0013-7227/99/$03.00/0
Endocrinology

Vol. 140, No. 11
Printed in U.S.A.

Rescue of the Skeletal Phenotype of Vitamin D Receptor-Ablated Mice in the Setting of Normal Mineral Ion Homeostasis: Formal Histomorphometric and Biomechanical Analyses*

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ABSTRACT

1,25-Dihydroxyvitamin D_3 has been shown to play an important role *in vitro* in regulating osteoblast gene transcription and promoting osteoclast differentiation. To address the role of the vitamin D receptor (VDR) in skeletal homeostasis, formal histomorphometric analyses were performed in VDR null mice in the setting of impaired mineral ion homeostasis as well as in VDR null mice in whom normal mineral ion homeostasis had been preserved. In hypocalcemic VDR null mice, there was an increase in bone volume as a result of a dramatic increase in osteoid. There was also an increase in the number of osteoblasts without a significant change in the number of osteoclasts. Examination of the growth plate revealed marked disorganization, with an increase in vascularity and matrix. Biomechanical parameters demonstrated increased bone fragility in the hypocalcemic VDR null mice.

In the VDR ablated mice in whom normal mineral ion homeostasis had been preserved, none of these measurements was significantly different from those in wild-type littermates raised under identical conditions. Notably, the morphology and width of the growth plate were indistinguishable from those in wild-type controls, demonstrating that a calcium/phosphorus/lactose-enriched diet started at 16 days of age in the VDR null mice permits the development of both normal morphology in the growth cartilage and adjacent metaphysis and normal biomechanical competence of cortical bone. Thus, the principle action of the VDR in skeletal growth, maturation, and remodeling is its role in intestinal calcium absorption. The skeletal consequences of VDR ablation are a result of impaired intestinal calcium absorption and/or the resultant secondary hyperparathyroidism and hypophosphatemia. (*Endocrinology* **140:** 4982–4987, 1999)

INVESTIGATIONS directed at clarifying the role of 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] on bone have been complicated by an inability to dissociate the effects of impaired mineral ion homeostasis and of vitamin D deficiency. The vitamin D receptor (VDR) has been shown to be present in the fetal rat at the time of mesenchymal condensation of skeletal tissues (1); however, there has been no critical role ascribed to 1,25-$(OH)_2D_3$ in the development and maturation of normal bone *in vivo*. It has been demonstrated that calcium and phosphate infusions can heal osteomalacic lesions in vitamin D-deficient animals (2). The calcium and phosphate infusions not only normalize mineralization lag times, but also are associated with an increase in trabecular bone volume, the molecular basis of which remains unclear (3). Healing of osteomalacic lesions with parenteral calcium has also been observed in humans with VDR mutations (4). These observations present a paradox between the seemingly inconsequential effects of vitamin D deficiency on bone *in vivo* and the important effects of 1,25-$(OH)_2D_3$ on osteoblast and osteoclast function *in vitro*. Mature osteoblasts have VDRs, and although investigations performed in vitamin D-deficient rats have failed to show significant differences in the bone content of several matrix proteins, including osteocalcin, relative to those in normal rats (5), *in vitro* studies have demonstrated that the transcription of the genes encoding several matrix proteins is regulated by 1,25-$(OH)_2D_3$. 1,25-$(OH)_2D_3$ has been shown to down-regulate the transcription of α1(I) collagen by osteoblasts (6). 1,25-$(OH)_2D_3$ is a major stimulus *in vitro* for the transcription of the gene encoding human and rat osteocalcin; however, it has been shown to inhibit the expression of the mouse genes (7). 1,25-$(OH)_2D_3$ induces the transcription of genes encoding other matrix proteins, including osteopontin (8).

1,25-$(OH)_2D_3$ is thought to play an important role in stimulating the differentiation of osteoclasts from monocyte-macrophage stem cell precursors *in vitro* (9) and is a key regulator of osteoclast differentiation factor expression (10). The effects of 1,25-$(OH)_2D_3$ deficiency on osteoclast number and function *in vivo* remain unclear. In vitamin D-deficient rats cured of their osteomalacic lesions by calcium and phosphate infusions, there was no significant decrease in osteoclast number relative to that in controls (3). However, decreases in osteoclast number and activity have been observed in osteomalacic vitamin D-deficient animals (11), suggesting that,

Received April 8, 1999.
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* This work was supported by Grant DK-46974 (to M.B.D.) and DE-04724 (to R.B.).

by direct or indirect mechanisms, vitamin D deficiency (or hypocalcemia and hypophosphatemia) may impair osteoclastic bone resorption.

