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Conventional and Tissue-Specific Inactivation of the 25-Hydroxyvitamin D-1α-hydroxylase (CYP27B1)

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Abstract Mutations in the human 25-hydroxyvitamin-D_3-1α-hydroxylase (CYP27B1) gene cause pseudo vitamin D deficiency rickets (PDDR). The kidney is the main site of expression of the *CYP27B1* gene, but expression has been documented in other cell types, including chondrocytes. We engineered a tissue-specific and a conventional knockout of CYP27B1 in mice. The conventional knockout strain reproduced the PDDR phenotype. Homozygote mutant animals were treated with 1,25$(OH)_2D_3$ or fed a high-calcium diet (2% calcium, 1.25% phosphate, 20% lactose) for 5 weeks post-weaning. Blood biochemistry revealed that both rescue treatments corrected the hypocalcemia and secondary hyperparathyroidism. Bone histomorphometry confirmed that rickets were cured. The rescue regimen restored the biomechanical properties of the bone tissue. Mice carrying the loxP-bearing allele were bred to transgenic animals expressing the Cre recombinase in chondrocytes under the control of the collagen type II promoter. Genotyping confirmed excision of exon 8 in chondrocytes. Serum biochemistry revealed that mineral ion homeostasis is normal in mutant animals. Preliminary observation of bone tissue from mutant mice did not reveal major changes to the growth plate. Precise histomorphometric analysis will be required to assess the impact of chondrocyte-specific inactivation of CYP27B1 on the maturation and function of growth plate cells in vivo. J. Cell. Biochem. 88: 245–251, 2003.

Key words: vitamin D hydroxylase; rickets; PDDR; knockout

Vitamin D, produced endogenously in the skin upon exposure to ultraviolet light (sunlight) [Holick, 1997], must be metabolized twice to be activated and function as a key regulator of mineral ion homeostasis. Vitamin D, bound to the vitamin D binding protein, (DBP), is transported to the liver where the enzyme vitamin D 25-hydroxylase (CYP27) adds a hydroxyl group on carbon 25 to produce 25-hydroxyvitamin D [25(OH)D] [Gascon-Barré, 1997]. The 25(OH)D metabolite also circulates in the bloodstream bound to DBP [Bikle et al., 1986]. It must be further hydroxylated in the kidney to gain hormonal bioactivity. Hydroxylation at position 1α by the enzyme 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) [Miller and Portale, 2000] converts 25(OH)D to 1α,25-dihydroxyvitamin D [1,25$(OH)_2$D], the active, hormonal form of vitamin D. Upon reaching target tissues, 1,25$(OH)_2$D binds to its specific receptor, the vitamin D receptor (VDR) [Haussler et al., 1998], to regulate the transcription of vitamin D target genes responsible for carrying out the physiological actions of 1,25$(OH)_2$D: mineral homeostasis, skeletal homeostasis, and cellular differentiation [Feldman et al., 1997].

The 1,25$(OH)_2$D hormone regulates calcium homeostasis by promoting calcium absorption from the intestinal lumen. The homeostatic feedback loop involves both 1,25$(OH)_2$D and parathyroid hormone (PTH). Decreases in blood calcium stimulate synthesis and secretion of PTH, which in turn leads to increased expression of the *CYP27B1* gene and augmented 1,25$(OH)_2$D synthesis that triggers intestinal calcium absorption. To prevent sustained production of 1,25$(OH)_2$D that would lead to hypercalcemia, the vitamin D hormone in turn inhibits PTH and *CYP27B1* gene expression [Heaney, 1997].

