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Osteoblasts Lacking the Vitamin D Receptor Display Enhanced Osteogenic Potential In Vitro

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Abstract 1,25-Dihydroxyvitamin D plays an important role in the regulation of osteoblast gene expression, regulating the expression of bone matrix proteins as well as that of Runx2, a key regulator of osteoblast differentiation. Studies in mice lacking the vitamin D receptor (VDR) have revealed that the actions of the VDR on the skeleton are not required in the setting of normal mineral ion homeostasis. Since paracrine and endocrine factors can compensate for gene defects in vivo, studies were performed to determine whether ablation of the VDR alters the program of osteoblast differentiation in vitro. Studies in primary calvarial cultures revealed that ablation of the VDR enhanced osteoblast differentiation. The cells from the VDR null mice exhibited an earlier onset and increased magnitude of alkaline phosphatase activity, as well as an earlier and sustained increase in mineralized matrix formation, demonstrating that this enhancement persists throughout the program of osteoblast differentiation. The expression of bone sialoprotein, which enhances mineralization, was also increased in the VDR null cultures. To determine whether the increase in osteoblast differentiation was associated with an increase in the number of osteogenic progenitors, the number of osteoblastic colony forming units (CFU-OB) was evaluated. There was a twofold increase in the number of CFU-OB in the cultures isolated from the VDR null mice. Furthermore, the VDR null CFU-OB demonstrated an earlier onset and higher magnitude of expression of alkaline phosphatase activity when compared to the CFU-OB from their wild-type control littermates. These studies demonstrate that the VDR attenuates osteoblast differentiation in vitro and suggest that other endocrine and paracrine factors modulate the effect of the VDR on osteoblast differentiation in vivo. J. Cell. Biochem. 94: 81–87, 2005. © 2004 Wiley-Liss, Inc.

Key words: vitamin D; receptor; osteoblast; differentiation; knockout

The biologically active metabolite of vitamin D, 1,25-dihydroxyvitamin D, is thought to exert its principal actions by binding to a nuclear receptor, the vitamin D receptor (VDR) [Haussler et al., 1998]. Numerous investigations have been performed in in vitro and in vivo models to examine the biological effects of 1,25-dihydroxyvitamin D and its receptor. These studies have demonstrated that deficiency of vitamin D leads to hypocalcemia, secondary to a defect in intestinal calcium absorption [Walters et al., 1992; Thomas and Demay, 2000]. This hypocalcemia leads to secondary hyperparathyroidism and resultant hypophosphatemia due to an increase in urinary phosphate excretion. The skeletal manifestations of vitamin D deficiency include a widened, disorganized growth plate in growing animals known as rickets, and osteomalacia, characterized by an increase in unmineralized bone matrix.

The generation of mice lacking functional VDRs has permitted investigations directed at clarifying the requirement for the genomic actions of 1,25-dihydroxyvitamin D in skeletal development and maturation. Mice lacking the VDR are indistinguishable from their wild-type littermates at birth [Li et al., 1997; Yoshizawa et al., 1997; Erben et al., 2002]. However, by 5 weeks of age, rickets and osteomalacia are observed [Li et al., 1997; Yoshizawa et al., 1997; Erben et al., 2002]. To determine whether these changes were due to absence of the receptor dependent actions of 1,25-dihydroxyvitamin D

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on the skeleton or were a consequence of secondary metabolic effects, the VDR null mice were fed a diet that prevents the development of abnormal mineral ion homeostasis. Institution of a diet high in calcium, phosphorus, and lactose before the onset of abnormalities in mineral ions are observed, prevented all detectable skeletal abnormalities in the VDR null mice [Li et al., 1998; Amling et al., 1999] These in vivo studies suggest that the VDR is not required for skeletal homeostasis. However, they do not exclude the possibility that the role of the VDR in the skeleton is redundant and that, in its absence, an alternative homeostatic pathway performs its normal functions. Notable in this respect is the effect of 1,25-dihydroxyvitamin D on the regulation of RANK ligand production by the osteoblast. Although 1,25-dihydroxyvitamin D has been shown to play an important role in inducing the synthesis of this key regulator of osteoclast differentiation [Yasuda et al., 1998], histomorphometric analyses in the normocalcemic VDR null mice revealed normal osteoclast numbers and resorption surfaces [Amling et al., 1999]. In vitro studies demonstrated that osteoblasts from VDR null mice could not support osteoclastogenesis when co-cultured with normal spleen cells and 1,25-dihydroxyvitamin D. However, when these co-cultures were performed with PTH and interleukin 1α, osteoclasts with bone resorbing activity were formed [Takeda et al., 1999]. These data clearly support the hypothesis that the VDR plays a key role in the skeleton but, that in its absence, other regulatory molecules including hormones and cytokines, are called upon to maintain skeletal homeostasis.