In addition to bony abnormalities, rachitic changes are seen in vitamin D deficiency. These disorganized growth plates are also observed in hypophosphatemic rickets, where affected individuals are normocalcemic but are thought to have inappropriately low serum levels of 1,25-$(OH)_2D_3$. To what extent the abnormal organization observed in the growth plates is a reflection of hypophosphatemia, hypocalcemia, secondary hyperparathyroidism, or vitamin D deficiency has not been clarified.

Investigations in normocalcemic mice lacking functional VDRs were undertaken to determine which *in vivo* effects of 1,25-$(OH)_2D_3$ on the skeleton were a consequence of impaired mineral ion homeostasis and which were secondary to the lack of nuclear actions of 1,25-$(OH)_2D_3$.

Materials and Methods

Animal maintenance

All studies performed were approved by the institutional animal care committee. VDR null mice (12) and control littermates were maintained in a virus- and parasite-free barrier facility and exposed to a 12-h light, 12-h dark cycle. The mice were fed autoclaved Purina rodent chow (5010, Ralston Purina Co., St. Louis, MO) containing 1% calcium, 0.67% phosphorus, 0% lactose, and 4.4 IU vitamin D/g (regular diet). To normalize the blood mineral ion levels of the VDR ablated mice (13), the animals were fed a γ-irradiated rescue chow (TD96348, Teklad, Madison, WI) containing 2% calcium, 1.25% phosphorus, and 20% lactose with 2.2 IU vitamin D/g from 16 days of age. This resulted in serum ionized calcium levels, phosphorus levels, and immunoreactive PTH levels in the VDR null mice (1.29 ± 0.02 mmol/liter, 8.5 ± 0.8 mg/dl, and 43.0 ± 4.9 pg/ml, respectively, at 70 days) that were indistinguishable from those in wild-type littermates fed the same diet (1.27 ± 0.01 mmol/liter, 8.3 ± 0.9 mg/dl, and 39.4 ± 9.3 pg/ml, respectively, at 70 days) (13). At 60 and 67 days of age, wild-type and homozygous VDR ablated male littermates were injected ip with 30 μg calcein/g BW.

Sample preparation

Male mice were killed at 70 days of age. After whole animal contact radiography (Faxitron, Phillips, Germany) and autopsy, bones were dissected out and fixed in 3.7% PBS-buffered formaldehyde for 18 h at 4 C. After dehydration, the undecalcified tibiae were embedded in methylmethacrylate, and 5-μm sections were cut in the sagittal plane on a rotation microtome (Cut 4060E, MicroTech, Munich, Germany) as previously described (14, 15). Sections were stained with toluidine blue and modified von Kossa or Goldner Trichrome and evaluated using a Carl Zeiss microscope (Carl Zeiss, Jena, Germany). For assessment of dynamic histomorphometric parameters, 12-μm thick sections were mounted unstained in Fluoromount (Electron Microscopy Sciences, Fort Washington, PA) to permit evaluation by fluorescent microscopy.

Histomorphometry

Quantitative histomorphometry was performed on toluidine blue-stained, undecalcified, proximal tibial sections. Experiments were performed in a blinded fashion. The sampling site was the metaphysis, starting 0.25 mm distal to the growth plate. For comparative histomorphometry, samples from 10 wild-type and 10 $VDR^{-/-}$ males were used: 5 males of each genotype fed regular chow and the same number of mice fed the rescue chow. Analysis of bone volume (percentage), osteoid volume (percentage), osteoid surface (percentage), osteoid thickness (microns), trabecular thickness (microns), trabecular number (per mm), trabecular separation (microns), osteoblast surface per bone surface (percentage), osteoblast number per bone perimeter (per mm), osteoclast surface per bone surface (percentage), osteoclast number per bone perimeter (per mm), and growth plate thickness (microns) was carried out according to standardized protocols (16) using the Osteomeasure histomorphometry system (Osteometrix, Atlanta, GA). For assessment of dynamic histomorphometric indexes, mice were injected with calcein according to a standard double labeling protocol (17). Fluorochrome measurements were made on two nonconsecutive 12-μm thick unstained sections per animal. Growth plate thickness was assessed by measuring the mean width of the entire growth plate, including resting, proliferating, and hypertrophic chondrocytes, in the longitudinal axis of the bone. Statistical analysis was performed using Student's t test, $P < 0.05$ was accepted as significant; *error bars* represent the SEM.

