Distinctive anabolic roles of 1,25-dihydroxyvitamin D₃ and parathyroid hormone in teeth and mandible versus long bones

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Abstract

To assess the roles of 1,25-dihydroxyvitamin D (1,25(OH)₂D) and parathyroid hormone (PTH) in hard tissue formation in oro-facial tissues, we examined the effect of either 1,25(OH)₂D or PTH deficiency on dentin and dental alveolar bone formation and mineralization in the mandibles, and osteoblastic bone formation in long bones of 1 α -hydroxylase knockout (1 α (OH)ase^{-/-}) mice. Compared with wild-type mice, the mineral density was decreased in the teeth and mandibles, and unmineralized dentin (predentin and biglycan immunopositive dentin) and unmineralized bone matrix in the dental alveolar bone were increased in 1 α (OH)ase^{-/-} mice. The dental volume, reparative dentin volume, and dentin sialoprotein immunopositive areas were reduced in 1 α (OH)ase^{-/-} mice. The cortical thickness, dental alveolar bone volume, and osteoblast number were all

decreased significantly in the mandibles; in contrast, the osteoblast number and surface were increased in the trabecular bone of the tibiae in 1 α (OH)ase^{-/-} mice consistent with their secondary hyperparathyroidism. The expression of PTH receptor and IGF1 was reduced slightly in mandibles, but enhanced significantly in the long bones in the 1 α (OH)ase^{-/-} mice. To control for the role of secondary hyperparathyroidism, we also examined teeth and mandibles in 6-week-old PTH^{-/-} mice. In these animals, dental and bone volumes in mandibles were not altered when compared with their wild-type littermates. These results suggest that 1,25(OH)₂D₃ plays an anabolic role in both dentin and dental alveolar bone as it does in long bones, whereas PTH acts predominantly in long bones rather than mandibular bone. *Journal of Endocrinology* (2009) **203**, 203–213

Introduction

Vitamin D is essential for the development and maintenance of a mineralized skeleton. Vitamin D deficiency results in rickets in children and osteomalacia in adults. Vitamin D-dependent rickets type I (VDDR-I) or pseudovitamin D deficiency rickets results from an autosomal recessive hereditary defect in vitamin D metabolism. Patients with VDDR-I have loss-of-function mutations in the gene encoding the enzyme 25-hydroxyvitamin D 1 α -hydroxylase (1 α (OH)ase) that is located on chromosome 12, resulting in decreased levels of 1,25-dihydroxyvitamin D (1,25(OH)₂D; Fu *et al.* 1997). Clinical features of patients may include hypotonia, weakness, convulsions, tetany, open fontanels, and severe rachitic and osteomalacic lesions, resulting in growth failure, rachitic rosary, and pathologic fractures; biochemical abnormalities include hypocalcemia, hypophosphatemia, and secondary hyperparathyroidism (Prader *et al.* 1961, Fraser *et al.* 1973, Vasilakis *et al.* 1980, Zambrano *et al.* 2003). Dental examinations by radiographs may reveal markedly

hypoplastic, yellowish-to-brownish enamel in all permanent teeth, malocclusion, chronic periodontal disease, large quadrangular pulp chambers, and short roots. Light microscopic and ultrastructural examinations showed abnormalities of dental hard tissues, affecting both enamel and dentin in VDDR-I patients (Zambrano *et al.* 2003). Studies of the molecular mechanisms of vitamin D actions resulting in the abnormalities in these patients are limited.

Panda *et al.* (2001) and Dardenne *et al.* (2001) have previously reported a mouse model of targeted ablation of the 1 α (OH)ase gene that results in 1 α ,25(OH)₂D deficiency. After weaning, mice fed regular mouse chow developed secondary hyperparathyroidism, retarded growth, and skeletal abnormalities characteristic of rickets. These abnormalities mimic those described in the human genetic disorder VDDR-I (Fraser *et al.* 1973, Eil *et al.* 1986). When the phenotype of 1 α (OH)ase^{-/-} mice was analyzed, we found that the skeletal phenotype was different before and after weaning. At 2 weeks of age, the trabecular volume and osteoblast numbers in the 1 α (OH)ase^{-/-} mice were

