Discussion

In adult *Opg*$^{-/-}$ mice, we observed erosion of the malleus, incus, and stapes. Furthermore, TRAP activity was detected in all the three ossicles and the otic capsule of *Opg*$^{-/-}$ mice, indicating that osteoclastic bone resorption of auditory ossicles is elevated. It is unclear whether certain specific areas within each ossicle are preferentially resorbed or not. We also observed that *Opg*$^{-/-}$ mice show progressive hearing loss. The precise mechanisms of hearing loss in these mice are currently unknown. Since hearing loss in each animal was progressive, sudden loss of articulation between auditory ossicles is not likely to be the cause of impairment. We observed extensive resorption of the malleal processus brevis in *Opg*$^{-/-}$ mice, but the malleal processus brevis is dispensable for hearing function [2]. In *Opg*$^{-/-}$ mice, we observed stapedial fixation, namely, the ligaments between the stapes and the otic capsule were replaced with bone tissue. Among the observed morphological alterations, such as thinning of the malleal manubrium and incus body, stapedial fixation is the most likely cause of hearing loss in *Opg*$^{-/-}$ mice. Clinically, hearing loss in otosclerosis can be cured by surgical intervention at the stapedial-cochlear junction, indicating the importance of this junction in hearing.

OPG deficiency has been found in patients with juvenile Paget's disease, which is also known as idiopathic hyperphosphatasia [23-27] and characterized by markedly increased bone turnover. Paget disease of bone is a distinct and much more common disease than juvenile Paget's disease, characterized by excessive osteoclastic bone resorption followed by compensatory increase in osteoblastic bone formation [28]. Curiously, hearing is affected in approximately 50% of cases of Paget disease of bone involving the skull [29]. Hearing loss in patients with otosclerosis and Paget disease of bone could be due to the increased bone formation, which narrows the internal auditory

canal and causes nerve atrophy. On the other hand, hearing loss has been positively correlated with loss of bone density in the otic capsule [30, 31]. Similarly, activating mutations in *TNFRSF11A,* which encodes RANK, cause expansile skeletal hyperphosphatasia, which features deafness in infancy or early childhood [32]. Familial expansile osteolysis, which is also caused by mutation in *TNFRSF11A* [33], is also associated with deafness.

Recent reports show that OPG is highly expressed in cochlear soft tissues and secreted into the perilymph and surrounding bone [34]. Furthermore, OPG levels increase with age in women [35-37]. The apparent lack of association between osteoporosis and hearing loss in humans [18] implies that the auditory ossicles and the otic capsule may be protected from osteoclastic bone resorption by OPG even in patients with postmenopausal and age-related osteoporosis. In conclusion, these data reveal a critical role for OPG as an "audioprotegerin" in maintaining quality of bone conduction by protecting auditory ossicles and the otic capsule from bone resorption. Impaired production of OPG in the temporal bone may be a risk factor for hearing loss.

Acknowledgments

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Fig. 1. Excess bone remodeling of auditory ossicles. (a) Biomicroscopic photographs showing auditory ossicles from 10-week-old wild-type (+/+), heterozygous (+/-) and *Opg*$^{-/-}$ (-/-) mice (n=6 or more for each genotype). Ossicles were stained for TRAP activity (red). Arrowheads indicate the malleal manubrium, incus body, and stapedial footplate. Scale bar, 100 μm. (b) Histological sections of auditory ossicles in the middle ear cavity from 15-week-old *Opg*$^{-/-}$ mice (n=2 per genotype). HE and TRAP activity staining are shown. TRAP-positive areas in the malleus (M), incus (I), and stapes (S) are indicated by arrowheads, and those in the otic capsule by arrows. tm, tympanic membrane. Scale bar, 100 μm. (c) The number of TRAP-positive MNCs in mallei from wild-type, heterozygous, and *Opg*$^{-/-}$ mice (n=5 for +/+, n=4 for +/- and -/-). #$p<0.001$.

Fig. 2. Histological sections of the stapedial-cochlear junction (arrowheads). Staining is hematoxylin eosin (HE) (n=3 for each genotype). Scale bar, 25 μm.

