Journal of Cellular Biochemistry 99:922–935 (2006)

Evidence That Both 1α,25-Dihydroxyvitamin D_3 and 24-Hydroxylated D_3 Enhance Human Osteoblast Differentiation and Mineralization

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Abstract Vitamin D plays a major role in the regulation of mineral homeostasis and affects bone metabolism. So far, detailed knowledge on the vitamin D endocrine system in human bone cells is limited. Here we investigated the direct effects of 1α,25-$(OH)_2D_3$ on osteoblast differentiation and mineralization. Also, we studied the impact of 24-hydroxylation, generally considered as the first step in the degradation pathway of vitamin D, as well as the role of the nuclear and presumed membrane vitamin D receptor (VDR). For this we used a human osteoblast cell line (SV-HFO) that has the potency to differentiate during culture forming a mineralized extracellular matrix in a 3-week period. Transcriptional analyses demonstrated that both 1α,25-$(OH)_2D_3$ and the 24-hydroxylated metabolites 24R,25-$(OH)_2D_3$ and 1α,24R,25-$(OH)_3D_3$ induced gene transcription. All metabolites dose-dependently increased alkaline phosphatase (ALP) activity and osteocalcin (OC) production (protein and RNA), and directly enhanced mineralization. 1α,24R,25-$(OH)_3D_3$ stimulated ALP activity and OC production most potently, while for mineralization it was equipotent to 1α,25-$(OH)_2D_3$. The nuclear VDR antagonist ZK159222 almost completely blocked the effects of all metabolites. Interestingly, 1β,25-$(OH)_2D_3$, an inhibitor of membrane effects of 1α,25-$(OH)_2D_3$ in the intestine, induced gene transcription and increased ALP activity, OC expression and mineralization. In conclusion, not only 1α,25-$(OH)_2D_3$, but also the presumed 24-hydroxylated ''degradation'' products stimulate differentiation of human osteoblasts. 1α,25-$(OH)_2D_3$ as well as the 24-hydroxylated metabolites directly enhance mineralization, with the nuclear VDR playing a central role. The intestinal antagonist 1β,25-$(OH)_2D_3$ acts in bone as an agonist and directly stimulates mineralization in a nuclear VDR-dependent way. J. Cell. Biochem. 99: 922–935, 2006. © 2006 Wiley-Liss, Inc.

Key words: vitamin D; 1α-hydroxylation; 24-hydroxylation; VDR; human osteoblasts; differentiation; mineralization

INTRODUCTION

Vitamin D is one of the major factors involved in calcium homeostasis via actions on intestine, kidney, parathyroid gland, and bone. The biologically most active vitamin D molecule is 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$). 1α,25-$(OH)_2D_3$ is formed after two hydroxylation steps of vitamin D_3 (cholecalciferol). The first step results in the formation of 25-hydroxyvitamin D_3 (25-$(OH)D_3$) in the liver. This molecule is transported to the kidney where it is hydroxylated at the 1α position by the renal cytochrome P450 enzyme 25-hydroxyvitamin D_3-1α-hydroxylase resulting in the formation of 1α,25-$(OH)_2D_3$. In the kidney 25-$(OH)D_3$ can also be hydroxylated at the C-24 position resulting in the formation of 24R,25-dihydroxyvitamin D_3 (24R,25-$(OH)_2D_3$) [Christakos et al., 2003]. The 24-hydroxylase (CYP24) is capable of hydroxylating the 24 position not only of 25-$(OH)D_3$ but also of 1α,25-$(OH)_2D_3$, resulting in the formation of 1α,24R,25-trihydroxyvitamin D_3

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Received 12 December 2005; Accepted 1 February 2006
DOI 10.1002/jcb.20875

(1α,24R,25-$(OH)_3D_3$). CYP24 is expressed in all vitamin D target tissues [Akeno et al., 1997].

Mainly from in vivo observations it is concluded that 1α,25-$(OH)_2D_3$ is important for bone mineralization, though it has been published that 1α,25-$(OH)_2D_3$ directly stimulates in vitro mineralization of bone [Miyahara et al., 2002c]. It is presumed that the vitamin D effects on mineralization are indirect via control of calcium (re)absorption in intestine and kidney. However, the vitamin D receptor (VDR) is present in osteoblasts and 1α,25-$(OH)_2D_3$ has direct effects on osteoblasts as shown by regulation of the expression of several genes [van Leeuwen et al., 2001b].