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Pseudo vitamin D deficiency rickets (PDDR) is a rare autosomal recessive disease characterized by growth retardation, failure to thrive, rickets, and osteomalacia [St-Arnaud and Glorieux, 2000]. Serum biochemistry reveals hypocalcemia, secondary hyperparathyroidism, and undetectable levels of $1,25(OH)_2D_3$. Measurements of circulating vitamin D metabolite levels and 1α-OHase enzymatic activity have long suggested that the disease is caused by inactivating mutations in the cytochrome P450 gene responsible for the synthesis of $1,25(OH)_2D_3$ [St-Arnaud and Glorieux, 2000]. The cloning of the 1α-OHase cDNA and gene [Fu et al., 1997a,b] have confirmed this hypothesis, first by mapping of the gene to the disease locus [Fu et al., 1997b; St-Arnaud et al., 1997], then by the identification of mutations in affected patients [Portale and Miller, 2000]. We have generated an animal model of PDDR by targeted inactivation of the 1α-OHase gene in mice [Dardenne et al., 2001]. The engineered mouse strain represents a valid animal model for the disease. The homozygous mutant animals present with the same clinical phenotype as patients with PDDR: retarded growth, failure to thrive, undetectable $1,25(OH)_2D_3$, hypocalcemia, secondary hyperparathyroidism, and bone anomalies that included rickets and osteomalacia [Dardenne et al., 2001].

The treatment of choice for PDDR patients is replacement therapy with $1,25(OH)_2D_3$ [Delvin et al., 1981]. It results in rapid and complete correction of the abnormal phenotype, restoring normocalcemia, eliminating secondary hyperparathyroidism, and features of rickets. The restoration of bone mineral content is equally rapid and histological evidence of healing has been documented [Delvin et al., 1981]. The same treatment has been used successfully to heal bone lesions in young X-linked hypophosphatemic male mice [Marie et al., 1982].

Mutation in the VDR gene results in a second form of vitamin D-related rickets called hereditary vitamin D resistant rickets (HVDRR), characterized by hypocalcemia, secondary hyperparathyroidism, rickets, osteomalacia, and alopecia [Malloy et al., 1999]. Several laboratories have developed a valid mouse model for this type of hereditary rickets [Li et al., 1997; Yoshizawa et al., 1997; Van Cromphaut et al., 2001]. Rescue of rickets has been successfully performed using a 2% calcium diet, 1.25% phosphorus, and 20% lactose, previously reported to prevent hypocalcemia in vitamin D-deficient rats. This diet completely normalized all biochemical and histomorphometric parameters in VDR-ablated animals [Li et al., 1998; Amling et al., 1999].

Here we report the rescue of the PDDR phenotype in mice homozygous for the CYP27B1 mutation. Both the $1,25(OH)_2D_3$ replacement therapy and the high-calcium diet regimen led to normalization of serological parameters and complete healing of the rickets phenotype, as assessed by both histomorphometric and biomechanical analyses.

The main site for the 1α-hydroxylation of 25(OH)D is the proximal tubule of the renal cortex [Brunette et al., 1978]. The expression of CYP27B1 enzyme has also been reported in osteoblasts [Turner et al., 1980], chondrocytes [Pedrozo et al., 1999a], keratinocytes [Fu et al., 1997a], and cells of the lympho-hematopoeitic system [Reichel et al., 1991]. The identification of these extra-renal sites of expression of the CYP27B1 enzyme has led investigators to hypothesize that local production of $1,25(OH)_2D$ could play an important autocrine or paracrine role in the differentiation or function of these cells [Boyan et al., 1997; Hewison and O'Riordan, 1997].

Most in vivo studies suggest that the primary function of $1,25(OH)_2D$ in cartilage is in the maintenance of extracellular calcium concentrations. In vitro studies, however, have demonstrated that growth plate chondrocytes exhibit differential responsiveness to $1,25(OH)_2D$ and $24,25(OH)_2D$ [Boyan et al., 1997]. These experiments revealed that the response of rat costochondral chondrocytes to vitamin D metabolites depends on the zone of maturation from which the cells were originally derived [Boyan et al., 1988]. The prehypertrophic and upper hypertrophic zones (defined as the 'growth zone' in this culture system) respond primarily to $1,25(OH)_2D$: the metabolite inhibits proliferation but stimulates alkaline phosphatase activity, collagen synthesis, and proteoglycan synthesis [Schwartz et al., 1995]. Additional effects of $1,25(OH)_2D$ on growth zone chondrocytes include increases in membrane fluidity, stimulation of arachidonic acid turnover, and activation protein kinase C activity [reviewed in Boyan et al., 1997]. The precise mechanisms involved in these effects are not clear, but nongenomic effects via a hypothetical membrane

receptor have been hypothesized [Boyan et al., 1999; Pedrozo et al., 1999b].

