measured using an OSTEOLINKS-DPD EIA kit (Sumitomo Seiyaku Biomedical Co., Ltd., Osaka). Also, the urinary parameters were corrected based on creatinine levels.

Measurement of Bone Mineral Density

The average BMD of the lumbar vertebrae (L2-L4) was measured by dual-energy X-ray absorptiometry (DCS-600, Aloka Co., Ltd., Tokyo). The trabecular structure of the cancellous bone in L5 was analyzed by micro-CT 40 in terms of bone mass (BV/TV; unit, %), trabecular surface area (BS/BV; unit, %), trabecular space (Tb.Sp; unit, mm), trabecular thickness (Tb.Th; unit, mm) and trabecular number (Tb.N; unit, /mm) [9, 10].

Measurement of mechanical properties

The mechanical strength of vertebral body (L5) was measured using a compression test. For the compression test, the planoparallel surfaces were obtained by removing the cranial and caudal ends of the vertebral specimen. From the vertebral body, a central cylinder with planoparallel ends and a height of approximately 5 mm was obtained. A compression force was applied to the specimen in the cranio-caudal direction using a steel disk, at a deformation rate of 2.5 mm/min [11]. The ultimate compressive load (max load, N) was calculated as the mechanical strength directly from the load-deformation curve.

Structural analysis using micro-computed tomography (mCT)

The micro-CT apparatus (μCT20) and the analyzing software used in this study were obtained from SCANCO Medical AG (Bassersdorf, Switzerland) [23]. The micro-CT

system has a micro X–ray source (10 μm, 25 keV) directed toward the sample. Quantitative modification of the X-ray beam by apatite crystals in the bone sample is then analyzed by a plane detector (CCD array; 1024 elements). The process is directed using a DEC a-station (Digital Equipment Corp., Marseille, France), and an open VMS system in cluster configuration is used to perform the 3D analysis. The whole spinal body (L5) was scanned in 250 slices (thickness, 13 microns) in the dorsoventral direction.

On the original 3D images, morphometric indices were directly determined from the binalized volume of interest (VOI) [24]. Three-dimensional reconstruction of bone was performed using the triangulation algorithm. The volume of trabecular bone (BV, mm^3) was calculated using tetrahedrons corresponding to the enclosed volume of the triangulated surface. The total tissue volume (TV, mm^3) is the volume of the whole examined sample. For comparison of samples with different sizes, measured values were normalized according to the bone volume fraction (BV/TV, %). Using the original application, the histomorphometric parameters, which were the trabecular number (Tb.N, 1/mm), the trabecular thickness (Tb.Th, μm) and the trabecular separation (Tb.Sp,μm), were directly measured on 3D images using the method described by Hildebrand *et al.* [25], not using the parallel plate model. Thus, the parameters Tb.Th, Tb.Sp, and Tb.N are model-independent indices, and are not biased by eventual deviations of the actual structure. For the non-metric parameters, the trabecular bone pattern factor (TBPf) and the structure model index (SMI) were computed using software provided with the micro-CT machine. The trabecular bone pattern factor (TBPf, 1/mm), which represents the ratio of concave to convex surfaces in 2D sections of trabeculae, was measured for each slice, and the mean value was determined

for each specimen [26]. The structure model index (SMI) is used to quantify the characteristic form of a three-dimensional structure consisting of plates and rods [27].

3.4 Statistical analysis

All data were expressed as the mean ± standard deviation (SD) or the mean ± standard error (SE). Analysis of variance (ANOVA) was performed using Statistic Analysis System (SAS) software. The significance of differences (in comparisons with OVX-vehicle group or Sham-operated group) was determined using Dunnett's multiple test. A p value less than 0.05 was considered to indicate a significant difference.

4. Results

1. Changes in body weight and biochemical markers (Table 1)

At the time of necropsy, body weight was significantly higher for the OVX control group than for the Sham group ($p<0.01$). While body weight was not markedly affected by ALF, body weight was significantly lower for the Ca4%P3.5% and Ca6%P5.2% groups (high-dose Ca administration with a Ca/P ratio of 1.2) than for the OVX control group ($p<0.01$). The level of serum UN was significantly higher for the Ca4%P1% and Ca6%P1% groups (Ca/P ratio of 4 and 6, respectively) than for the OVX control group ($p<0.05$), and the level of serum CRE was significantly higher for the Ca4%P1% group than for the OVX control group ($p<0.05$). While OVX significantly increased the level of urinary DPD (DPD/CRE, a bone resorption marker) ($p<0.001$), the level of urinary DPD was significantly lower for all 3 ALF groups than for the OVX control group ($p<0.001$), and the level of urinary DPD was significantly lower for the Ca4%P1% group (Ca/P ratio of 4) than for the OVX control group ($p<0.05$).