The 24-hydroxylated metabolites were considered to be only the first degradation products of the catabolic pathway finally leading to excretion of calcitroic acid and were supposed to have no biological function other than controlling the production of 1α,25-$(OH)_2D_3$ [van Leeuwen et al., 2001a]. Studies with rachitic rats [Tanaka et al., 1979], post-menopausal women [Riis et al., 1986], vitamin D-deficient rats [Wang et al., 1993], 24-hydroxylase KO mice [St Arnaud et al., 2000], and 24.24-difluoro-25-hydroxy vitamin D_3 [Parfitt et al., 1984] supported this view. However, many other (animal) studies did show a positive involvement of 24R,25-$(OH)_2D_3$ and 1α,24R,25-$(OH)_3D_3$ in bone biology [Ornoy et al., 1978; Galus et al., 1980; Tam et al., 1986; Nakamura et al., 1992a,b; Yamate et al., 1994; Ono et al., 1996; Erben et al., 1997; Seo et al., 1997; Seo and Norman, 1997; Boyan et al., 2001; Schwartz et al., 2001], or an influence on the 1α,25-$(OH)_2D_3$ effect [Henry and Norman, 1978; Rambeck et al., 1988; Yamato et al., 1993; Birkenhager-Frenkel et al., 1995; Yamamoto et al., 1998]. At present, it is unknown whether these 24-hydroxylated vitamin D metabolites are biologically active in human osteoblasts and exert effects on mineralization by human osteoblasts and if so whether they act via the nuclear vitamin D receptor (VDR).

1α,25-$(OH)_2D_3$ acts via the VDR which is a member of the nuclear receptor family. The affinity for the VDR differs among the different metabolites. Compared to 1α,25-$(OH)_2D_3$, 1α,24R,25-$(OH)_3D_3$ has about a 10-fold lower affinity for the rat [Wang et al., 1993] and also the chicken [Eisman and DeLuca, 1977] intestinal VDR while 24R,25-$(OH)_2D_3$ has been reported to have about a 100-fold lower affinity for the nuclear VDR [Bouillon et al., 1995]. However, it is still unclear whether 24R,25-$(OH)_2D_3$ indeed uses the same nuclear VDR as 1α,25-$(OH)_2D_3$. Specific binding sites for 24R,25-$(OH)_2D_3$ located in the membrane have been postulated based on studies on rapid effects of 24R,25-$(OH)_2D_3$ on intracellular-signaling pathways [Norman et al., 2002a]. In rat endochondral chondrocytes the presence of a specific 24R,25-$(OH)_2D_3$ membrane receptor has been described [Pedrozo et al., 1999] and a distinct membrane receptor is also found in osteoblasts [Boyan et al., 2002], and in this latter study also a membrane receptor for 1α,25-$(OH)_2D_3$ has been postulated.

The aim of this study is threefold. First, to assess whether 1α,25-$(OH)_2D_3$ directly enhances matrix mineralization by human osteoblasts and stimulates human osteoblast differentiation. Second, to establish the effect of the 24-hydroxylated vitamin D molecules, 24R,25-$(OH)_2D_3$ and 1α,24R,25-$(OH)_3D_3$ on osteoblast differentiation, and matrix mineralization. Third, to examine the involvement of the nuclear VDR. For this, we used the in vitro differentiating human osteoblast cell line SV-HFO [Chiba et al., 1993; Arts et al., 1997; Weyts et al., 2003]. These cells proceed in a 3-week period through a tightly controlled process of differentiation eventually resulting in the formation of a mineralized matrix.

MATERIALS AND METHODS

Cell Culture

SV-HFO cells were seeded in a density of 10×10^3 vital cells per cm^2 in phenol-red free α-Minimal Essential Medium (α-MEM Gibco BRL, Paisley, UK), pH 7.5, supplemented with 20 mM HEPES (Sigma, St. Louis, MI), streptavidin/penicillin, 1.8 mM $CaCl_2 \cdot 2H_2O$ (Sigma) and 2% heat-inactivated charcoal-treated FCS at 37°C and 5% CO_2 in a humidified atmosphere. Medium was replaced every 2–3 days supplemented with freshly diluted 1 μM dexamethasone (9α-Fluoro-16α-methylprednisolone, Sigma) and 10 mM β-glycerophosphate (Sigma). From day 2 onwards, cells were cultured in the continuous presence of 1α,25-$(OH)_2D_3$, 24R,25-$(OH)_2D_3$ or 1α,24R,25-$(OH)_3D_3$ with or without ZK159222 or 1β,25-$(OH)_2D_3$. 1α,25-$(OH)_2D_3$, 1α,24R,25-$(OH)_3D_3$ and 1β,25-$(OH)_2D_3$ were generously provided by Dr. L. Binderup, Leo Pharmaceuticals, Ballerup, Denmark. 24R,25-$(OH)_2D_3$ was kindly

provided by Dr. J.P. van der Velde, Solvay Pharmaceuticals, Weesp, The Netherlands and ZK195222 by Dr. A. Steinmeyer, Schering AG, Berlin, Germany. SV-HFO cells and culture supernatant from duplicate wells were collected at days 7, 9, 12, 14, 16, 19, and 21 of culture. Medium was stored at −20°C and cells were scraped in PBS containing 0.1% triton X-100 and stored in −80°C. Prior to use, cell lysates were sonicated on ice in a sonifier cell disrupter for 2×15 s.