Fig. 3. Radiographical analysis of auditory ossicles and tibia. (a) Representative μCT images of the malleus from 8-week-old mice (n=5 for +/- and n=5 for -/-). (b) Malleal cortical thickness of mice analyzed in (a). (c) Tibial cortical BMD of mice analyzed in (a). #$p<0.001$, versus heterozygous mice.

Fig. 4. Progressive increase in ABR thresholds of *Opg*$^{-/-}$ mice. Data points for thresholds at 2, 4, 12, and 20 kHz in *Opg*$^{-/-}$ mice and heterozygous controls at the ages of 6 (n=5 each), 10 (n=7 each) and 15 (n=7 each) weeks. Significant differences between 15-week-old *Opg*$^{-/-}$ and heterozygous control mice are indicated with asterisks (*$p<0.05$, **$p<0.01$).

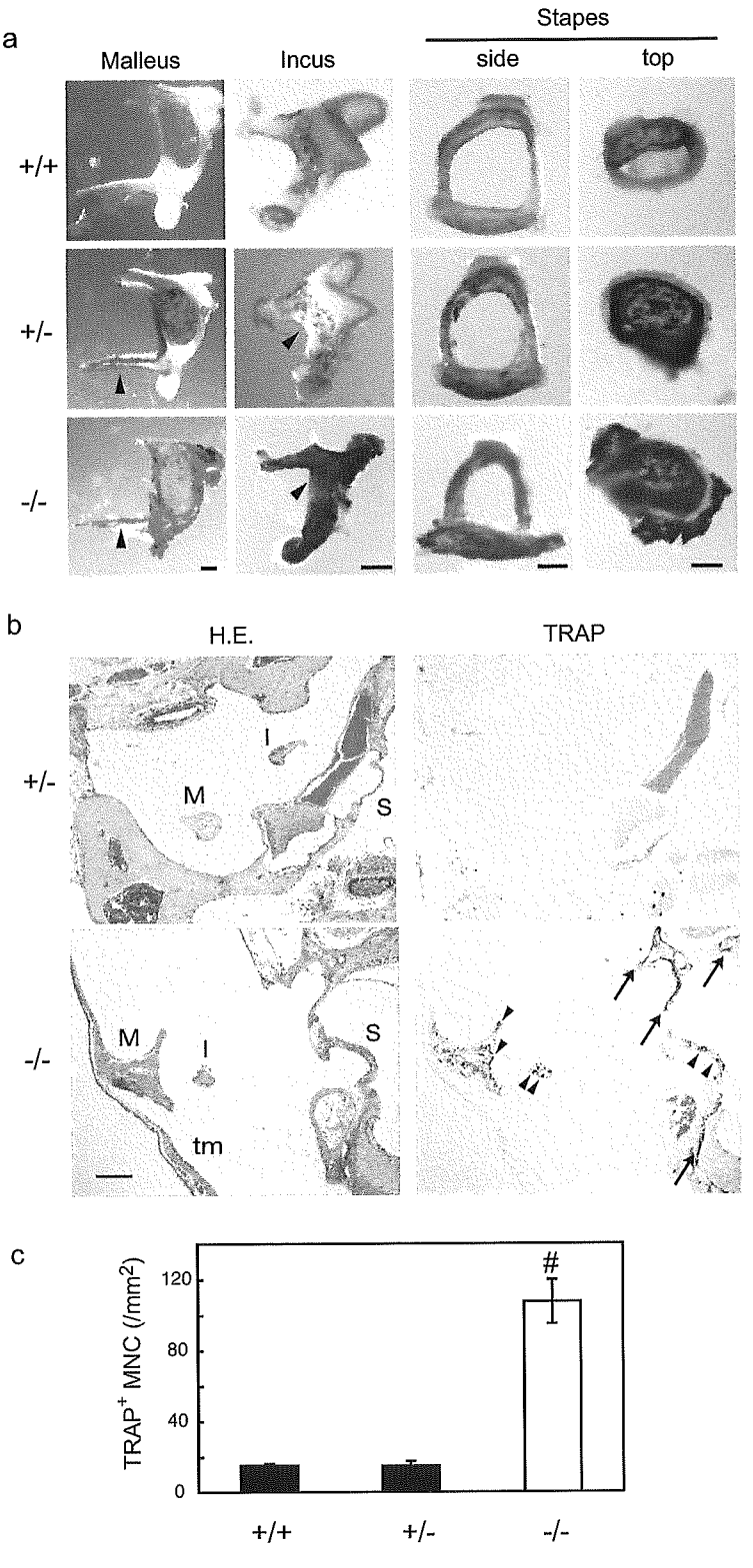

Fig. 1 Kanzaki et al.

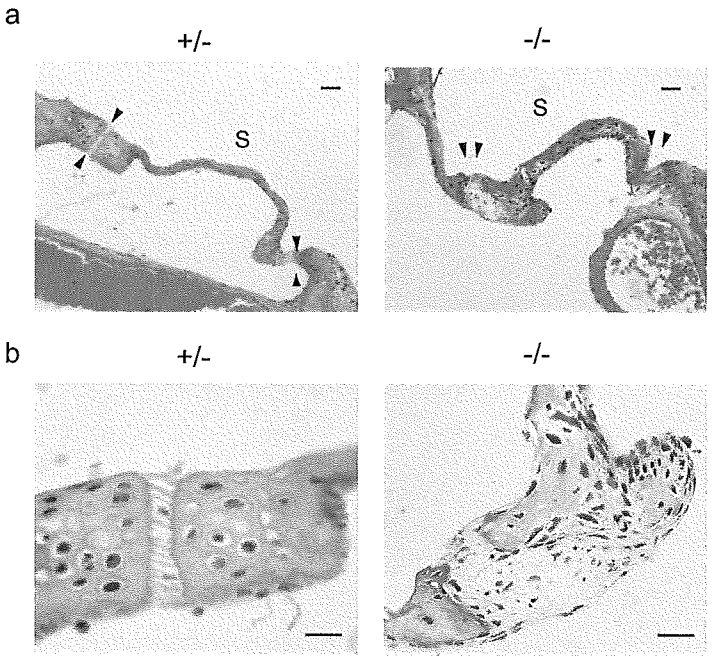

Fig. 2 Kanzaki et al.

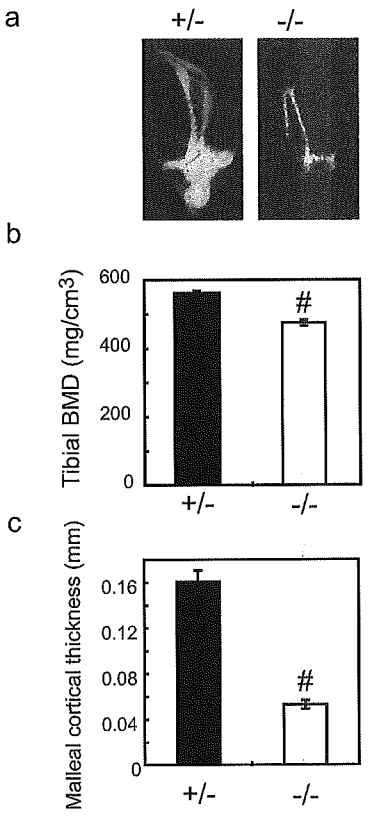

Fig. 3 Kanzaki et al.