Other models demonstrate that 1,25-dihydroxyvitamin D and its receptor have significant effects on the osteoblast in vivo. Mice engineered to overexpress the VDR in mature osteoblasts demonstrate an increase in cortical and trabecular bone volume, demonstrating significant anabolic effects of the VDR on bone [Gardiner et al., 2000]. In contrast, other studies suggest that high levels of 1,25-dihydroxyvitamin D impair mineralization of bone: the osteomalacia seen in 24-hydroxylase null mice, born of affected mothers, is prevented by making these mice null for the VDR as well [St-Arnaud et al., 2000]. These observations suggest that the high levels of 1,25-dihydroxyvitamin D in the 24-hydroxylase null fetuses of an affected mother, impair mineralization during development by a VDR dependent mechanism. Notably, 24-hydroxylase null pups born to a metabolically normal (heterozygous) mother are unaffected.

In addition to these in vivo data, in vitro analyses using both clonal osteosarcoma cell lines and primary osteoblasts have demonstrated a key role for 1,25-dihydroxyvitamin D in the regulation of genes encoding bone matrix proteins, including type I collagen [Lichter et al., 1989] and osteocalcin [Demay et al., 1990], the two most abundant matrix proteins. 1,25-dihydroxyvitamin D induces the expression of RANK ligand [Yasuda et al., 1998], a molecule secreted by the osteoblast, that is the key regulator of osteoclast differentiation. However, questions regarding the essential role of vitamin D metabolites and the VDR in the regulation of osteoblast differentiation and matrix protein production remain unanswered. To address this matter, the program of osteoblast differentiation was examined in primary calvarial osteoblasts isolated from VDR null mice. This well established model permitted studies directed at examining the effect of VDR ablation on the osteoblast phenotype, in the absence of systemic or paracrine factors that could compensate for absence of the VDR in vivo.

MATERIALS AND METHODS

Animal Maintenance

All studies were approved by the institutional animal care committee. Mice were maintained in a virus and parasite-free barrier facility and exposed to a 12-h light/dark cycle. Analyses were performed using wild-type and VDR knockout littermates obtained from matings of heterozygous mice.

Calvarial Osteoblast Isolation

Calvaria were isolated from 3-day-old wild-type and VDR knockout mice according to a standard protocol [Divieti et al., 1998; Chen and Fry, 1999]. The frontal and parietal bones were dissected free of connective tissue and rinsed in ice cold digestion buffer (αMEM, 0.1% BSA, 25 mM HEPES pH 7.4). Calvarial osteoblasts were obtained by sequential 15-min digestions with type I and type II collagenase in a 1:3 ratio (Worthington Biochemical Corp, Freehold, NJ). Cells from the first two digestions were discarded. The cells from digestions III–VI were

pooled and plated in α-MEM supplemented with 10% FBS and 1% penicillin/streptomycin (Gibco, Grand Island, NY). The following day, adherent cells were trypsinized and plated at a density of 5×10^3 cells/cm^2 in media supplemented with10 mM β-glycerolphosphate and 50 μg/ml ascorbic acid (Sigma, St. Louis, MO) and cultured for the times indicated.

Alkaline Phosphatase Activity

The alkaline phosphatase activity of cell lysates was assayed in buffer (50 mM Tris-HCl, pH 7.6, and 0.1% Triton X-100) containing 1.5M 2-amino-2-methyl-1-propanol (Sigma) for 30 min at 37°C using *p*-nitrophenyl phosphate (Sigma) as a substrate [Gori et al., 2001]. The release of *p*-nitrophenol was monitored by measuring absorbance at 405 nm. All results were normalized for protein content (BCA Assay, Pierce, Rockford, IL) and reported as nmol PNPP/30 min/μg protein.