decreased, and the osteoid volume was not increased significantly (Xue *et al.* 2005). In contrast, at 4 months of age, the trabecular volume, osteoblast number, and osteoid volume were all increased significantly in the $1\alpha(\text{OH})\text{ase}^{-/-}$ mice even on a high calcium diet containing 1.5% calcium in the drinking water (Panda *et al.* 2004). These differences were thought to result from the elevations in circulating parathyroid hormone (PTH). The serum PTH was increased 0.5-fold at 2 weeks of age (Xue *et al.* 2005), but 30-fold at 4 months of age (Panda *et al.* 2004), in the $1\alpha(\text{OH})\text{ase}^{-/-}$ mice compared with their wild-type counterparts. This possibility was confirmed in the $1\alpha(\text{OH})\text{ase}^{-/-}$ mice fed a 'rescue diet' containing 2% calcium, 1.25% phosphorus, and 20% lactose. After serum PTH and calcium levels were normalized, skeletal mineralization was normalized and the trabecular volume and osteoblast number were reduced in the $1\alpha(\text{OH})\text{ase}^{-/-}$ mice (Panda *et al.* 2004). These findings suggest that the secondary hyperparathyroidism occurring in the $1\alpha(\text{OH})\text{ase}^{-/-}$ mice on the normal diet was responsible for the increased bone formation. Moreover, the bone volume of the normocalcemic $1\alpha(\text{OH})\text{ase}^{-/-}$ mice on the rescue diet was less than that observed in the wild-type mice, suggesting that $1\alpha,25(\text{OH})_2\text{D}$ was necessary for baseline bone formation. By comparing mice with targeted disruption of the genes encoding either PTH or $1\alpha(\text{OH})\text{ase}$ or both ($\text{PTH}^{-/-}1\alpha(\text{OH})\text{ase}^{-/-}$ mice), we found that PTH plays a major role in appositional bone growth, whereas $1,25(\text{OH})_2\text{D}_3$ acts predominantly on endochondral bone formation, and both play collaborative roles in modulating skeletal and calcium homeostasis (Xue *et al.* 2005). However, the relative contribution of $1,25(\text{OH})_2\text{D}_3$ and PTH to dentin formation and intramembranous bone formation remains unknown.

To determine whether endogenous $1,25(\text{OH})_2\text{D}$ plays a distinctive role in mandibles relative to long bones, we examined the effect of $1,25(\text{OH})_2\text{D}$ deficiency on the dentin and dental alveolar bone formation, and mineralization in the mandibles and compared osteoblastic bone formation in mandibles and tibiae in $1\alpha(\text{OH})\text{ase}^{-/-}$ mice. Our data indicate that $1,25(\text{OH})_2\text{D}$ plays a more dominant role than PTH in dentin and dental alveolar bone formation in mandibles.

Materials and Methods

1 $\alpha(\text{OH})\text{ase}$ and PTH null mice and genotyping

The generation of $1\alpha(\text{OH})\text{ase}^{-/-}$ and $\text{PTH}^{-/-}$ mice and mouse genotyping were described previously (Panda *et al.* 2001, Xue *et al.* 2005). The mice were fed autoclaved rodent chow containing 1% calcium and 0.67% phosphorus. All animal experiments were approved by the Institutional Animal Care and Use Committee. Mutant mice and control littermates were maintained in a virus- and parasite-free barrier facility and exposed to a 12 h light:12 h darkness. Six-week-old wild-type and $1\alpha(\text{OH})\text{ase}^{-/-}$ siblings, and 6-week-old wild-type and $\text{PTH}^{-/-}$ siblings were used for experiments.

Biochemistry

Serum calcium and phosphorus were determined by autoanalyzer (Beckman Synchron 67; Beckman Instruments, Brea, CA, USA). Serum intact PTH was measured using an ELISA (Immutopics, San Clemente, CA, USA).

Radiography

Mandibles were removed and dissected free of soft tissue. Contact radiographs were taken using a Faxitron model 805 radiographic inspection system (Faxitron Contact, Faxitron, Ethicon, Norderstedt, Germany) (22 kV voltage and 4 min exposure time). X-Omat TL film (Eastman Kodak Co.) was used and processed routinely.

Micro-computed tomography

Mandibles were fixed overnight in 70% ethanol and analyzed by micro-computed tomography (micro-CT) with a SkyScan 1072 scanner and associated analysis software (SkyScan, Antwerp, Belgium) as described. Briefly, image acquisition was performed at 100 kV and 98 mA with a 0.98 rotation between frames. During scanning, the samples were enclosed in tightly fitting plastic wrap to prevent movement and dehydration. Thresholding was applied to the images to segment the bone from the background. Two-dimensional images were used to generate three-dimensional renderings using the 3D Creator software (Skyscan, Aartselaar, Belgium) supplied with the instrument. The resolution of the micro-CT images is 18.2 μm .

Histology

Mandibles and tibiae were removed and fixed in PLP fixative (2% paraformaldehyde containing 0.075 M lysine and 0.01 M sodium periodate) overnight at 4 °C, and processed histologically as described (Miao *et al.* 2001). Mandibles and proximal ends of tibiae were decalcified in EDTA glycerol solution for 5–7 days at 4 °C. Decalcified samples were dehydrated and embedded in paraffin, and 5 μm sections were cut on a rotary microtome. The sections were stained with hematoxylin and eosin (H&E), histochemically for total collagen, tartrate-resistant acid phosphatase (TRAP) or immunohistochemically as described below. Alternatively, undecalcified mandibles were embedded in LR White acrylic resin (London Resin Company Ltd, London, UK), and 1- μm sections were cut on an ultramicrotome. These sections were stained for mineral with the von Kossa staining procedure and counterstained with toluidine blue.

