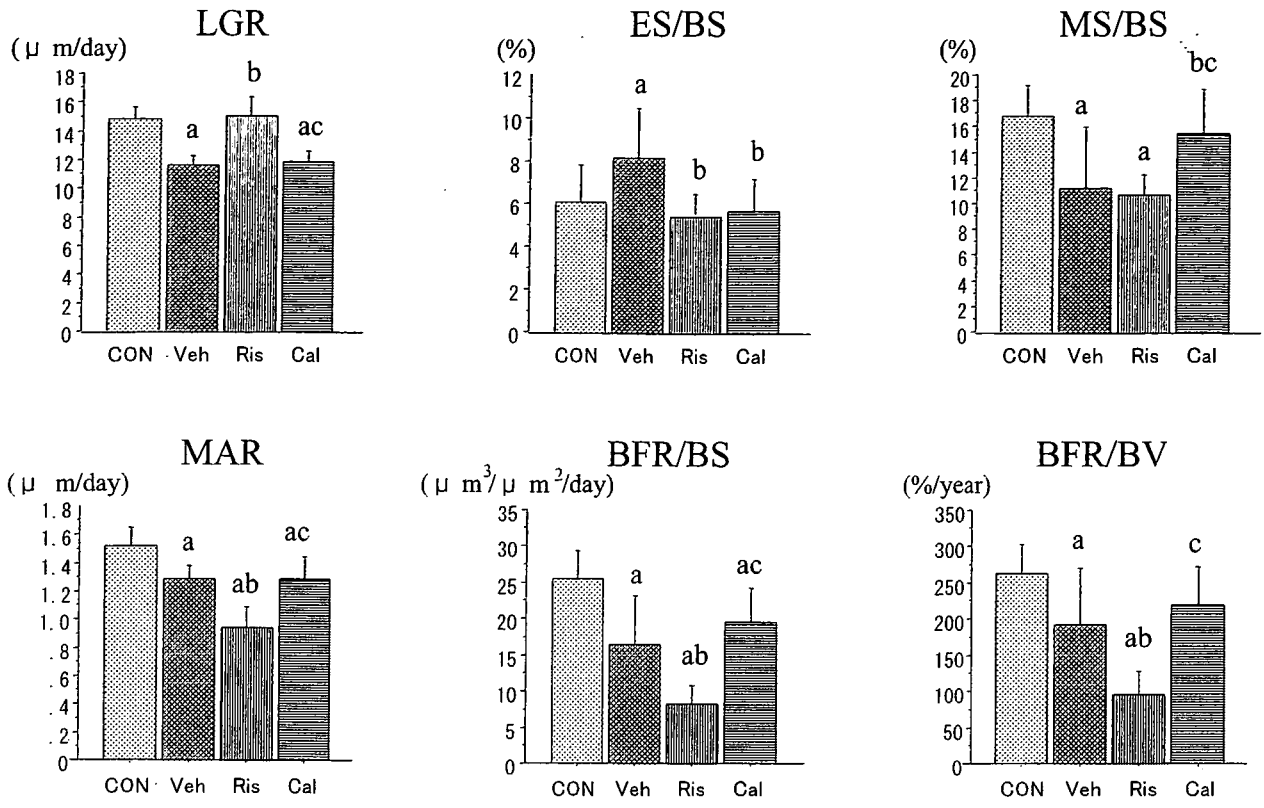

Fig. 2. Bone histomorphometric analysis of cancellous bone of the proximal tibial metaphysis –Formative and resorptive variables– Data are expressed as mean ± SD. ANOVA with Fisher's PLSD test was used to compare the data among the groups. CON: intact controls; Veh: GC(glucocorticoid)+Vehicle; Ris: GC+Risedronate; Cal: GC+Calcitriol. a, significant vs CON; b, significant vs Veh; c, significant vs Ris. LGR: longitudinal growth rate; ES: eroded surface; BS: bone surface; MS: mineralizing surface; MAR: mineral apposition rate; BFR: bone formation rate; BV: bone volume.

bone growth and femoral bone length, and reduces the mechanical strength of the lumbar spine [5, 6, 15–17, 22]. GC-induced osteopenia has been associated with decreased bone formation, with the effects on bone resorption in terms of osteoclastic activity being inconsistent. Bone resorption has been reported to be affected more severely by higher doses of GCs [10], suggesting that higher daily or total doses of GC may decrease osteoclastic activity. Thus, the inconsistent effects on bone resorption observed may have been due to the inconsistent daily and total doses of GC. The key histological feature of corticosteroid-induced cancellous bone loss is reduction in Tb Th, reflecting suppressed bone formation [12].

In our study, the high-dose GC administration decreased cancellous BV/TV, Tb N, and Tb Th, primarily due to decreased bone formation and increased bone resorption. The GC administration also decreased LGR and tibial length. These results suggest that in the early (4 weeks) phase of the high-dose GC administration, bone resorption is increased and bone formation is decreased, resulting in cancellous osteopenia and a decrease in the longitudinal growth of long bones. Because 4-month-old rats were used, growth-related alterations of cancellous bone needed to be considered.

The efficacy of Ris or Cal against cancellous bone loss in ovariectomized rats has been well documented. Ris was shown to suppress bone resorption and prevent cancellous bone loss in ovariectomized rats [4, 18], and Cal with calcium supplementation was shown to decrease bone resorption and increase cancellous bone mass in ovariectomized osteopenic rats [12]. However, very few studies have reported the effect of Ris or Cal on cancellous bone mass in GC-treated rats.

In our study, Ris treatment markedly increased cancellous BV/TV and Tb N above the control level. These