We have engineered a strain of mice that allows tissue-specific inactivation of the *CYP27B1* gene [Dardenne et al., 2001] using the Cre/lox technology [Lewandoski, 2001]. We have crossed this strain to transgenic mice expressing the Cre recombinase in chondrocytes to specifically ablate the *CYP27B1* gene in growth plate cells and examine the role of local production of $1,25(OH)_2D$ in chondrocytic differentiation and function.

MATERIALS AND METHODS

Rescue Regimens

All procedures involving animals were previously approved by the Institutional Animal Care Committee. For hormone replacement therapy, animals received daily subcutaneous injections of $1,25(OH)_2D_3$ (a generous gift from Dr. Milan Uskokovic, Hoffmann-LaRoche, Nutley, NJ). A 'rescue' concentration of 500 pg/g body weight (BW) was administered from 21 days of age until 35 days of age, followed by a 'maintenance' dose of 100 pg/g BW (s.c.) from 35 days of age until they reached the age of 60 days. Injections were given in a vehicle consisting of 0.1% ethanol and 99.9% propylene glycol. For dietary rescue, the control groups mice were fed the regular autoclaved rodent chow (#5075, Charles River Laboratories, St-Constant, QC) containing 0.97% calcium, 0.85% phosphorus, 0% lactose, and 4.4 IU vitamin D/g (control diet). In order to normalize blood mineral ion levels of the CYP27B1-ablated mice, the animals were fed a γ-irradiated diet (TD96348, Teklad, Madison, WI) containing 2% calcium, 1.25% phosphorus, 20% lactose, and 2.2 IU vitamin D/g from 21 days of age (rescue diet) until sacrifice.

Bone Histology

We collected femurs from −/− mutants and +/− control littermates at 60 days of age. Bones were dissected, fixed overnight in 4% paraformaldehyde, and embedded in methylmethacrylate. Sections of 6 μm were deplastified and stained by von Kossa [Dickson, 1984].

Biomechanical Analysis

Femurs were collected in normal saline solution and mounted in a modified three-point bending test apparatus. The ends of the bone were rested on two fulcra separated by 1 cm, and a load was applied from above to the anterior midshaft, midway between the two fulcra. Compressive load was applied using a servo-hydraulic Instron™ test machine at a constant rate of 5 mm/min, and data were acquired at a sampling rate of 250 Hz. Testing progressed until the femur fractured or had been deflected 5 mm from the horizontal resting position. The maximum applied load was obtained by calculating the average of the maximal readings of the load cell over the test duration. The ultimate breaking force (maximum load) and ultimate deformation (maximum displacement) were determined directly from the curve. Young's modulus (stiffness) was calculated as the slope of the force-deformation curve through its linear portion.

Genotyping

Genotype analysis was performed using tail DNA. The genomic DNA was digested with the BamHI restriction endonuclease, and analyzed by Southern blotting with probes for the floxed CYP27B1 allele [Dardenne et al., 2001] and for the Cre transgene.

RESULTS

Blood biochemistry analysis revealed that both rescue treatments corrected the hypocalcemia, hypophosphatemia, and secondary hyperparathyroidism (data not shown). Femurs from all animals were collected at 60 days of age following 5½ weeks of treatment. Histological analysis was performed on von Kossa-stained 6 μm sections. Mutant $CYP27B1^{-/-}$ mice develop rickets from weaning and the severity of rickets and osteomalacia is amplified as mice grow [Dardenne et al., 2001]. Mineralization is also dramatically impaired (Fig. 1C). The mineralization defect and the severe disorganization of the growth plate in $CYP27B1^{-/-}$ mice at 8½ weeks (Fig. 1C) was corrected when they were treated with $1,25(OH)_2D_3$ (Fig. 1D) or when they were fed the high calcium diet (data not shown).