Histochemical staining for total collagen and TRAP

Total collagen was detected in paraffin sections using a modified method of Lopez-De Leon & Rojkind (Panda *et al.* 2004). Dewaxed sections were exposed to 1% sirius red in saturated picric acid for 1 h. After washing with distilled

water, the sections were counterstained with hematoxylin and mounted with Biomount medium. Enzyme histochemistry for TRAP was performed using a modification of a previously described protocol (Miao & Scutt 2002). Dewaxed sections were preincubated for 20 min in buffer containing 50 mM sodium acetate and 40 mM sodium tartrate at pH 5.0. Sections were then incubated for 15 min at room temperature in the same buffer containing 2.5 mg/ml naphthol AS-MX phosphate (Sigma) in dimethylformamide as substrate and 0.5 mg/ml fast garnet GBC (Sigma) as a color indicator for the reaction product. After washing with distilled water, the sections were counterstained with methyl green and mounted in Kaiser's glycerol jelly.

Immunohistochemical staining

Immunohistochemical staining for biglycan, dentin sialoprotein (DSP), PTH receptor (PTHrP), and insulin-like growth factor 1 (IGF1) using the avidin–biotin–peroxidase complex technique with affinity-purified rabbit anti-mouse biglycan (LF-106) antibody (courtesy of Dr L W Fisher, NIDCR, NIH, Bethesda, MD, USA), affinity-purified rabbit anti-mouse DSP (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), affinity-purified mouse anti-PTH/PTH related protein receptor (Clone 3D1.1, Upstate, Syracuse, NY, USA) and goat anti-mouse IGF1 antibody (Santa Cruz) was carried out as described previously (Bai *et al.* 2007). Briefly, dewaxed and rehydrated paraffin-embedded sections were incubated with methanol–hydrogen peroxide (1:10) to block endogenous peroxidase activity and then washed in Tris-buffered saline (pH 7.6). The slides were then incubated with the primary antibodies overnight at room temperature. After rinsing with Tris-buffered saline for 15 min, tissues were incubated with biotinylated secondary antibody (Sigma). Sections were then washed and incubated with the Vectastain ABC-AP reagent or the Vectastain Elite ABC reagent (Vector Laboratories, Burlingame, CA, USA) for 45 min. After washing, red pigmentation to demarcate regions of immunostaining was produced by a 10- to 15-min treatment with Fast Red TR/Naphthol AS-MX phosphate (containing 1 mM levamisole as endogenous alkaline phosphatase inhibitor; Sigma), or brown pigmentation was likewise produced using 3,3'-diaminobenzidine (2.5 mg/ml). After washing with distilled water, the sections were counterstained with methyl green, and mounted with Kaiser's glycerol jelly or with Mayer's hematoxylin, dehydrated in graded ethanol and xylene, and mounted with Biomount medium.

Western blot analysis

Proteins were extracted from mandibles and long bones and quantitated using a commercial kit (Bio-Rad). Thirty micrograms protein samples were fractionated by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblotting was carried out as described (Xue *et al.* 2005) using antibodies against PTH/PTHrP receptor (Clone 3D1.1,

Upstate), IGF1 (Santa Cruz), and β -tubulin (Santa Cruz). Bands were visualized using ECL chemiluminescence (Amersham) and quantitated by Scion Image Beta 4.02 (Scion Corporation, NIH, MD, USA).

Computer-assisted image analysis

Histological images were photographed with a Sony digital camera. Images of micrographs from single sections were digitally recorded using a rectangular template, and recordings were processed and analyzed using Northern Eclipse image analysis software as described (Miao *et al.* 2001, 2002, Xue *et al.* 2006).

Statistical analysis

Data from image analysis are presented as means $\pm$ S.E.M. Statistical comparisons were made using Student's *t*-test, with $P < 0.05$ being considered significant.

Results

Effect of 1,25(OH)₂D deficiency on mineralization in teeth and mandibles

By the measurement of serum calcium, phosphorus, and PTH levels, we confirmed that $1\alpha(\text{OH})\text{ase}^{-/-}$ mice displayed hypocalcemia (Fig. 1A), hypophosphatemia (Fig. 1B), and hyperparathyroidism (Fig. 1C). To determine whether 1,25(OH)₂D₃ deficiency affects the mineralization of teeth and mandibles, the teeth and mandibles of 6-week-old wild-type and $1\alpha(\text{OH})\text{ase}^{-/-}$ mice were examined by radiography, micro-CT scanning, and von Kossa staining. As shown in Fig. 1, in $1\alpha(\text{OH})\text{ase}^{-/-}$ mice radiolucency was increased in all teeth including molars and incisors and in the mandible except for the mandibular condylar process compared with their wild-type littermates (Fig. 1D). Comparison between wild-type and $1\alpha(\text{OH})\text{ase}^{-/-}$ littermates of micro-CT scanned sections through the incisor before the first molar, and through the first, second, and third molars showed that the mineralized tooth volume in incisor and molars and the mineralized cortical and alveolar bone volume in mandibles were decreased in $1\alpha(\text{OH})\text{ase}^{-/-}$ mice (Fig. 1E). The mineralization defects in teeth and alveolar bone were also evident in $1\alpha(\text{OH})\text{ase}^{-/-}$ mice by von Kossa staining (Fig. 1F).