Biomechanical testing

Both femurs were dissected free of soft tissue and stored in 50% ethanol-saline. They were transferred to isotonic saline and stored at 4 C for 12 h before testing. A three-point bending test was performed as previously described (18, 19), using a commercial high precision instrument (Z2.5/TN 1S testing machine, Zwick GmbH & Co., Ulm, Germany). In brief, the ends of the bone were supported on two fulcra separated by 5 mm. With the posterior aspect of the femur resting on the fulcra, a load was applied from above to the anterior midshaft midway between the two fulcra, at a constant speed of 10 mm/min to failure. A chart recorder was used to generate a force-deformation curve. The ultimate force (maximum load) and the ultimate deformation (maximum displacement) were determined directly from the curve. The stiffness was assessed as the slope of the force-deformation curve through its linear region. Experiments were performed in a blinded fashion.

Results

VDR null mice fed regular chow develop osteomalacia by 35 days of age (data not shown), and by 70 days of age, greater than 85% of their bone surface is covered with osteoid (Fig. 1A, osteoid surface/bone surface). At this time, their osteoid volume is 30-fold greater than that of control littermates fed the same diet (Fig. 1A, osteoid volume/bone volume). This increase in osteoid volume is associated with a 5-fold increase in bone volume (bone volume/tissue volume), resulting in a bone volume/tissue volume that is 145-fold elevated (26.21 ± 1.62 in VDR null mice *vs.* 0.18 ± 0.06 in wild-type mice). When VDR null mice and wild-type littermates are placed on the rescue diet at 16 days of age, before the development of impaired mineral ion homeostasis, no increased osteoid is seen in the knockout mice, and their bone volume (bone volume/tissue volume) is indistinguishable from that of wild-type littermates fed the same diet (Fig. 1B). The osteoid thickness is also normalized by the rescue diet. Of note, the 2-fold increase in trabecular thickness and trabecular number seen in the hypocalcemic knockout mice is normalized in the VDR ablated mice with normal mineral ion homeostasis (Fig. 1). The trabecular separation was decreased 5-fold in the hypocalcemic VDR ablated mice. Once again, this parameter was normalized by the rescue diet (Fig. 1, A and B).

To address whether the increase in bone volume and trabecular thickness was associated with altered cellular composition, osteoblast and osteoclast numbers were assessed in wild-type and VDR null littermates in the setting of normal and impaired mineral ion homeostasis. There was an increase in osteoblast number in the hypocalcemic VDR ablated mice that was normalized by the rescue diet (Fig. 2, A and B). There was a parallel increase in the surface of bone covered by osteoblasts (osteoblast surface/bone surface) in the hypocalcemic VDR ablated mice that was normalized by the rescue diet (Fig. 2). The change in the number of oste-