Biomechanical analysis showed that femurs from vehicle-treated $CYP27B1^{-/-}$ mice had a fourfold decrease in ultimate load and stiffness when compared to +/− controls (Fig. 2). Treatment of $CYP27B1^{+/-}$ animals with $1,25(OH)_2D_3$ had no effect on biomechanical parameters, while the same treatment restored maximal

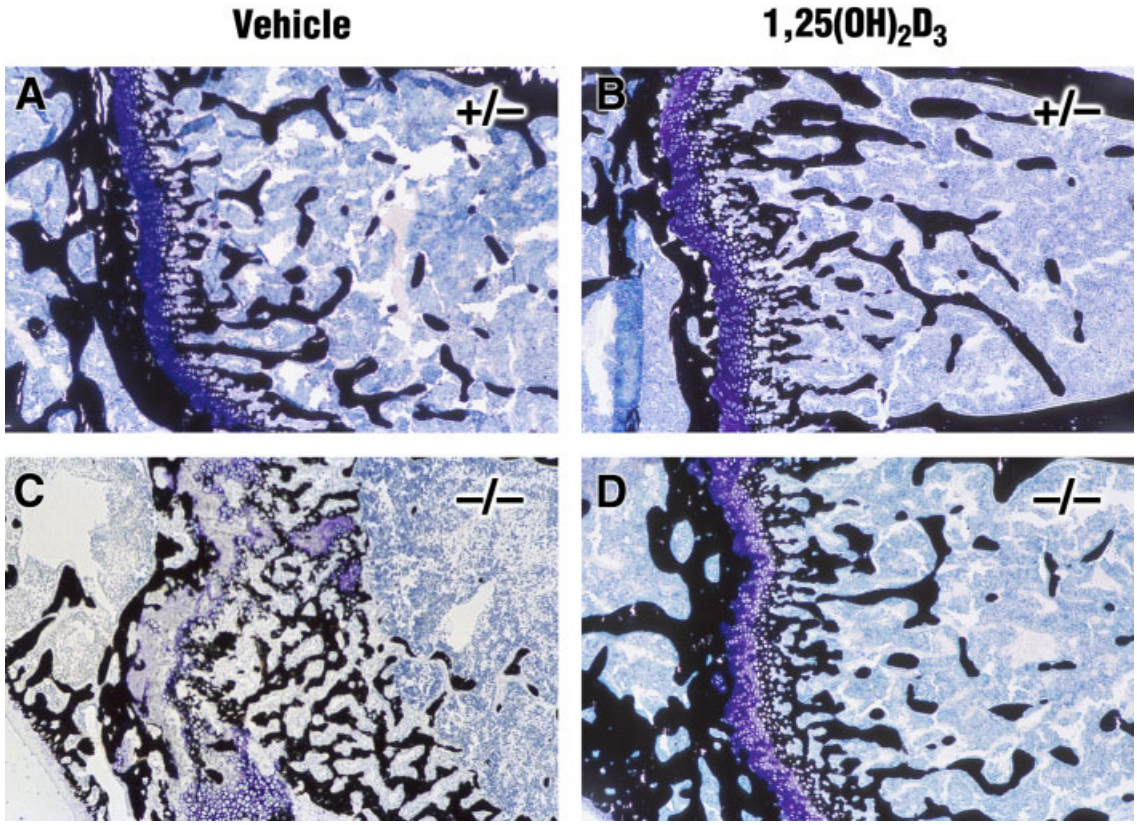

Fig. 1. Rickets and mineralization defect are prevented in CYP27B1$^{-/-}$ mice by treatment with 1,25(OH)$_2$D$_3$. Sagittal sections through the epiphysis of femur from 60 days-old heterozygous (+/–) and homozygous mutant (–/–) mice treated (**B** and **D**) or not (**A** and **C**) with 1,25(OH)$_2$D$_3$. The lower mineral content and complete disorganization of the growth plate displayed by CYP27B1$^{-/-}$ mice was corrected after they received 1,25(OH)$_2$D$_3$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

load and stiffness of bones from CYP27B1$^{-/-}$ mice to values measured in +/– littermates (Fig. 2). Because of the softness and pliability of the femurs from vehicle-treated 1α-OHase$^{-/-}$ mice, the ultimate deformation could not be measured in these samples. Treatment with 1,25(OH)$_2$D$_3$, however, normalized ultimate deformation to control values (data not shown).