Distinctive effect of 1,25(OH)₂D deficiency on trabecular bone volume in long bones and on tooth volume and cortical and alveolar bone volume in mandibles

Our previous study demonstrated that trabecular volume and osteoblast number were increased significantly in the $1\alpha(\text{OH})\text{ase}^{-/-}$ mice at 7 weeks of age (Panda *et al.* 2001) and at 4 months of age even on a high calcium diet containing 1.5% calcium in the drinking water (Panda *et al.* 2004). In this

Figure 1 Effect of 1,25(OH)₂D deficiency on the mineralization of teeth and mandibles. (A) Serum calcium, (B) phosphorus, and (C) PTH. (D) Representative contact radiographs of the mandibles from 6-week-old wild-type (WT) and 1α(OH)ase^{-/-} mice. (E) Representative micro-CT scanned sections through the incisor before the first molar (In), and through the first (1st), second (2nd) and third (3rd) molars from wild-type and 1α(OH)ase^{-/-} mice. (F) Representative micrographs from undecalcified sections through the first molars stained by the von Kossa procedure as described in Materials and Methods and photographed. Scale bar represents 50 μm. ***P*<0.01; ****P*<0.001 relative to the wild-type mice.

study, we confirmed that trabecular volume was increased significantly in the 1α(OH)ase^{-/-} mice at 6 weeks of age (Fig. 2A and B). To determine whether 1,25(OH)₂D₃ deficiency affects the tooth volume and the cortical and alveolar bone volumes in mandibles, paraffin sections through the first molars were stained with H&E (Fig. 2C) and histochemically for total collagen (Fig. 2D). The root wall thickness of the first molars and incisors and the cortical thickness and dental alveolar bone volume were determined by computer-assisted image analysis. Total (mineralized and unmineralized) dental volume and the cortical and alveolar bone volumes in mandibles were decreased in 1α(OH)ase^{-/-} mice compared with their wild-type littermates (Fig. 2C and D). Quantitative data showed that the root wall thickness of the first molars (Fig. 2E) and incisors (Fig. 2F), the cortical

thickness (Fig. 2G) and alveolar bone volume (Fig. 2H) were all decreased significantly in 1α(OH)ase^{-/-} mice compared with their wild-type littermates.

Effect of 1,25(OH)₂D deficiency on predentin maturation and dentin formation

To determine whether the decreased tooth mineralization and volume were associated with predentin maturation and dentin

Figure 2 Distinctive effect of 1,25(OH)₂D deficiency on tooth volume and on cortical and alveolar bone volume in mandibles. (A) Representative micrographs of decalcified paraffin-embedded sections of proximal ends of tibiae from 6-week-old wild-type (WT) and 1α(OH)ase^{-/-} mice stained with hematoxylin and eosin (H&E). (B) Trabecular bone volume relative to tissue volume (BV/TV, %). Representative micrographs of decalcified paraffin-embedded sections through the first molars from 6-week-old wild-type and 1α(OH)ase^{-/-} mice stained (C) with H&E, and (D) with serious red for total collagen as described in Materials and Methods. Scale bars represent 100 μm. (E) The root wall thickness of the first molars, (F) the root wall thickness of incisors, (G) the cortical thickness, and (H) alveolar bone volume of the mandibles were measured. Each value is the mean ± S.E.M. of determinations in six animals of each group. **P*<0.05; ***P*<0.01; ****P*<0.001 relative to the wild-type mice.

Figure 3 Effect of 1,25(OH)₂D deficiency on predentin maturation and dentin formation. Paraffin-embedded sections through the first molars and the incisors from 6-week-old wild-type (WT) and 1α(OH)ase^{-/-} mice stained with H&E (A and C), immunohistochemically for biglycan (E and G) and dentin sialoprotein (DSP, I) and photographed. Scale bars represent 50 μm. Representative H&E staining of micrographs of (A) the root walls of the first molars and (C) the incisors. Quantitative thickness of predentin in the root walls of (B) the first molars and (D) the incisors. Representative micrographs of the root wall of (E) the first molars and (G) the incisors stained immunohistochemically for biglycan, and (I) the root wall of the first molars stained immunohistochemically for DSP. Quantitative biglycan immunopositive areas in (F) the first molars and (H) the incisors. (J) Quantitative DSP immunopositive areas in the first molars. Each value is the mean ± S.E.M. of determinations in six animals of each group. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 relative to the wild-type mice.