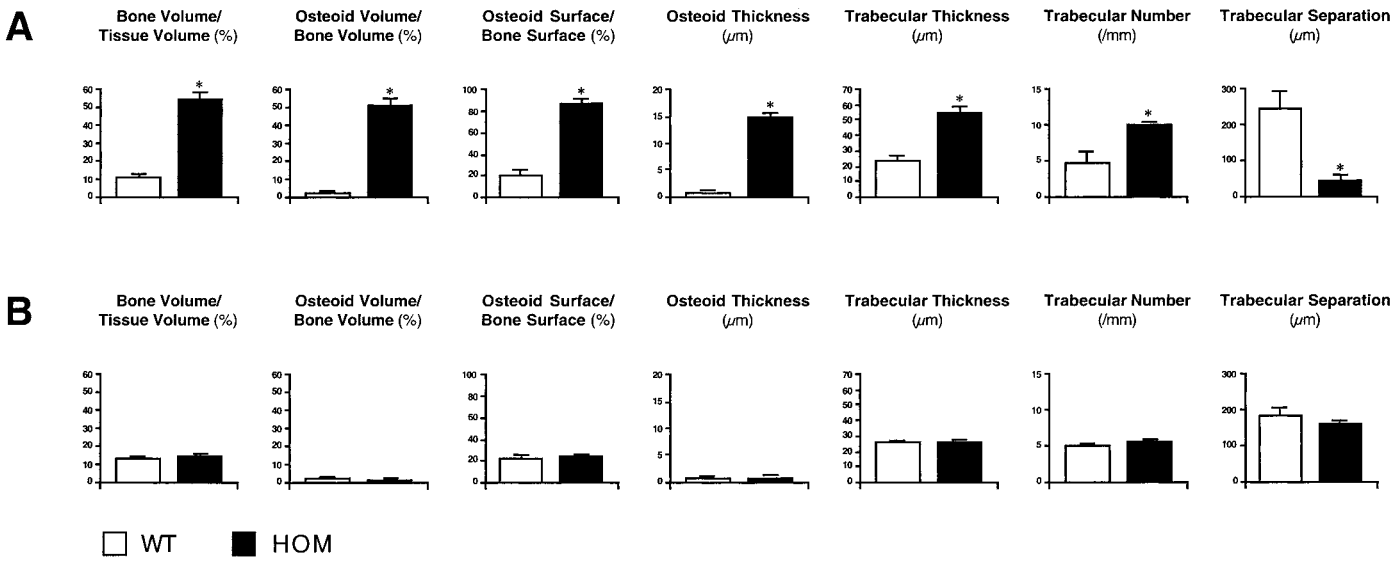

FIG. 1. A, There is a marked increase in bone volume, due to increased osteoid, in hypocalcemic VDR ablated mice. B, Prevention of abnormal mineral ion homeostasis results a normalization of these indexes. wt, Wild-type; hom, homozygous. *, $P < 0.05$. *Bars* represent the mean and SEM of determinations from five animals of each phenotype.