MG-63 and SaOS-2 human osteoblast-like sarcoma cells were cultured in α-MEM (see above) and 10% heat-inactivated FCS and stimulated for 24 h with 1α,25-$(OH)_2D_3$, 24R,25-$(OH)_2D_3$, 1α,24R,25-$(OH)_3D_3$ and 1β,25-$(OH)_2D_3$.

DNA Measurement and Alkaline Phosphatase Activity

SV-HFO cell lysates were treated with heparin and RNAse A (50 μg/ml in PBS) for 30 min at 37°C. DNA content was measured according to the ethidium bromide method of Karsten and Wollenberger [1977]. Alkaline phosphatase (ALP) activity was assayed by determining the release of paranitrophenol from paranitrophenylphosphate (20 mM in 1 M diethanolamin buffer supplemented with 1 mM $MgCl_2$ at pH 9.8) in the cell lysates for 10 min at 37°C [Lowry et al., 1954]. Adding 0.1 M NaOH stopped the reaction and absorption was measured at 405 nm using a Packard Spectra Count. Results were adjusted for DNA content of the corresponding cell lysates.

Osteocalcin

Osteocalcin (OC) was determined in medium and matrix extracts. Matrix extracts were prepared by incubating scraped cells and matrix overnight with 0.25 M HCl at 4°C, after which the matrix extracts were neutralized. Next OC in medium and matrix extracts was assayed by radioimmunoassay by incubating overnight with ^{125}Iodide-labeled human OC and anti-human OC. Anti-human OC and human OC were kindly provided by Dr. C.M. Gundberg, Department of Orthopaedics and Rehabilitation, School of Medicine, New Haven, Connecticut. Results were adjusted for DNA content of the corresponding cell lysates.

Mineralization

SV-HFO cell lysates were incubated overnight with 0.25 M HCl at 4°C. Calcium content was colorimetrically determined after addition of 1 M ethanolamine buffer (pH 10.6) 0.35 mM *o*-cresolphtalein complexone, 19.8 mM 8-hydroxyquinoline; and 0.6 mM hydrochloric acid at 595 nm (Packard Spectra Count). Results were adjusted for DNA content of the corresponding cell lysates.

Gene Expression

At days 7, 14, and 21, cells were washed with PBS and total RNA was isolated using RNA-Bee solution (Tel-Test, Friendswood, TX) according to the manufacturer's protocol. RNA was washed and precipitated overnight at −20°C with equal volumes of 100 mM EDTA and 8 M LiCl to remove Ca^{2+}. One μg of RNA was reverse transcribed into cDNA, using both 0.5 μg oligo$(dT)_{18}$, 0.2 μg random hexamer primers and Moloney murine leukemia virus according to the protocol of the manufacturer (MBI fermentas, St. Leon-Rot, Germany). Quantitative real-time PCR was carried out using an ABI Prism 7700 sequence detection system (ABI Foster City, CA).

Reactions were performed in 25 μl volumes using a qPCR^{TM} core kit (Eurogentec, Seraing, Belgium). Primer and probe concentrations and sequences were as follows: human OC, 1,000 nM forward, 5′-CAGGAGGGCAGCGAGGTA-3′; 1,000 nM reverse primer, 5′-TGGGGCTC-CCAGCCA-3′, and 200 nM probe 5′-FAM-TGATACAGGTAGCGCCTG-TAMRA-3′. Human 24-hydroxylase (CYP24): 600 nM forward primer, 5′-CAA-ACC-GTG-GAA-GGC-CTA-TC-3′; 600 nM reverse primer, 5′-AGT-CTT-CCC-CTT-CCA-GGA-TCA-3′, and 300 nM probe, 5′-ACT-ACC-GCA-AAG-AAG-GCT-ACG-GGC-TG-3′. The amount of human glyceraldehyde-3-phosphate dehydrogenase mRNA (GAPDH) (150 nM forward, 5′-ATGGGGAAGGTGAA-GGTCG-3′, 150 nM reverse 5′-TAAAAGCAG-CCCTGGTGACC-3′, and 150 nM probe 5′-FAM-CGCCCAATACG ACCAAATCCGTTGAC-TA-MRA-3′) was used as internal control to normalize for possible differences in RNA extraction and degradation as well as efficiency of the cDNA synthesis. Data are presented as relative mRNA levels calculated by the equation $2^{-\triangle Ct}$ ($\triangle Ct = Ct$ of target gene minus Ct of GAPDH).