formation, the thickness of predentin in the first molars and incisors, the area of reparative dentin, and the immunoreactivity of DSP in the first molar were assessed by histology and immunohistochemistry. As seen in H&E sections, the

thickness of predentin was increased significantly in the first molars (Fig. 3A and B) and incisors (Fig. 3C and D) in $1\alpha(\text{OH})\text{ase}^{-/-}$ mice compared with their wild-type littermates. Positive immunoreactivity for biglycan was detected in

the region of the predentin (Fig. 3E and G). The biglycan-positive area was increased significantly in the first molars (Fig. 3E and F) and incisors (Fig. 3G and H) in $1\alpha(\text{OH})\text{ase}^{-/-}$ mice compared with their wild-type littermates. Positive immunoreactivity for DSP was detected in the predentin and dentin in the molars (Fig. 3I). The DSP-positive area was decreased dramatically in the first molars in $1\alpha(\text{OH})\text{ase}^{-/-}$ mice compared with their wild-type littermates (Fig. 3I and J).

Distinctive effect of $1,25(\text{OH})_2\text{D}$ deficiency on osteoblastic bone formation in the mandibles and long bones

We previously reported that the increased trabecular bone volume was associated with the increased osteoblast number in the $1\alpha(\text{OH})\text{ase}^{-/-}$ mice with secondary hyperparathyroidism (Panda *et al.* 2001, 2004). To determine whether decreased cortical and alveolar bone volume was associated with diminished osteoblastic bone formation in the mandibles, the osteoblast number and surface were measured on the H&E-stained dental alveolar bone by histomorphometric analyses. Results showed that the osteoblast number and surface were reduced in the dental alveolar bone of mandibles from $1\alpha(\text{OH})\text{ase}^{-/-}$ mice (Fig. 4A, D and E). We also confirmed that the osteoblast number and surface were increased in the trabecular bone of tibiae from $1\alpha(\text{OH})\text{ase}^{-/-}$ mice (Fig. 4A, D and E). The increased trabecular bone volume and osteoblast numbers in long bones in the $1\alpha(\text{OH})\text{ase}^{-/-}$ mice, which have been shown to result from elevations in circulating PTH (Panda *et al.* 2004), may be mediated by IGF1; therefore, we examined the expression of PTHR and IGF1 in the dental alveolar bone of mandibles and in the trabecular bone of tibiae. Positive immunoreactivity for PTHR (Fig. 4B) and IGF1 (Fig. 4C) was detected in the osteoblasts, and the PTHR- and IGF1-positive areas (Fig. 4F and G respectively) were slightly decreased in the dental alveolar bone of the mandibles, but increased significantly in the trabecular bone of tibiae in $1\alpha(\text{OH})\text{ase}^{-/-}$ mice compared with their wild-type littermates. These alterations in PTHR and IGF1 expression in the mandibles and long bones were confirmed by western blots (Fig. 4H–J). To assess whether the decreased dental alveolar bone volume was associated with increased osteoclastic bone resorption, the

paraffin sections were stained histochemically for TRAP, and the TRAP-positive osteoclast number and surface were measured by histomorphometric analyses. The data showed that the TRAP-positive osteoclast number and surface were decreased in the dental alveolar bone in $1\alpha(\text{OH})\text{ase}^{-/-}$ mice compared with their wild-type littermates (Fig. 4K–M).

Distinctive effect of PTH deficiency on trabecular bone volume in long bones and on tooth volume and cortical and alveolar bone volume in mandibles

Our previous study demonstrated that trabecular volume was increased dramatically in the $\text{PTH}^{-/-}$ mice at 4 months of age due to reduced bone turnover (Miao *et al.* 2004). In this study, we confirmed that trabecular volume was increased significantly in the $\text{PTH}^{-/-}$ mice at 6 weeks of age (Fig. 2A and B), although trabecular bone volume was not increased as dramatically as at 4 months of age. To determine whether PTH deficiency affects the tooth volume and the cortical and alveolar bone volume in mandibles, paraffin sections through the first molars of 6-week-old $\text{PTH}^{-/-}$ mice were stained with H&E (Fig. 5C) and histochemically for total collagen (Fig. 5D). The root wall thickness of the first molars and incisors and the cortical thickness and dental alveolar bone volume were determined by computer-assisted image analysis. Total (mineralized and unmineralized) dental volume and the cortical and alveolar bone volume in mandibles were not altered significantly in $\text{PTH}^{-/-}$ mice compared with their wild-type littermates (Fig. 5C and D). Quantitative data showed that the root wall thickness of the first molars (Fig. 5E) and incisors (Fig. 5F), the cortical thickness (Fig. 5G), and alveolar bone volume (Fig. 5H) were all not altered significantly in $\text{PTH}^{-/-}$ mice compared with their wild-type littermates.