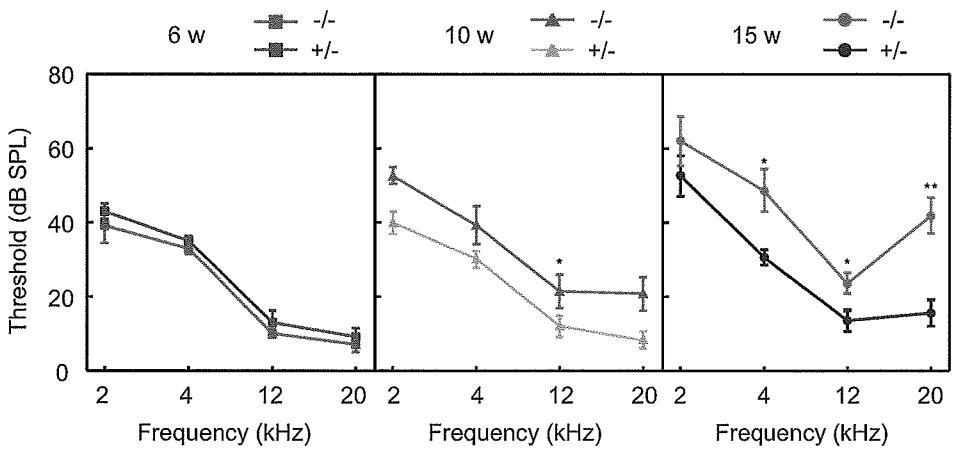

Fig. 4 Kanzaki et al.

Calcium supplementation does not reproduce the pharmacological efficacy of alfacalcidol for the treatment of osteoporosis in rats

Abbreviated running title: Alfacalcidol versus calcium supplement therapy in rat osteoporosis model

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1. Abstract

The purpose of this study was to assess whether a nutritional supply of calcium could be substituted for ALF administration in preventing bone loss due to estrogen deficiency. Female Wistar-Imamichi rats (8 months old) were ovariectomized (OVX) or sham operated. The OVX rats received ALF administration (0.025, 0.5 or 0.1 μg/kg, p.o. 5 times a week), a calcium-enriched diet containing 2, 4 or 6%Ca (Ca/P ratio of 2, 4 and 6, respectively), or a Ca-P enriched diet (Ca/P ratio of 1.2). After 12 weeks of treatment, all rats were sacrificed to harvest the spine, serum, and urine samples. Neither the ALF treatment nor the Ca supplement caused hypercalcemia. In the spine, ALF prevented decreases in bone mineral density (BMD) and compressive strength of lumbar spine induced by OVX. Micro-CT analysis confirmed that ALF significantly improved the trabecular bone pattern factor (TBPf) and the structure model index (SMI), and suppressed bone destruction. In contrast (and of particular interest), high-dose Ca administration did not have marked effects on bone fragility. Also, when both Ca and P were administered in high doses, body weight did not increase, BMD and strength decreased dose-dependently, urinary P significantly increased, and serum parathyroid hormone (PTH) increased, suggesting involvement of hyperparathyroidism. Together, it is difficult to adjust the Ca supply through diet alone without disrupting the balance between serum Ca and P levels. Consequently, we conclude that ALF is beneficial for the treatment of osteoporosis, which is not achieved by the use of a Ca supplement.

Key words: osteoporosis--alfacalcidol--calcium--ovariectomy

2. Introduction

In the elderly, the level of bone formation tends to be low, and the elderly are in a state of secondary hyperparathyroidism because the response of vitamin D receptors in the small intestine is weakened [1], vitamin D levels are deficient, and Ca intake is insufficient. Consequently, the level of bone resorption is elevated [2], and bone mass decreases. Treatments for osteoporosis have included bisphosphonate, calcium, estrogen, vitamin D and calcitonin. These findings suggest that supplementation of Ca and native vitamin D would be an effective treatment for Ca and vitamin D deficiency. Active vitamin D_3 is one of the regulatory hormones of Ca, and alfacalcidol ($1\alpha(OH)D_3$; ALF), a prodrug of active vitamin D_3, is widely used to treat various metabolic bone diseases, such as, rickets, osteomalacia, renal osteodystrophy and osteoporosis [3,4]. In Japan, ALF is frequently used to treat osteoporosis [5, 6]. We previously used osteoporotic rats with estrogen deficiency to study the pharmacological effects of ALF on reduced bone mass, but did not examine the effects of Alf as a supplement in cases of Ca and vitamin D deficiency [7, 8]. In those studies, compared to native vitamin D, ALF more effectively increased bone mass and mechanical strength at given serum Ca or urinary Ca levels (or intestinal Ca absorption). Also in those studies, an extremely large dose of natural vitamin D was required to achieve an increase in BMD comparable to that produced by ALF; such high-dose administration of vitamin D could cause hypercalcemia, hypercalciuria and degradation of bone quality [8]. The pharmacological effects of active vitamin D cannot be achieved by supplementation of native vitamin D. Native vitamin D does not express its biological actions in this form, because its biological actions are due to the activities of its metabolites, which are

synthesized in the body.