The high calcium, high phosphorus, high lactose diet also corrected the biomechanical properties of bones from mutant animals. Maximum load (Fig. 3), stiffness, and ultimate deformation (data not shown) were normalized when CYP27B1$^{-/-}$ mice were fed the rescue diet from weaning.

Analysis of the direct role of vitamin D metabolites in chondrocyte differentiation and function in whole animals is complicated due to the abnormal mineral ion homeostasis that accompanies vitamin D deficiency. The 'floxed' *CYP27B1* (gene flanked by two loxP sites) strain that we have engineered, [Dardenne et al., 2001] provides a unique tool to study the putative paracrine role of 1,25(OH)$_2$D in growth plate chondrocyte maturation and function. We have obtained transgenic mice expressing the Cre recombinase under the control of the α_1(II) collagen promoter (targeted ablation in chondrocytes), a kind gift from Dr. Gérard Karsenty, Baylor College of Medicine, Houston, TX. We have crossed the CYP27B1 'floxed' mice to the Cre-expressing transgenic mice and have successfully achieved excision in chondrocytes (Fig. 4). Serum biochemistry confirmed that mineral ion homeostasis was not perturbed in mutant animals (not shown). Preliminary observation of bone tissue from mutant mice did not reveal major changes to the growth plate (data not shown).

DISCUSSION

Replacement therapy with 1,25(OH)$_2$D$_3$ is the treatment of choice for PDDR [Delvin et al.,

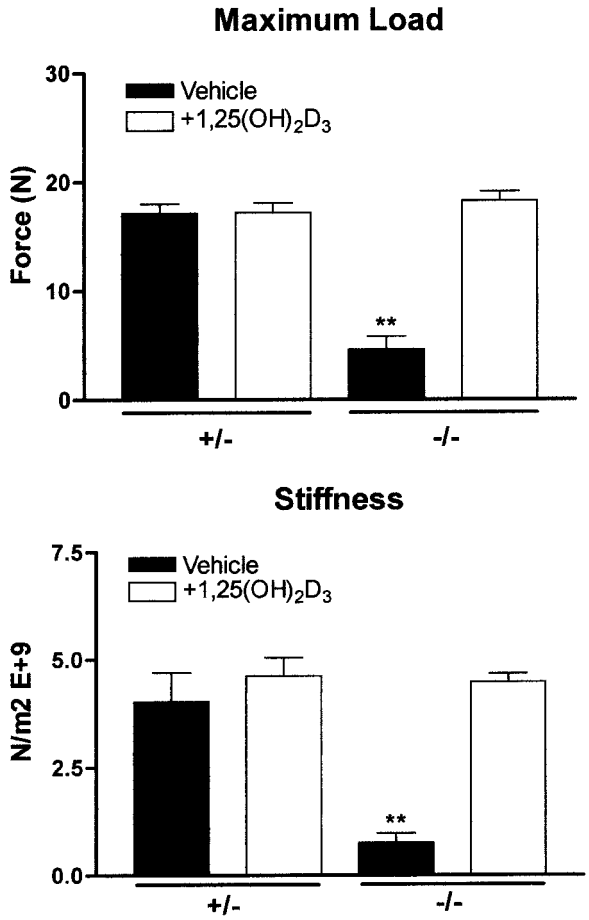

Fig. 2. $1,25(OH)_2D_3$-replacement therapy normalizes biomechanical properties of bones from CYP27B1$^{-/-}$ animals. Upper panel, ultimate load; lower panel, stiffness. The dramatic reduction in ultimate load and stiffness in mutant (−/−) animals was corrected by the treatment.

1981]. Our results show that in an animal model of PDDR, treatment with the active, hormonal form of vitamin D also effectively normalized biochemical parameters, restored the biomechanical properties of bones, and cured rickets and osteomalacia.