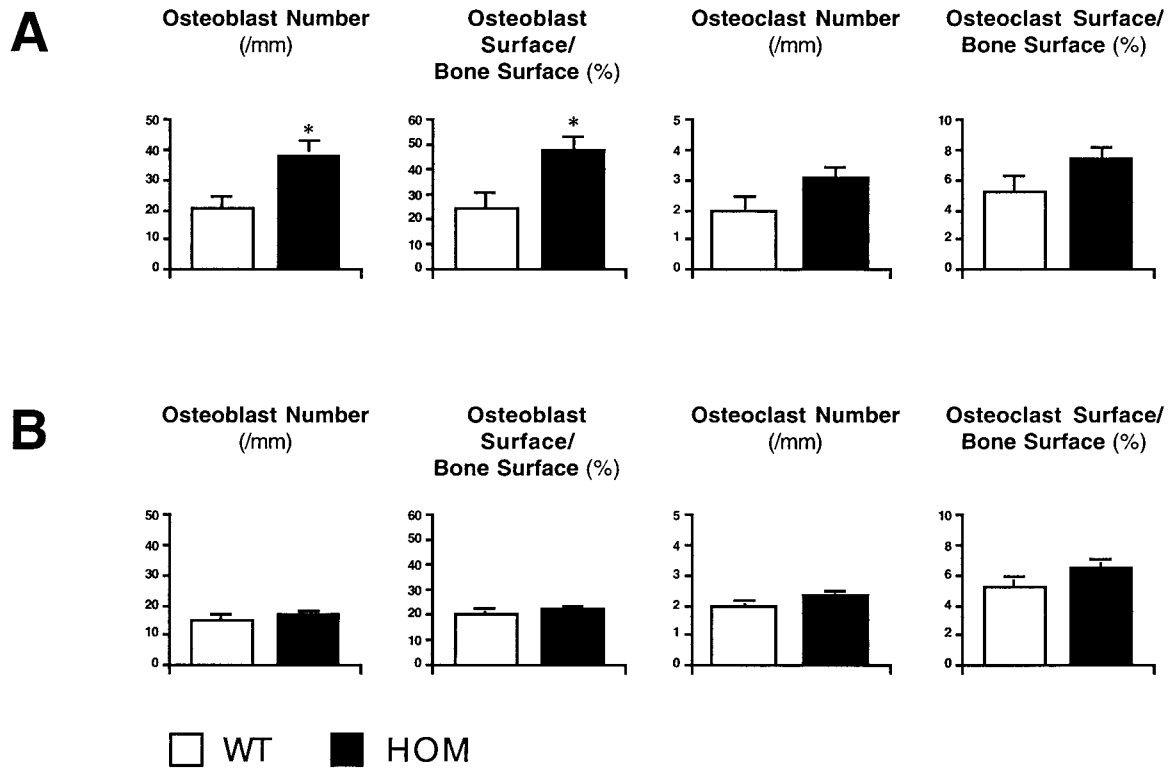

FIG. 2. A, There is an increase in osteoblast number in the hypocalcemic VDR ablated mice. These mice do not have a statistically significant increase in osteoclast number or surface. B, Prevention of abnormal mineral ion homeostasis results in normalization of osteoblast number and surface in the VDR ablated mice. wt, Wild-type; hom, homozygous. *, $P < 0.05$. *Bars* represent the mean and SEM of determinations from five animals of each phenotype.

oclasts and the surface of bone covered by osteoclasts (osteoclast surface/bone surface) was not statistically significant in the hypocalcemic or normocalcemic VDR ablated mice (Fig. 2).

Double calcein labeling was performed to assess the mineral apposition rate in the VDR ablated mice. As demonstrated in Fig. 3, no crisp labels were seen in the bones of the hypocalcemic knockout mice (Fig. 3C). The mineral apposition rates of the normocalcemic mice were identical regardless of genotype or diet (Fig. 3B, wild-type, regular diet; Fig. 3E, wild-type rescue diet; Fig. 3F, VDR null rescue diet).