The main target of active vitamin D is the intestine, and vitamin D increases the intestinal absorption of Ca. Therefore, the effects of ALF on bone tissue are assumed to be purely due to an increase in the intestinal absorption of Ca. Hence, in the present study, we focused on changes in intestinal absorption of Ca after ALF administration, and investigated whether the protective effects of ALF on bone tissue could be achieved by simply increasing the supply of Ca in the intestinal tract.

3. Methods

3.1 Reagents

The ALF preparation was dissolved and diluted using medium-chain triglyceride (MCT) (vehicle) to a predetermined concentration, and was stored at 4°C in the dark until use. The ALF dose was 1 mL/kg body weight. Seven diets containing different amounts of Ca and P were prepared (A, Ca 1.2% and P 1.04%; B, Ca 2.0% and P 1.04%; C, Ca 2.0% and P 1.74%; D, Ca 4.0% and P 1.04%; E, Ca 4.0% and P 3.48%; F, Ca 6.0% and P 1.04%; G, Ca 6.0% and P 5.22%; Japan Clea, gamma-ray sterilized). The contents of dietary components other than Ca and P were the same as those of the regular diet, CE-2, and these diets were stored at room temperature after delivery until use. The amount of food consumed was measured every week.

3.2 Experimental procedures

All animal experiments were conducted according to the animal study guidelines established by Chugai Pharmaceutical Co., Ltd. Eight-month-old female Wistar-Imamichi rats, purchased from the Institute for Animal Reproduction (Ibaraki), were used. During the study, the rats were placed in individual stainless steel wire cages and had free access to tap water and solid feed (CE-2, Clea Japan). Based on body weight, the rats were divided into 11 groups of 7 rats each. In 10 of the 11 groups, bilateral ovariectomy (OVX) was performed via a skin incision on the back under etherization; in the remaining group, a sham operation (skin incision) was performed. Diet A (Ca 1.2% and P 1.04%) was given to

4 of the 10 OVX groups, and the other 6 OVX groups were given either Diet B (Ca 2.0% and P 1.04%), Diet C (Ca 2.0% and P 1.74%), Diet D (Ca 4.0% and P 1.04%), Diet E (Ca 4.0% and P 3.48%), Diet F (Ca 6.0% and P 1.04%), or Diet G (Ca 6.0% and P 5.22%). Of the 4 groups that were given Diet A, 3 groups were orally administered 0.025, 0.05 or 0.1 μg/kg of ALF 5 times weekly for 12 weeks, and the remaining group was orally administered MCT only (OVX control group). In the SHAM and OVX control groups, 1 mL/kg MCT was administered orally 5 times weekly for 12 weeks. The body weight of the rats was measured every week, and the dosage of each agent was calculated based on the most recent body weight. Starting 24 hours before the final administration, the rats were fasted, and a 24-hour urine sample was collected and stored frozen at -80°C. Under etherization, the rats were necropsied by drawing blood from the abdominal aorta, and the serum was separated and stored frozen at -80°C. After collecting the blood sample, the rats were exsanguinated, and the lumbar vertebrae (L2-L4 and L5) were excised. The fifth lumbar vertebral body (L5) was stored frozen before measurement of its bone strength, while L2-L4 was fixed in 70% ethanol to measure BMD.

3.3 Measurements

Biochemical Analysis

The levels of serum Ca, P, urea nitrogen (UN) and creatinine (CRE) and urinary Ca, P and CRE were measured using an automatic analyzer (Hitachi 7070; Hitachi Co., Ltd., Tokyo). The level of serum parathyroid hormone (PTH) was measured using a rat intact PTH ELISA kit (Immutopics Inc.), and the level of urinary deoxypyridinoline (DPD) was