Discussion

Our previous study demonstrated that trabecular volume, osteoblast number, and osteoid volume were all increased significantly in the $1\alpha(\text{OH})\text{ase}^{-/-}$ mice at 7 weeks of age (Panda *et al.* 2001) and at 4 months of age even on a high calcium diet containing 1.5% calcium in drinking water

Figure 4 Distinctive effects of the $1,25(\text{OH})_2\text{D}$ deficient state on osteoblastic bone formation in the mandibles and long bones. Representative micrographs of paraffin-embedded sections of mandibles and tibiae from 6-week-old wild-type (WT) and $1\alpha(\text{OH})\text{ase}^{-/-}$ mice stained with H&E (A), immunohistochemically for PTHR (B) and IGF1 (C) and photographed. Scale bars represent 50 μm . (D) Number of osteoblasts per mm bone parameter (N.Ob/B.Pm, #/mm) and (E) the surface of osteoblasts relative to the bone surface (Ob.S/B.S, %) were determined in the dental alveolar bone and the metaphyseal regions of H&E-stained mandibles and tibiae respectively. (F) PTHR and (G) IGF1 immunopositive areas as a percent of the tissue areas were determined in the dental alveolar bone and the metaphyseal regions of H&E-stained mandibles and tibiae respectively. (H) Western blots of mandible and long bone extracts for expression of PTHR and IGF1. β -Tubulin was used as loading control for western blots. (I) PTHR and (J) IGF1 protein levels relative to β -tubulin protein level were assessed by densitometric analysis and expressed relative to levels of mandibles of the wild-type mice. (K) Representative micrographs of paraffin-embedded sections of mandibles stained histochemically for tartrate-resistant acid phosphatase activity (TRAP) and photographed. Scale bars represent 50 μm . (L) Number of TRAP-positive osteoclasts per mm bone parameter (N.Oc/B.Pm, #/mm) and (M) the surface of osteoclasts relative to the bone surface (Oc.S/B.S, %) were determined in the dental alveolar bone of H&E-stained mandibles. Each value is the mean $\pm$ S.E.M. of determinations in six animals of each group. * $P < 0.05$ relative to the wild-type mice.

Figure 5 Effect of PTH deficiency on tooth volume and on cortical and alveolar bone volume in mandibles. (A) Representative micrographs of decalcified paraffin-embedded sections of proximal ends of tibiae from 6-week-old wild-type (WT) and PTH^{-/-} mice stained with hematoxylin and eosin (H&E). (B) Trabecular bone volume relative to tissue volume (BV/TV, %). Representative micrographs of decalcified paraffin-embedded sections through the first molars from 6-week-old WT and PTH^{-/-} mice stained (C) with HE, and (D) with serious red for total collagen as described in Materials and Methods. Scale bars represent 100 μm. (E) The root wall thickness of the first molars, (F) the root wall thickness of incisors, (G) the cortical thickness, and (H) alveolar bone volume of the mandibles were measured. Each value is the mean ± S.E.M. of determinations in six animals of each group. ***P* < 0.01 relative to the wild-type mice.

(Panda *et al.* 2004). In the present study, we examined the specific effect of 1,25(OH)₂D deficiency on the dentin and dental alveolar bone formation and mineralization in the mandibles and compared the osteoblastic bone formation in mandibles with that in tibiae from 6-week-old 1α(OH)ase^{-/-} mice. Our data confirmed that the hypomineralization in both teeth and mandibles was consistent with that in long bones in the 1α(OH)ase^{-/-} mice after weaning (Dardenne *et al.* 2001, Panda *et al.* 2001, 2004). In contrast to long bones with increased trabecular bone volume and

osteoblast number, however, the dental volume and dental alveolar bone volume were reduced significantly in 6-week-old 1α(OH)ase^{-/-} mice. These results indicate that the bone formation processes in dentin and dental alveolar bone of the mandibles are distinctly regulated in the 1,25(OH)₂D₃-deficient state relative to the processes driving formation in trabecular bone in long bones.