Mineralized Matrix Formation

Cells were fixed in 70% ethanol for 1 h at room temperature, washed with PBS and stained for 10 min with 40 mM Alizarin Red S pH 4.2 (Sigma) at room temperature. The cells were washed with dH_2O five times and then with PBS for 15 min to remove background staining. Quantitation of Alizarin Red staining was performed by elution with 10% (w/v) cetylpyridium chloride (Sigma) for 10 min at room temperature and measuring absorbance at 570 nm [Gori et al., 2001]. For evaluation of calcium deposition into the matrix, the calcium deposited into similarly treated cultures was solubilized with 0.6 N HCl for 6 h at room temperature. The samples and a standard curve of calcium carbonate were reacted with methylthymol blue and measured spectrophotometrically at 620 nm [Gindler and King, 1972].

Northern Analyses

Wild-type and VDR knockout calvarial osteoblasts were plated at a density of 1×10^4 cells/cm^2 and cultured for the times indicated. Total RNA was prepared using Tri reagent (Sigma) according to the manufacturer's instructions. Ten micrograms of RNA was resolved on 1% agarose/formaldehyde gels and transferred overnight onto Biotrans membranes (ICN, Aurora, OH) by capillary blotting. Hybridization was performed using α ^{32}P dATP labeled cDNA probes and Ultra Hyb buffer (Ambion, Austin, TX). A cDNA probe for GAPDH was used to normalize for RNA loading.

Colony Formation Assays

Primary calvarial osteoblasts were trypsinized the day after isolation and seeded at a concentration of 450 cells/cm^2. At selected time intervals post plating, CFU-OB and CFU-F were detected by alkaline phosphatase (Sigma) and crystal violet staining, respectively. Colonies greater than 1 mm in diameter were counted with a dissecting microscope [Bellows et al., 1986].

RESULTS

To address whether the VDR plays a regulatory role in the program of osteoblast differentiation, primary calvarial osteoblasts were isolated from 3-day-old VDR null pups and their wild-type littermates. Absence of the VDR did not alter the proliferation rate of these cultures. Analyses examining the time of onset and degree of expression of markers of osteoblast differentiation were performed. The acquisition of the osteoblast phenotype is characterized by the ability of these cells to synthesize alkaline phosphatase and an increase in the specific activity of this enzyme is directly correlated with a more differentiated state [Stein and Lian, 1993]. Cultures from the VDR null mice demonstrate a significant and sustained increase in alkaline phosphatase activity, when compared to those of their wild-type littermates (Fig. 1).

The expression of type I collagen, an early marker of osteoblast differentiation that is transcriptionally repressed by 1,25-dihydroxyvitamin D [Harrison et al., 1989], was not altered in the VDR null osteoblasts (Fig. 2A). To determine whether this apparent enhancement in the differentiation of VDR null osteoblasts was secondary to an increase in the expression of Runx2, a key determinant of osteoblast differentiation, mRNA levels for this transcription factor were examined. As shown in Figure 2B, Runx2 expression was unaffected by VDR status. Runx2 is transcriptionally repressed by 1,25-dihydroxyvitamin D [Drissi et al., 2002], as is expression of the mouse osteocalcin gene [Ducy and Karsenty, 1995], a marker of terminal differentiation. Unlike Runx2, and consistent with an acceleration in the differentiation

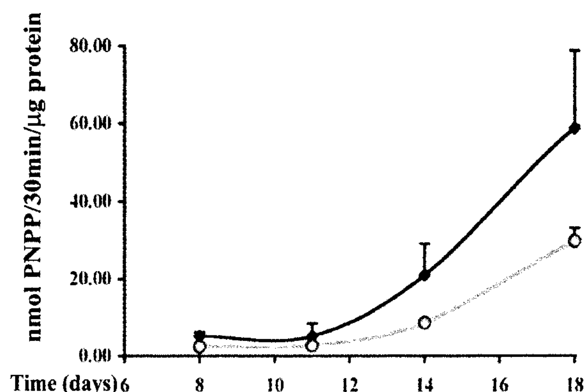

Fig. 1. Alkaline phosphatase activity. Alkaline phosphatase activity of calvarial osteoblasts isolated from vitamin D receptor (VDR) null mice (closed symbol) and wild-type (open symbol) littermates was assessed from 8 to 18 days in culture. The results are the mean ± SD of two independent calvarial preparations. Data shown are representative of that obtained with four independent calvarial preparations. $P < 0.05$ by multiple measures ANOVA between VDR null and wild-type osteoblasts.