2. Changes in the level of serum parathyroid hormone (Figure 1)

At weeks 4 and 12, the level of serum PTH was higher for the OVX control group than for the Sham group. However, at week 4, the level of serum PTH was significantly lower for the high-dose ALF (0.1 μg/kg) group than for the OVX control group ($p<0.001$), and at week 12, the level of serum PTH was significantly lower for the middle (0.05 μg/kg) and high-dose ALF groups than for the OVX control group ($p<0.01$). There was no marked difference in the level of serum PTH between the high-dose Ca groups (Ca/P ratio of 2, 4 or

6) and the OVX control group. However, at week 4, the level of serum PTH was significantly higher for the high-dose Ca and P groups (Ca/P ratio of 1.2) than for the OVX control group, but there was no marked difference at week 24.

3. Changes in the levels of serum calcium and phosphorous (Figure 2)

The level of serum Ca was significantly lower for the OVX control group than for the Sham group ($p<0.01$). While the level of serum Ca was significantly higher for the middle and high-dose ALF groups than for the OVX control group ($p<0.01$), there was no marked difference between the middle and high-dose ALF groups and the Sham Group. While there was no marked difference in the level of serum calcium between the high-dose Ca groups (Ca/P ratio of 2, 4 or 6) and the OVX control group, the level of serum calcium was significantly lower for the Ca4%P3.5% and Ca6%P5.2% groups (high Ca and P-dose groups with a Ca/P ratio of 1.2) than for the OVX control group ($p<0.05$).

While OVX decreased the level of serum P, the level of serum P was significantly higher for the high-dose ALF group than for the OVX control group ($p<0.05$). While there was no marked difference in the level of serum P between the high-dose Ca and P groups (Ca/P ratio of 1.2) and the OVX control group, the level of serum P was significantly higher for the Ca4%P1% and Ca6%P1% groups (Ca/P ratio of 4 and 6, respectively) than for the OVX control group ($p<0.01$).

4. Changes in the levels of urinary calcium and phosphorous (Figure 3)

While OVX did not have a marked effect on the level of urinary calcium (Ca/Cre),

ALF increased the level of urinary calcium in a dose-dependent manner, and there was a significant difference between the OVX control group and the moderate- and high-dose ALF groups ($p<0.001$). Also, there was no significant difference in the level of urinary calcium between the high-dose Ca and P groups (Ca/P ratio of 1.2) and the OVX control group, but the level of serum Ca was significantly higher for the Ca4%P1% and Ca6%P1% groups (Ca/P ratio of 4 and 6, respectively) than for the OVX control group ($p<0.01$ and 0.05, respectively).

OVX and ALF did not have a marked effect on the level of urinary P (P/Cre). The level of urinary P was significantly lower for the high-dose Ca groups (Ca/P ratio of 2, 4 or 6) than for the OVX control group ($p<0.05$ 0.001 and 0.001, respectively). Furthermore, the level of urinary P was significantly higher for the high-dose Ca and P groups with a Ca/P ratio of 1.2 (Ca4%P3.5% and Ca6%P5.2% groups) than for the OVX control group ($p<0.001$).

5. Effects on the bone mineral density of the lumbar vertebra (Figure 4)

The average BMD of the lumbar vertebra (L2-L4) was significantly lower for the OVX control group than for the Sham group ($p<0.001$), but ALF increased the BMD in a dose-dependent manner. The BMD was significantly higher for the high-dose ALF (0.1 μg/kg) group than for the OVX control group ($p<0.001$). There was no significant difference in BMD between the OVX control group and the high-dose Ca groups (Ca/P ratio of 2, 4 or 6). However, the BMD was significantly lower for the high-dose Ca and P groups (Ca/P ratio of 1.2; Ca4%P3.5% and Ca6%P5.2% groups) than for the OVX control

group ($p<0.05$ and 0.001, respectively).

6. Effects on the mechanical strength of the lumbar vertebra (Figure 5)

While the mechanical strength of the lumbar vertebra (L5) was significantly lower for the OVX control group than for the Sham group ($p<0.01$), ALF increased bone strength in a dose-dependent manner. The bone strength was significantly higher for the high-dose ALF (0.1 μg/kg) group than for the OVX control group ($p<0.05$). However, there was no marked difference in bone strength between the OVX control group and any of the high-dose Ca groups or the high-dose Ca and P groups, and dose-dependency was not observed.