Skeletal mineralization defects have been observed in both VDDR-I patients (Fraser *et al.* 1973, Vasilakis *et al.* 1980) and vitamin D-deficient experimental animals (Dardenne *et al.* 2001, Panda *et al.* 2001, 2004). The dental manifestations of VDDR-I have not been reported in detail until recently (Zambrano *et al.* 2003), and the hard dental tissue alterations of 1α(OH)ase^{-/-} mice have not been previously described in the literature. In the reported case of VDDR-I, hypomineralization of teeth was observed by radiographs (Zambrano *et al.* 2003). In the present study in 1α(OH)ase^{-/-} mice, we detected decreased mineral density in teeth and mandibles by radiography and by micro-CT scanning, and increased unmineralized dentin and unmineralized bone matrix in dental alveolar bone by von Kossa staining. Furthermore, we observed an increase in biglycan immunopositive area in 1α(OH)ase^{-/-} mice consistent with increased predentin thickness. Our results therefore suggest that the mineralization defects in teeth and mandibles caused by 1,25(OH)₂D₃ deficiency are similar to those that occur in long bones. The mineralization defect and alterations of the predentin and biglycan protein expression in teeth of the 1α(OH)ase^{-/-} mice found here were comparable to those observed in vitamin D receptor (VDR) gene knockout mice (Lezot *et al.* 2002, Davideau *et al.* 2004, Zhang *et al.* 2007). Although these findings suggest that VDR-mediated actions of 1,25(OH)₂D₃ play an essential role in dental mineralization, several studies indicate that the principal function of vitamin D in mineralization of bone is to provide adequate calcium and phosphorus in the extracellular fluid space. Thus, when vitamin D-deficient rats have been infused with high calcium and high phosphorus for several days, it was found that the bones had little evidence of rickets (Holick *et al.* 1992). Similarly, when vitamin D-deficient rats were fed a high calcium, high phosphorus diet, histological studies of the skeleton did not reveal any skeletal abnormalities consistent with either osteomalacia or rickets (Holtrop *et al.* 1986). When a child with severe rickets caused by the rare hereditary disease VDDR-II (also known as hereditary resistance to 1,25(OH)₂D), which is caused by loss-of-function mutations in the VDR, was infused with calcium for 7 months, her skeleton began to mineralize normally (Balsan *et al.* 1986). Our recent studies showed that mineralization of both cartilage and bone was severely impaired in the 1α(OH)ase^{-/-}, VDR^{-/-}, and 1α(OH)ase^{-/-}VDR^{-/-} mice that were hypocalcemic on either a lactose-free normal diet or a high calcium diet (Panda *et al.* 2001, 2004). However, cartilage and bone mineralization were normalized in all mutants when hypocalcemia was eliminated by a high lactose, high calcium, high phosphorus 'rescue' diet

(Panda *et al.* 2004). Consequently, the major determinant of mineralization of both bones and teeth appears to be the ambient concentration of extracellular calcium and phosphate, and the $1,25(\text{OH})_2\text{D}/\text{VDR}$ system appears to play no direct role in this process.

In contrast to consistency in the mode of regulation of mineralization of bones and teeth, our studies in adult $1\alpha(\text{OH})\text{ase}^{-/-}$ mice show discordance between the regulation of dental volume and dental alveolar bone volume in mandibles versus trabecular volume in long bones. Our previous study showed that trabecular volume and osteoblast numbers in long bones were increased significantly in $1\alpha(\text{OH})\text{ase}^{-/-}$ mice on a normal or even on a high calcium intake (Panda *et al.* 2004). This increase disappeared when elevated concentrations of circulating PTH were normalized and is therefore attributable to the anabolic effect of endogenous PTH. However, in the present study, we found that the dental volume, the reparative dentin volume, and DSP immunopositive area were all reduced below wild-type levels, even though endogenous PTH levels are highly elevated in the $1\alpha(\text{OH})\text{ase}^{-/-}$ mice on the high calcium intake. The cortical thickness, the dental alveolar bone volume, and osteoblast numbers were also all decreased significantly in the mandibles of $1\alpha(\text{OH})\text{ase}^{-/-}$ mice. We showed previously that trabecular volume and osteoblasts were reduced in long bones of 2-week-old $1\alpha(\text{OH})\text{ase}^{-/-}$ mice (Xue *et al.* 2005) and of 4-month-old $1\alpha(\text{OH})\text{ase}^{-/-}$ mice on a rescue diet when the serum calcium, phosphorus, and PTH were normalized (Panda *et al.* 2004). Therefore, $1,25(\text{OH})_2\text{D}$ appears to exert an anabolic effect, which is necessary to sustain basal bone-forming activity in long bones. We also demonstrated previously that exogenous $1,25(\text{OH})_2\text{D}_3$ increased both trabecular and cortical bone volume, augmented both osteoblast number and type I collagen deposition in bone matrix, and up-regulated expression levels of the osteoblastic genes including alkaline phosphatase, type I collagen, and osteocalcin (Xue *et al.* 2006). Consequently, the reduction in dental volume and dental alveolar bone below wild-type levels in the present study, even in the presence of secondary hyperparathyroidism is consistent with the absence of this bone anabolic effect of $1,25(\text{OH})_2\text{D}$. During dentin formation, VDR is mainly present in subodontoblastic cells (Davideau *et al.* 1996). However, VDR expression in odontoblasts and ameloblasts, cells involved in matrix deposition and mineralization, appears to be induced by $1,25(\text{OH})_2\text{D}$ injection (Davideau *et al.* 1996), suggesting that these cells are target cells for $1,25(\text{OH})_2\text{D}$. This is in keeping with a role for $1,25(\text{OH})_2\text{D}$ in dental homeostasis and consistent with our finding that $1,25(\text{OH})_2\text{D}$ is required for anabolic activity in dental and dental alveolar bone as well as in long bones.