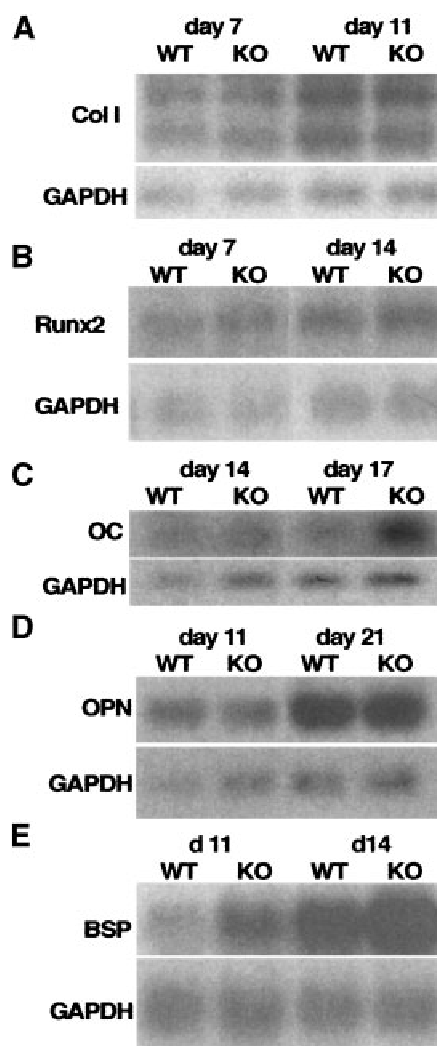

Fig. 2. mRNA expression. RNA isolated from VDR null and wild-type osteoblasts was transferred to nylon membranes and hybridized with probes for type I collagen (Col I, **A**), Runx2 (**B**), osteocalcin (OC, **C**), osteopontin (OPN, **D**), or BSP (**E**). Control hybridization with a GAPDH probe was performed to verify equal RNA loading. The data presented is representative of that obtained from three independent calvarial preparations.

of the VDR null osteoblasts, osteocalcin mRNA expression by the VDR null osteoblasts was increased 3.5 ± 0.2-fold by 17 days, compared to expression in cultures of wild-type osteoblasts (Fig. 2C).

The genes encoding osteopontin and bone sialoprotein (BSP), proteins that are thought to, respectively, attenuate and promote matrix mineralization, are regulated by 1,25-dihydroxyvitamin D: osteopontin being induced [Noda et al., 1990] and BSP being repressed [Bellows et al., 1999]. The expression of the mRNAs encoding these genes was, therefore, examined. Osteopontin mRNA levels of the VDR null cultures were unaltered from 11 to 21 days (Fig. 2D), whereas the expression of BSP was dramatically increased at both 11 and 14 days in the VDR null cultures (Fig. 2E).

The final stage of osteoblast differentiation is the formation of mineralized matrix nodules. These nodules can easily be detected by staining fixed cultures with Alizarin Red S. Cultures isolated from the VDR null mice demonstrated an earlier appearance and persistent increase in the number of Alizarin red staining nodules when compared to those isolated from their wild-type littermates. Elution of the Alizarin red S bound to the nodules confirmed this acceleration in mineralized matrix formation by the VDR null osteoblasts (Fig. 3). Quantitation of calcium deposited into the cultures similarly revealed a 2.2 ± 0.6-fold and a 1.4 ± 0.3-fold increase in the calcium content of the VDR null osteoblasts at 24 and 35 days, respectively.