7. Analysis of the trabecular structure of the vertebra by micro-CT (Table 2 and Figure 6)

Figure 6 shows the trabecular structure in the cancellous region of L5, as assessed by micro-CT. OVX caused loss of trabecular connectivity, and caused the trabecular structures to become rough. However, in the ALF groups, the bone structure was maintained and reinforced. Of the 3 high-dose Ca groups, slight improvement was seen in the Ca6% group (Ca/P ratio of 6), but there was no marked difference between the OVX control group and the Ca6%P5.2% group (Ca/P ratio of 1.2).

In order to investigate the effects of ALF and high-dose Ca administration on the trabecular structure of L5 in greater detail, a three-dimensional analysis was conducted by micro-CT to calculate various parameters; Table 2 shows the results. The BV/TV and Tb.N were significantly lower for the OVX control group ($p<0.001$), while TBPf (an indicator of

trabecular connectivity) and SMI (an indicator of trabecular morphology) were significantly higher ($p<0.001$), suggesting that OVX decreased the bone mass, damaged the trabecular structure, disrupted the trabecular connectivity, and caused rod-like structures to form. ALF improved these parameters in a dose-dependent manner: compared to the OVX control group, 0.1 μg/kg ALF caused a significant improvement in the BV/TV, Tb.N and TBPf ($p<0.01$, $p<0.001$ and $p<0.01$, respectively). There were no marked differences in any of the parameters between the OVX control group and the high-dose Ca and P groups (Ca/P ratio of 2, 4 or 6). However, compared to the OVX control group, the BV/TV and Tb.N were significantly lower for the Ca6%P5.2% group (Ca/P ratio of 1.2) ($p<0.01$), while TBPf and SMI were significantly higher ($p<0.05$).

5. Discussion

In Ca and vitamin D deficiency, vitamin D supplementation (like Ca supplementation) increases bone mass by facilitating the intestinal absorption of Ca, which in turn increases the level of serum Ca [13, 14, 15]. Hence, vitamin D administration can serve as supplementary therapy in cases of vitamin D deficiency.

The present study investigated whether the beneficial effects of ALF (an active vitamin D drug), as a therapeutic agent for osteoporosis rather than a supplement for treatment of Ca and vitamin D deficiency, on bone tissue could be achieved by administering a large dose of Ca. The results show that the preventive effects of ALF against bone fragility in aged OVX rats could not be achieved by supplementation with a large dose of Ca, even when the dose of P was adjusted.

ALF suppressed the OVX-induced decreases in the BMD of the lumbar vertebrae (L2-L4) and increased the compressive bone strength of L5 in a dose-dependent manner. The lumbar BMD and mechanical strength were significantly higher for the high-dose ALF group than for the OVX control group. Micro-CT was performed to analyze the trabecular structure of the lumbar vertebra, and the results show that OVX significantly decreased the BV/TV and Tb.N and increased TBPf and SMI, confirming that estrogen deficiency causes a change from plate-like trabecular structures to rod-like structures and reduces trabecular connectivity. However, ALF improved these parameters in a dose-dependent manner, suggesting that ALF has suppressive effects on the destruction of trabecular structures in the lumbar spine. These effects of ALF were exerted while maintaining the level of serum Ca at a normal level without inducing hypercalcemia or body weight changes. Also, OVX

increased the level of urinary DPD, a bone resorption marker, but ALF significantly suppressed the urinary excretion of DPD in a dose-dependent manner. Past studies have shown that the mechanism of preventive effects of ALF against bone loss is based on the suppression of bone resorption and the maintenance and stimulation of bone formation. This is different from the mechanism of the most common anti-resorptive agent, estrogen (17β-estradiol), in that estrogen suppresses both bone resorption and formation [16].

High doses of Ca (Ca/P ratio of 2 to 6) did not markedly affect BMD or mechanical strength of the lumbar vertebra following OVX. In terms of the trabecular parameters, the high doses of Ca supplementation (Ca/P ratio of 2 to 6) did not improve trabecular structure. Also, a significant decrease in the level of urinary P was observed, suggesting that P absorption was suppressed. While the levels of UN and CRE increased, there was no marked difference in body weight between the high-Ca-dose groups and the OVX control group, and there was no dose-dependency in the suppression of bone resorption, in terms of DPD. Thus, when Ca intake is increased and P intake is unchanged, bone mass does not increase. Heaney et al. reported that, even in clinical patients, excessive Ca intake suppressed P absorption, indicating that high Ca intake is an inappropriate treatment for osteoporosis, because Ca and P easily form complexes with each other [17]. Increases in bone mass are due to the formation of osteoid and hydroxyapatite, and hydroxyapatite is formed by the binding of Ca to P. Therefore, efficient absorption of Ca and P is essential for increasing bone mass [18, 19, 20], as the present results confirm.