Although CYP27B1 null mice, fed with the rescue diet present no detectable level of $1,25(OH)_2D_3$, their calcium serum concentration was back to normal level (data not shown). It is likely that in animals, fed with the high calcium rescue diet, calcium entry in the intestine involves passive intake mechanisms. Normalization of mineral homeostasis, using the 2% calcium, 1.25% phosphorus, and 20% lactose rescue diet, cured rickets and osteomalacia, and normalized the biomechanical properties of the bones of CYP27B1$^{-/-}$ animals receiving this diet (Fig. 3, data not shown). A

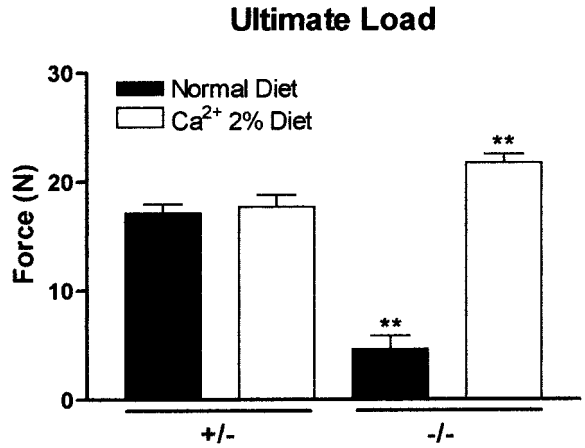

Fig. 3. Prevention of abnormal mineral ion homeostasis by feeding with a high calcium 'rescue' diet corrects the biomechanical parameters of bones from CYP27B1$^{-/-}$ mice. Control (+/−) and mutant (−/−) mice were fed a high calcium, high phosphorus, high lactose diet from weaning until sacrifice at 8 weeks.

comparable effect was previously described in VDR-ablated mice [Amling et al., 1999].

The growth plate of adult CYP27B1$^{-/-}$ animals is severely disorganized [Dardenne et al., 2001], rendering measurement of their width impossible (Fig. 1). Replacement therapy corrected growth plate architecture (Fig. 1). It has been shown that CYP27B1 is expressed in growth plate chondrocytes [Pedrozo et al., 1999a; Schwartz et al., 2001] and suggested that local production of $1,25(OH)_2D_3$ could play

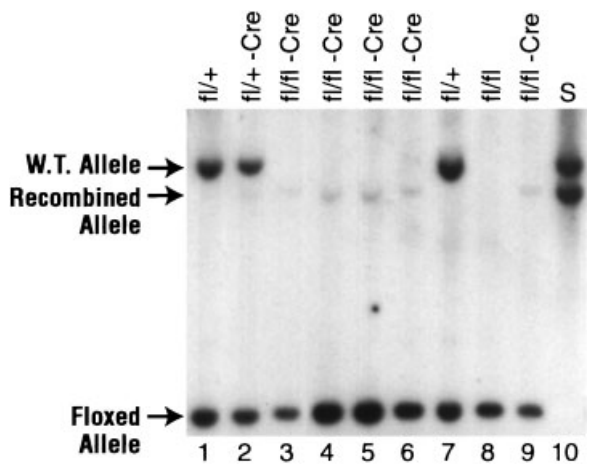

Fig. 4. Excision of the CYP27B1 floxed allele by the Col II-Cre transgene. Southern blot of tail DNA from 3-week-old mice. The tip of the tail is mostly comprised cartilage at this age, so this is a good tissue to examine Col II-Cre-mediated excision. The presence of the recombined allele band is proof of excision by Cre. Deduced genotypes are indicated at the top of each lane. S, position size markers for the wild-type and recombined alleles.

a significant autocrine or paracrine role in the regulation of chondrocyte maturation [Boyan et al., 1997]. These effects have been mostly studied in vitro and could be difficult to assess in animals, where systemic manipulation of vitamin D status provokes marked imbalances of mineral ion homeostasis. The targeted mouse model that we have engineered [Dardenne et al., 2001] allowed to selectively inactivate CYP27B1 in growth plate chondrocytes by crossing the mice with transgenic animals expressing the Cre recombinase in chondrocytes under the control of the type II collagen gene promoter [Long et al., 2001; Schipani et al., 2001]. These genetic manipulations will permit to examine the putative autocrine/paracrine roles of $1,25(OH)_2D_3$ in the growth plate under conditions of normal mineral ion homeostasis. While the first examination of the bones from mutant animals did not reveal any gross abnormalities, precise histomorphometric analysis will be required to assess the impact of chondrocyte-specific inactivation of CYP27B1 on the maturation and function of growth plate cells in vivo.

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