The increase in osteogenic potential observed in the cultures from the VDR null mice could be due to an acceleration in the differentiation of osteoblasts, an increase in the number of osteogenic precursors, or both. When plated at very low densities, 0.3% of cells isolated from fetal rat calvaria have been shown to be osteoprogenitors that form discrete bone nodules [Bellows and Aubin, 1989]. To determine whether the number and the differentiation of osteoprogenitors in the calvarial cultures from the VDR null mice differed from those of their

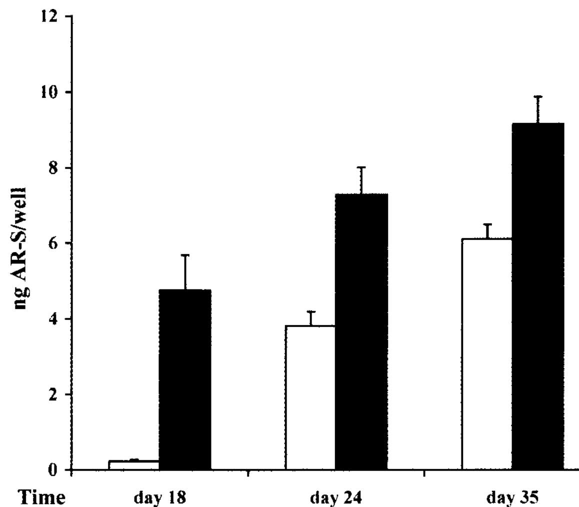

Fig. 3. Mineral deposition. Alizarin red S staining of calvarial osteoblasts from VDR null mice (closed bars) and wild-type (open bars) littermates was assessed from 18 to 35 days in culture. Following staining, the Alizarin Red S dye was eluted and quantitated spectrophotometrically. The results are the mean ± SD of two independent calvarial preparations. Data shown are representative of that obtained with four independent calvarial preparations. $P < 0.05$ by multiple measures ANOVA between VDR null and wild-type osteoblasts.

wild-type littermates, colony formation assays were performed. Colonies of osteoblastic progenitors (CFU-OB) were identified by alkaline phosphatase staining and total number of colonies, (CFU-F), were identified by crystal violet staining. As shown in Figure 4, the ratio of CFU-OB/CFU-F was increased in the cultures from the VDR null mice relative to those of their control littermates. Furthermore, there was

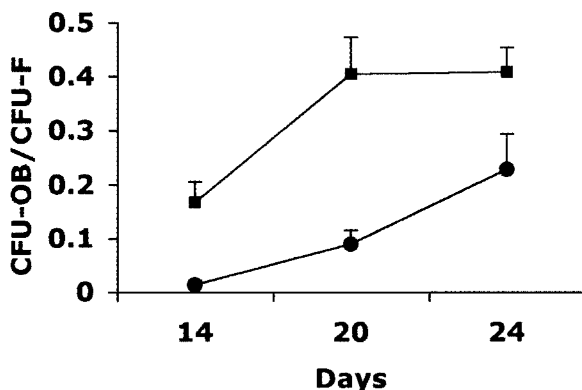

Fig. 4. Evaluation of CFU-OB. Primary calvarial osteoblasts were seeded at low density and cultured for 24 days. At the indicated time points, CFU-OB were detected by alkaline phosphatase staining and normalized for total CFUs (CFU-F) by staining with crystal violet. WT osteoblasts are represented by the circles and VDR KO osteoblasts by the squares. Data represent the mean ± SEM of four independent experiments.

an earlier appearance of alkaline phosphatase positive CFU-OB colonies in the cultures from the VDR null mice, confirming accelerated osteoblast differentiation by these cells.

DISCUSSION

The studies presented herein demonstrate that the VDR serves to attenuate the differentiation of primary calvarial osteoblasts in vitro. These results, which suggest that the VDR is a negative regulator of osteoblast activity, are in striking contrast to investigations examining the effects of VDR overexpression in vivo. These latter studies demonstrate that overexpression of the VDR in mature osteoblasts results in an increase in both cortical and trabecular bone [Gardiner et al., 2000]. The time of onset of enhanced VDR action in this model may explain the differences in the phenotypes observed. Absence of the VDR during the early stages of commitment may enhance the differentiation of cells into the osteoblastic lineage, whereas its overexpression during terminal differentiation increases osteoblast activity and decreases bone resorption in vivo. Alternatively, paracrine and endocrine factors may modulate the in vivo consequences of VDR overexpression under the control of the osteocalcin promoter.