When the Ca/P ratio was maintained at 1.2 and the doses of Ca and P were both increased, body weight did not increase and the BMD and bone strength of the lumbar

spine decreased in a Ca dose-dependent manner. Also, there was no dose-dependency in the suppression of bone resorption, and an analysis of trabecular structure showed that the degree of decrease in BV/TV and Tb.N and the degree of increase in TBPf and SMI were greater for the groups with a Ca/P ratio of 1.2 than for the OVX control group. Further destruction of bone structure was seen in the Ca6% group. At week 4, the level of serum PTH was significantly elevated in the Ca6% group, suggesting that hyperparathyroidism aggravates reduction of bone mass. Several studies have found that excessive P intake accelerates bone resorption via elevation of the level of PTH, thus lowering the Ca and P content of bone [19, 20, 21]. The present results also confirm that high P intake increases the degree of reduction of bone mass, thus clarifying the importance of an appropriate balance between Ca and P intake.

The present findings show that ALF increased bone mass and intestinal absorption of Ca and P, and the pharmacological effect of ALF on bone loss in the present osteoporotic rat model was not achieved by administering a large amount of Ca. We previously compared the effects of ALF and native vitamin D on bone tissue in cases of Ca and vitamin D sufficiency, and found that, unlike native vitamin D, ALF acted independently from Ca [8]. When ALF was administered to parathyroidectomized rats in which the level of serum PTH was maintained at a constant level by continuously injecting PTH, the BMD of the femur significantly increased while the levels of serum Ca and P were maintained within a normal range [8]. These findings suggest that ALF directly affects bone tissue irrespective of intestinal absorption of Ca, without suppressing secretion of PTH.

The present results suggest that, if OVX rats are given a diet containing normal levels of Ca and vitamin D, even when a high dose of Ca is administered orally, the absorption of Ca and P reaches a plateau, and that even if the dose of P is adjusted, high-dose Ca administration cannot counteract decreases in BMD or mechanical strength of the lumbar vertebrae. It is difficult to adjust the Ca supply through diet alone without disrupting the balance between serum Ca and P levels. Together, the present findings indicate that the beneficial effect of alfacalcidol on bone dynamics is not reproduced by calcium therapy combined with modulation of phosphorus supply in an ovariectomized rat model of osteoporosis.

FIGURE LEGENDS

Fig.1 Effects of alfacalcidol and high calcium diets on serum PTH level in OVX rats.

Starting 2 weeks after OVX, either ALF or high-dose Ca was administered orally to rats, and serum samples were collected 4 (A) and 12 (B) weeks after the start of oral administration to measure the level of serum parathyroid hormone. Each value represents the mean ± SE (n = 6–7). $^{**}p<0.01$, $^{***}p<0.001$ compared with the OVX-control group (Dunnett's t-test). Note that serum PTH level was significantly increased by 4-week treatment with high intake of Ca and P when the Ca/P ratio was 1.2. PTH: parathyroid hormone

Fig.2 Effects of alfacalcidol and high calcium diets on serum biochemical parameters in OVX rats.

Starting 2 weeks after OVX, either ALF or high-dose Ca was administered orally to rats for 12 weeks, and serum Ca (A) and P (B) were measured as described in MATERIALS AND METHODS. Each value represents the mean ± SD (n = 6–7). $^{*}p<0.05$, $^{**}p<0.01$ compared with the OVX-control group. $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$ compared with the SHAM-control group (Tukey-Kramer test). Note that serum Ca was significantly lower in the groups fed a diet containing >4% Ca (3.5% P) when the Ca/P ratio was 1.2.

Fig.3 Effects of alfacalcidol and high calcium diets on urinary biochemical parameters in OVX rats.

Starting 2 weeks after OVX, either ALF or high-dose Ca was administered orally to rats for 12 weeks, and urinary Ca (A) and P (B) excretion were measured as described in MATERIALS AND METHODS. Data for Ca and P are corrected for urinary creatinine (CRE) concentrations. Each value represents the mean ± SD (n = 6–7). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared with the OVX-control group (Tukey-Kramer test). In B, note that high Ca intake significantly depressed urinary Pi excretion in a dose-dependent manner. In contrast, in the groups fed a diet containing >4% Ca (3.5% P) when the Ca/P ratio was

maintained at 1.2, the urinary Pi/Cr value was twice the value of the OVX control group ($p<0.001$).