To determine whether normalization of mineral ions in the

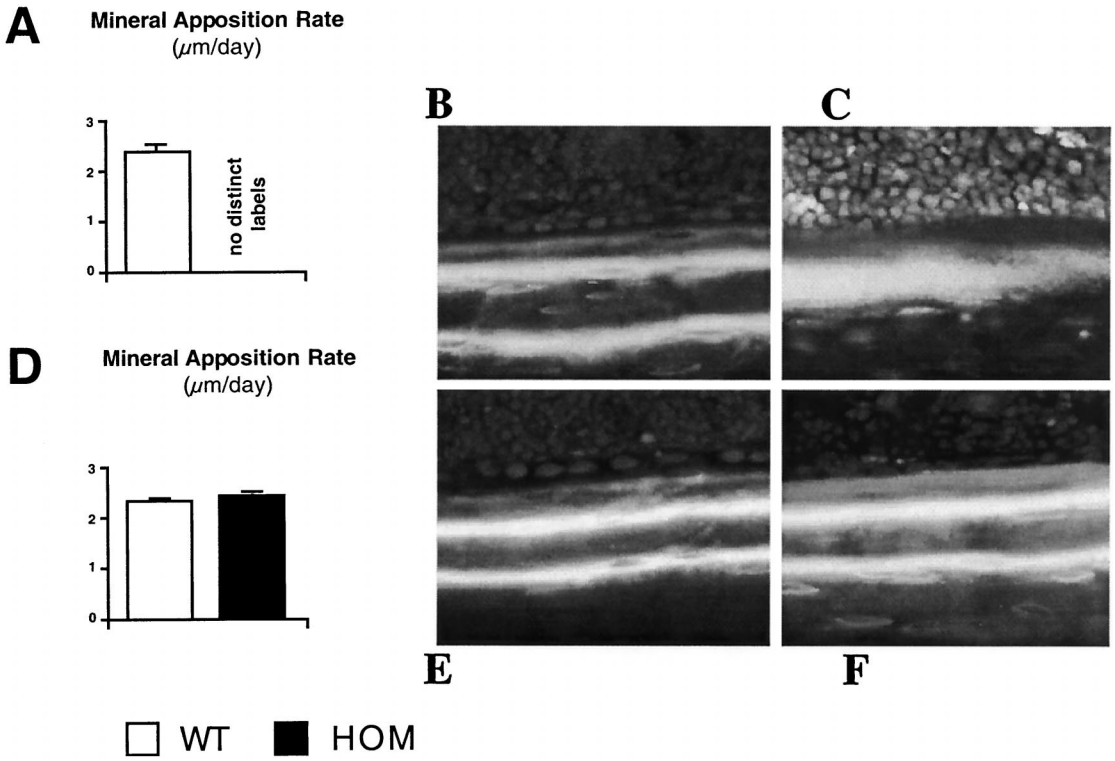

FIG. 3. Mineral apposition is markedly impaired in hypocalcemic VDR ablated mice. Mineral apposition rate was determined by the distance between the midpoint of two calcein labels injected 7 days apart. A–C, Hypocalcemic VDR ablated mice demonstrate a diffuse calcein label (A and C), indicating a disorderly deposition of mineral during the labeling interval. B, E, and F, The normocalcemic VDR ablated mice had a mineral apposition rate (MAR) indistinguishable from that of wild-type littermates fed either diet. Magnification, ×400. wt, Wild-type; hom, homozygous. *, $P < 0.05$. *Bars* represent the mean and SEM of determinations from five animals of each phenotype.

VDR null mice maintained biomechanical integrity, formal biomechanical analyses were performed. As shown in Fig. 4A, there was a dramatic decrease in ultimate load in the hypocalcemic VDR null mice. The stiffness of the bones from the hypocalcemic VDR null mice was reduced 7-fold, along with a 3-fold increase in ultimate deformation. The bones of the normocalcemic knockout mice, however, have biomechanical properties indistinguishable from those of control littermates fed the same diet (Fig. 4B).

Rickets is a classic feature of vitamin D deficiency in growing animals. Rachitic changes in the VDR knockout mice are seen as early as 24 days (data not shown), and by 70 days, there is a marked expansion and a 48% increase in the length of the growth plate (Fig. 5, A and C) associated with disorganization of the chondrocyte columns and increased matrix. Normalization of mineral ion homeostasis prevents growth plate abnormalities in the VDR ablated mice (Fig. 5, D and F), demonstrating that the nuclear VDR is not essential for the maintenance of a normal growth plate.

Discussion

1,25-$(OH)_2D_3$ is the major steroid hormone thought to play a role in the regulation of mineral ion homeostasis. Mice with targeted ablation of the nuclear VDR develop hypocalcemia, hyperparathyroidism, rickets, and osteomalacia (13) (20). Prevention of abnormal mineral ion homeostasis in the VDR ablated mice has permitted investigations aimed at clarifying the features of 1,25-$(OH)_2D_3$ deficiency or VDR deficiency that are a direct consequence of impaired receptor-dependent actions (13). The studies reported herein demonstrate that in the absence of a functional VDR, marked skeletal pathology is observed in association with disruption of mineral ion homeostasis. There is a severe impairment of bone mineralization, characterized by a dramatic increase in osteoid and impaired calcein deposition into newly formed bone. It is of interest that the VDR ablated mice have an increase in bone volume/tissue volume. The initial hypothesis proposed to explain this observation was that the increase in the bone matrix was a reflection of dysregulated matrix protein biosynthesis, perhaps reflecting lack of down-regulation of α1(I) collagen by the receptor-dependent actions of 1,25-$(OH)_2D_3$. Disorderly deposition of 1,25-$(OH)_2D_3$-regulated bone matrix proteins or abnormal ratios of these proteins could lead to abnormal modeling, matrix accumulation, and biomechanical fragility. However, this hypothesis was disproven by the studies of VDR ablated mice with normal mineral ion homeostasis. These mice have serum levels of calcium and phosphorus that are indistinguishable from those of control wild-type and heterozygous littermates fed the same diet. The normalization of bone volume by preventing osteomalacia was not predicted by the studies of Weinstein *et al.* (3). Their studies, however, were directed at curing established osteomalacic lesions rather than preventing them. It is possible, therefore, that more prolonged studies (months rather than a week) with similar design would have permitted a normal coupling of bone