The question raised in the present study is why the trabecular bone volume and osteoblast numbers are markedly increased in long bones (Dardenne *et al.* 2001, Panda *et al.* 2001, 2004), but not in mandibles of the $1\alpha(\text{OH})\text{ase}^{-/-}$ mice with increased circulating PTH. We previously

compared the relative contributions of PTH and $1,25(\text{OH})_2\text{D}$ to bone anabolism by analyzing and comparing the skeletal phenotypes of $1\alpha(\text{OH})\text{ase}^{-/-}$ mice and $\text{PTH}^{-/-}$ mice (Xue *et al.* 2005). Reduced osteoblastic bone formation in the metaphyseal region was seen in both mutants; however, PTH deficiency caused a more marked reduction, whereas $1\alpha(\text{OH})\text{ase}$ ablation caused a smaller reduction in trabecular bone volume. Therefore, although both $1,25(\text{OH})_2\text{D}$ and PTH can affect trabecular bone growth, PTH plays a greater role in appositional bone formation than $1,25(\text{OH})_2\text{D}$. Consequently, the secondary hyperparathyroidism in hypocalcemic $1\alpha(\text{OH})\text{ase}^{-/-}$ mice is in keeping with this anabolic effect of PTH to increase trabecular bone volume and osteoblast numbers in long bones. Nevertheless, this did not explain the absence in our present studies of an anabolic effect of high circulating PTH in teeth and mandibles.

The results of previous reports on the action of PTH on dentin formation are inconsistent. One previous report showed that PTH stimulated dentin apposition in the thyroparathyroidectomized rat in a dose-dependent manner (Turnbull *et al.* 1983), but another study showed that intermittent PTH treatment had no effect on dentin formation in aged ovariectomized rats (Miller *et al.* 1997). Although intermittent PTH treatment was shown to stimulate mandibular and alveolar crest bone formation in aged ovariectomized rats (Miller *et al.* 1997), the increased mineral apposition rate was much less in mandibles than in long bones (Hunziker *et al.* 2000). Therefore, these findings suggest that the sensitivity of skeletal tissues to PTH may vary according to their embryological origin (Yonaga 1978). The mandibular condylar process is derived from endochondral bone formation, whereas the mandibular body develops by intramembranous bone formation (Lee *et al.* 2001). Our data showed that the bone mineral density was decreased in mandibular bodies, but not in the mandibular condylar process in the $1\alpha(\text{OH})\text{ase}^{-/-}$ mice consistent with an effect of PTH on bone derived by endochondral ossification but not on bone of intramembranous origin. The dental alveolar bone volume and osteoblast numbers were decreased in mandibles of the $1,25(\text{OH})_2\text{D}$ deficient animals, whereas the trabecular bone volume and osteoblast number were increased in long bones in the same $1\alpha(\text{OH})\text{ase}^{-/-}$ mice with secondary hyperparathyroidism. In the present study, we confirmed that trabecular bone volume was increased significantly in long bones in PTH-deficient mice at 6 weeks of age and demonstrated that the dental and dental alveolar bone volumes in mandibles were not altered in PTH-deficient mice. These results therefore indicate that the mandibles are at least less sensitive to the anabolic actions of increased endogenous PTH than are long bones, and that $1,25(\text{OH})_2\text{D}_3$ plays a greater role in mandibular bone formation than PTH.

Given that PTH has a direct effect on the activity of the osteoblast, a cell known to have the PTHR (Halloran *et al.* 1997), and that IGF1 is required for the skeletal anabolic

actions of PTH (Bikle *et al.* 2002, Yamaguchi *et al.* 2005, Wang *et al.* 2007), we examined the expression of PTHR and IGF1 in the mandibles and long bones by immunohistochemistry and western blots. Our results revealed that the levels of PTHR and IGF1 expressed in osteoblasts were reduced slightly in the dental alveolar bone of mandibles, but were enhanced in the trabecular bone of tibiae in the adult $1\alpha(\text{OH})\text{ase}^{-/-}$ mice. These alterations in PTHR and IGF1 between mandibles and long bones were confirmed by western blots. These findings suggest that the differential sensitivity of the mandibles and long bones to PTH may be associated with differential expression of PTHR and subsequent regulation of IGF1 by PTH.

In summary, our study suggests that endogenous $1,25(\text{OH})_2\text{D}$ plays an anabolic role in dentin formation and dental alveolar bone formation in mandibles as it does in long bones, whereas PTH exerts an anabolic role predominantly in bone derived by endochondral ossification. This selective role of PTH may be contributed to by site-specific differences in PTHR and IGF1 expression.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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