Fig.4 Alfacalcidol increases the lumbar spine BMD more than does high Ca diet in OVX rats.

Starting 2 weeks after OVX, either ALF or high-dose Ca was administered orally to rats for 12 weeks, and spinal BMD was measured as described in MATERIALS AND METHODS. Each value represents the mean ± SD (n = 6–7). *$p<0.05$, ***$p<0.001$ compared with the OVX-control group (Tukey-Kramer test). Note a dose of 0.1μg/kg ALF prevented OVX-induced bone loss ($p<0.001$), but Ca supplementation did not. When the Ca/P ratio of the diet was maintained at 1.2, high intake of dietary P caused significant bone loss as long as the Ca content remained ≤2%.

Fig.5 Alfacalcidol improves the mechanical strength at lumbar spine more than does high Ca diet in OVX rats.

Starting 2 weeks after OVX, either ALF or high-dose Ca was administered orally to rats for 12 weeks, and spinal strength was measured as described in MATERIALS AND METHODS. Each value represents the mean ± SD (n = 6–7). *$p<0.05$, **$p<0.01$ compared with the OVX-control group (Tukey-Kramer test). Note that a dose of 0.1μg/kg ALF prevented the decrease in bone strength caused by OVX ($p<0.001$), but Ca supplementation did not prevent the decrease in bone strength regardless of the Ca/P ratio in the diet.

Fig.6 Three-dimensional trabecular microarchitectural images of L5 vertebral body using micro-CT analysis.

Starting 2 weeks after OVX, either ALF or high-dose Ca was administered orally to rats for 12 weeks, and the structural changes in fifth lumbar vertebra was analyzed by micro-CT as described in MATERIALS AND METHODS. (A) Sham control group, (B) OVX control group, (C) ALF-0.1μg/kg treated group, (D) Ca6%-group (Ca/P ratio of 6), (E) Ca6%P5.2%- group (Ca/P ratio of 1.2). Note that a dose of 0.1μg/kg ALF markedly

increased the number of plate-like structures in trabecular bone.

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Table 1 body weight and biochemical markers

groups			body weight (g)	biochemical markers		
				serum		urine
operation	diet	agent		UN (mg/dL)	CRE (mg/dL)	DPD/CRE (nM/mM)
SHAM	A : Ca 1.2%, P 1.0%	vehicle	310 ± 12**	15.7 ± 1.4	0.58 ± 0.04	43.4 ± 8.8***
OVX	A : Ca 1.2%, P 1.0%	vehicle	391 ± 24	14.8 ± 1.8	0.60 ± 0.06	122.4 ± 29.7
OVX	B : Ca 2.0%, P 1.0%	vehicle	387 ± 35	16.3 ± 1.5	0.65 ± 0.05	115.6 ± 11.6
OVX	C : Ca 4.0%, P 1.0%	vehicle	372 ± 27	23.2 ± 4.7*	0.74 ± 0.15*	79.7 ± 16.1*
OVX	D : Ca 6.0%, P 1.0%	vehicle	359 ± 21	21.7 ± 6.3*	0.70 ± 0.08	141.4 ± 41.3
OVX	E : Ca 2.0%, P 1.8%	vehicle	371 ± 45	17.9 ± 5.2	0.60 ± 0.09	119.5 ± 30.0
OVX	F : Ca 4.0%, P 3.5%	vehicle	341 ± 10**	18.3 ± 1.8	0.60 ± 0.06	128.9 ± 35.2
OVX	G : Ca 6.0%, P 5.2%	vehicle	333 ± 26**	17.5 ± 2.7	0.57 ± 0.05	132.3 ± 24.6
OVX	A : Ca 1.2%, P 1.0%	ALF 0.025μg/kg	376 ± 26	15.9 ± 1.8	0.60 ± 0.06	70.3 ± 8.2***
OVX	A : Ca 1.2%, P 1.0%	ALF 0.05μg/kg	385 ± 22	14.4 ± 2.1	0.63 ± 0.05	55.0 ± 17.7***
OVX	A : Ca 1.2%, P 1.0%	ALF 0.1μg/kg	365 ± 28	15.5 ± 1.2	0.63 ± 0.05	33.2 ± 5.6***

UN : urea nitrogen, CRE : creatinine, DPD : deoxypyridinoline
Mean±SD (n=6-7) *p<0.05, **p<0.01, ***P<0.001 vs. 1.2%Ca OVX-control group
(Dunnett's t test)

Fig. 1