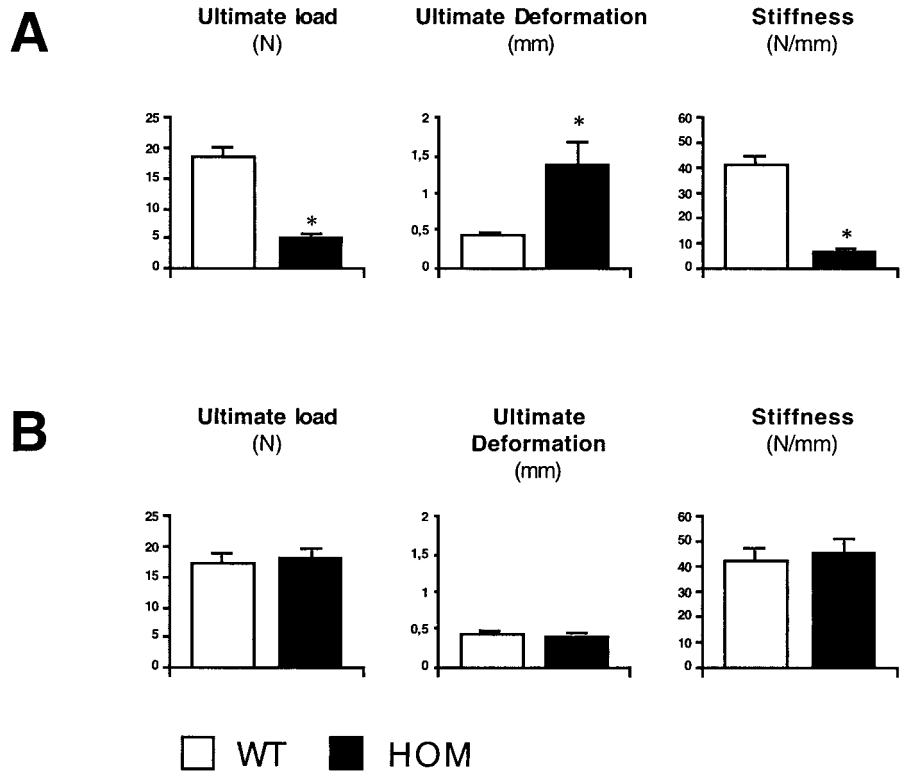

FIG. 4. A, The bones of the hypocalcemic VDR ablated mice are biomechanically abnormal; there is a dramatic decrease in ultimate load and stiffness. B. Prevention of abnormal mineral ion homeostasis results in normalization of these indexes. wt, Wild-type; hom, homozygous. *, $P < 0.05$. *Bars* represent the mean and SEM of determinations from five animals of each phenotype.