The results of investigations examining the effects of 1,25 dihydroxyvitamin D on osteoblast differentiation in vitro are dependent upon the species examined and the time course of hormone treatment. In the extensively studied rat calvarial model, culturing cells in the presence of 1,25 dihydroxyvitamin D inhibits bone nodule formation [Ishida et al., 1993]. However, studies in mouse models demonstrate that treatment with 1,25 dihydroxyvitamin D promotes osteoblastic differentiation of 3T3 cells and embryonic stem cells [Shui and Scutt, 2002; zur Nieden et al., 2003]. Although studies of the lineage progression of these cells in response to 1,25-dihydroxyvitamin D may not be generalizable to other models, 1,25-dihydroxyvitamin D has also been shown increase alkaline phosphatase activity, the size of mineralized matrix nodules and osteocalcin expression in primary mouse calvarial osteoblasts [Chen and Fry, 1999]. This increase in osteocalcin synthesis reflects an increase in the state of cellular differentiation since, unlike the rat and human osteocalcin genes, which are induced

by 1,25 dihydroxyvitamin D [Demay et al., 1989; Morrison et al., 1989], the mouse osteocalcin gene is transcriptionally repressed by 1,25 dihydroxyvitamin D [Ducy and Karsenty, 1995]. Similarly, 1,25-dihydroxyvitamin D has been shown to promote the osteogenic differentiation of human bone marrow stromal cells, reflected by an increase in alkaline phosphatase activity and osteocalcin production [Liu et al., 1999]. These data are particularly intriguing, given the observation that the key regulator of osteoblast differentiation, Runx2, is transcriptionally repressed by 1,25-dihydroxyvitamin D [Drissi et al., 2002]. In our culture model, we were unable to demonstrate a change in Runx2 mRNA levels in the absence of the VDR, nor could we observe an alteration in the expression of two other genes regulated by 1,25-dihydroxyvitamin D, col I, and osteopontin, suggesting that trace amounts of 1,25-dihydroxyvitamin D that may be present in the media, were not responsible for the effects observed.

Investigations addressing the effects of the VDR and PTH/PTHrP receptor on bone formation and resorption have demonstrated different effects on cortical and trabecular bone. Mice expressing a constitutively active PTH/PTHrP receptor gene in osteoblasts, demonstrate a marked increase in trabecular bone formation accompanied by a decrease in cortical bone [Calvi et al., 2001]. Overexpression of the VDR in mature osteoblasts in mice results in increased bone volume secondary to an increase in cortical bone formation and a decrease in trabecular osteoclast resorption surface [Gardiner et al., 2000]. Further investigations in marrow stromal cells isolated from the VDR null mice will be required to determine whether ablation of the VDR results in an analogous or disparate phenotype in osteoblasts that give rise to intramembranous versus endocortical bone.

The studies reported herein demonstrate that absence of the VDR increases osteoblast commitment and/or differentiation in vitro. These data are not entirely consistent with in vivo studies that fail to demonstrate any histological, biomechanical, or histomorphometric abnormalities in the skeletons of VDR null mice with normal mineral ion homeostasis [Li et al., 1998; Amling et al., 1999]. However, hypocalcemic VDR null mice have a dramatic increase in bone matrix, characterized by a fivefold increase in bone volume/tissue volume and twofold increase in trabecular thickness due to a dramatic increase in unmineralized matrix [Amling et al., 1999]. This increase in matrix could reflect a reduction in bone turnover due to the inability of osteoclasts to resorb unmineralized bone, could be a direct result of VDR deficiency, or alternatively could be a consequence of the secondary hyperparathyroidism. It is important to note that our studies were performed in the offspring of metabolically normal heterozygous females, prior to the development of secondary hyperparathyroidism in the VDR null offspring from which the osteoblasts were isolated, removing the potential confounding variable of secondary hyperparathyroidism. Characterization of the molecular mechanism by which the absence of the VDR promotes osteoblast differentiation in vitro, and characterization of the paracrine/endocrine factors that maintain a normal skeleton in vivo in the normocalcemic VDR null mice will further our understanding not only of the molecular actions of the unliganded VDR, but will clarify components of the complex homeostatic pathway that regulates skeletal differentiation and maturation.

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