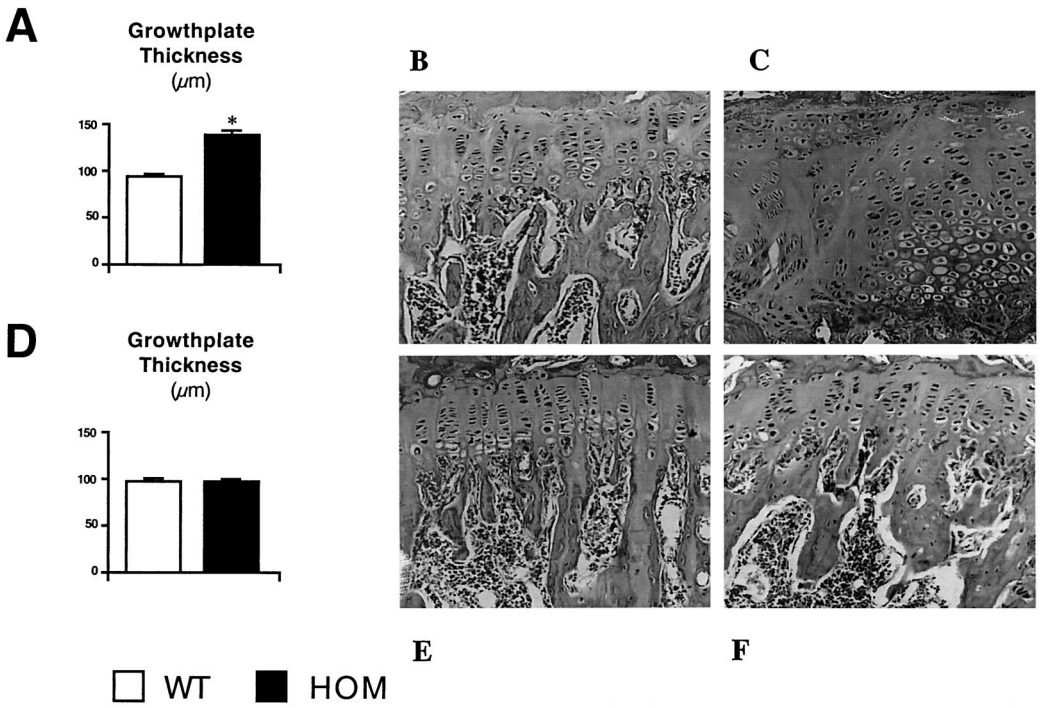

FIG. 5. Rickets is not seen in the normocalcemic VDR ablated mice. The growth plate thickness was measured as the mean perpendicular width of the complete growth plate, including resting, proliferating, and hypertrophic chondrocytes. There was a marked increase in the thickness of the growth plate in the hypocalcemic VDR ablated mice (A and C), which was not seen in the VDR ablated mice with normal mineral ion homeostasis (D and F). wt, Wild-type; hom, homozygous. *, $P < 0.05$. *Bars* represent the mean and SEM of determinations from five animals of each phenotype. Magnification, ×10.

formation and resorption and resulted in normalization of bone volume in the normocalcemic vitamin D-deficient rats.

All of the histomorphometric and biomechanical parameters evaluated in the normocalcemic receptor ablated mice were indistinguishable from those of wild-type littermates fed the same rescue diet. Furthermore, the development of

classic rachitic changes in the hypocalcemic VDR null mice was not observed in VDR null mice with normal mineral ion homeostasis. These data demonstrate that the receptor-dependent actions of 1,25-$(OH)_2D_3$ are not required for the development or maintenance of normal bone. They do, however, raise additional questions regarding the pathophysiology of the increased bone volume and of the rachitic changes.

The increase in bone volume observed in the hypocalcemic VDR null mice may reflect an increase in bone matrix protein synthesis as a result of the hyperparathyroidism, hypocalcemia, or hypophosphatemia, as these findings are not present in the VDR ablated mice with normal mineral ions and normal bone volume. It has previously been shown that PTH administration to hypophysectomized female rats results in increased bone formation, primarily due to an increase in osteoblast number (21); however, continuous exposure of the skeleton to high levels of PTH results in bone loss due to high bone turnover (22). The sustained elevation in PTH levels in the hypocalcemic VDR null mice, therefore, would be expected to result in a marked increase in bone turnover, which is reflected in the increased number of osteoblasts. The bone volume of these animals is paradoxically increased. This increase in bone volume may be a consequence of impaired bone resorption in the setting of continued bone formation. Alternatively, osteoclast function may be impaired by the profound hypocalcemia. However, previous studies have demonstrated that vitamin D-deficient females are able to mobilize normal amounts of calcium from their skeleton during pregnancy and lactation (23). This may, however, represent a unique physiological state. In the VDR ablated mice, despite increased PTH levels, no significant increase in the number of osteoclasts is observed in the hypocalcemic state. It has been demonstrated that both PTH and 1,25-$(OH)_2D_3$ increase messenger RNA levels for osteoclast differentiating factor (10); therefore, in the setting of hyperparathyroidism, one would have anticipated that osteoclast number would be elevated in the VDR null mice. However, this was not observed in our studies. It is likely, therefore, that the increase in bone volume in these mice is secondary to lack of resorption of osteoid in the setting of continued bone formation. In an analogous fashion, the expansion of the growth plate could be a result of impaired osteoclast/chondroclast function in the region of the primary spongiosa in the setting of continued matrix synthesis and delayed disappearance of hypertrophic chondrocytes.

These studies demonstrate that the nuclear effects of 1,25-$(OH)_2D_3$ are not required for normal skeletal development or modeling. Although 1,25-$(OH)_2D_3$ has been shown to have significant effects on the function of and genes expressed by osteoclasts and osteoblasts, other factors, such as PTH, may compensate and preserve skeletal homeostasis in the absence of a functional VDR. Further investigations will be required, including studies in vitamin D-deficient/VDR ablated mice, to ultimately prove that 1,25-$(OH)_2D_3$ is not required for skeletal homeostasis and that important skeletal effects of this steroid hormone are not mediated by a second nuclear VDR or by nongenomic actions.

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