Osteocalcin-Promoter GFP Analysis

For these studies a human osteoblast cell line stably transfected with an OC promoter GFP reporter construct was generated on basis of the

human osteoblastic cell line MG-63. The proximal promoter for human OC was a generous gift from Edith Gardiner, Garvan Institute, Sydney, Australia. A 1.3 kb BamHI fragment of the human OC promoter was digested from the obtained pGOSCAS vector and cloned in front of the sequence for the enhanced green fluorescence protein (EGFP) in Clontech's pEGFP-N3 vector of which the CMV promoter was removed using AsnI and Eco47III. All cloning steps were checked by sequencing. MG63 cells were cultured in RPMI containing 10% FCS and 1 mM of both penicillin and streptomycin. At 50% confluence, cells were transfected in 6-well plates with the above mentioned plasmid using Fugene (Roche) following the manufacturers protocol. Twenty-four hours post-transfection, medium was replaced by the basic medium containing 500 μg/ml neomycin to select for transfected cells. Fluorescent colonies were identified following 3 weeks of neomycin treatment by fluorescence microscopy, picked and cultured separately. Genomic DNA from several clones was isolated and checked for the presence of GFP fragments of the appropriate size using PCR. The clone OC.12 was selected for further studies. GFP expression was monitored using flow cytometry.

Luciferase Activity

Four hours after medium replacement, SV-HFO cultures were transiently transfected with 200 ng of the rat atrial natriuretic factor VDRE (generously provided by Prof. Carsten Carlberg, University of Kuopio, Finland) [Kahlen and Carlberg, 1996] luciferase reporter plasmid, using FuGENE6 transfection reagent according to the manufacturer's protocol (Roche, Basel, Switzerland). One day after transfection, SV-HFO cultures were treated with the vitamin D metabolites. After 24 h, the cells were lysed in 100–200 μl 1× lysis buffer (Promega) for 20 min with gentle shaking. Luciferase activity was measured using 25 μl cell lysate and the Steady-Glo Luciferase Assay System (Promega). Luciferase values were corrected for luciferase activity of empty pGL3-enhancer vector.

Statistical Analysis

A repeated measures ANOVA with a Bonferroni post-test was used to compare the effects of the treatment with the vitamin D metabolites and/or antagonists during time of SV-HFO culture. One-way ANOVA with a Bonferroni post-test was used to compare the effects of the treatments at one time point. Data are expressed as mean ± standard deviation (SD). *P*-values of less than 0.05 were considered significant.

RESULTS

Osteoblast Differentiation

Human osteoblasts displayed ALP activity with a maximum at day 14 of culture. 1α,25-$(OH)_2D_3$ (10^{-8} M) significantly increased ALP activity at all time-points during culture ($P < 0.01$) (Fig. 1). We tested the C24-hydroxylated compounds at concentrations reflecting their relative receptor affinity to 1μ,25-$(OH)_2D_3$ [Eisman and DeLuca, 1977; Wang et al., 1993; Bouillon et al., 1995; Muralidharan et al., 1997]. Both 24R,25-$(OH)_2D_3$ (10^{-6} M) and 1α,24R,25-$(OH)_3D_3$ (10^{-7} M) significantly increased ALP activity ($P < 0.01$ and $P < 0.001$, respectively) (Fig. 1). The effects of the different vitamin D metabolites on ALP activity were dose-dependent (data not shown).

1α,25-$(OH)_2D_3$ (10^{-8} M) significantly increased OC content in the matrix ($P < 0.05$) (Fig. 2A) as well as in the culture supernatant (data not shown). Both 24R,25-$(OH)_2D_3$ (10^{-6} M) and 1α,24R,25-$(OH)_3D_3$ (10^{-7} M) significantly increased OC levels in the matrix (both $P < 0.001$) (Fig. 2A) and supernatant (data not shown). The effect of both metabolites was as strong or even stronger as 1α,25-$(OH)_2D_3$. These effects of all vitamin D metabolites on OC were dose dependent (Fig. 2B). The DNA content of SV-HFO cells

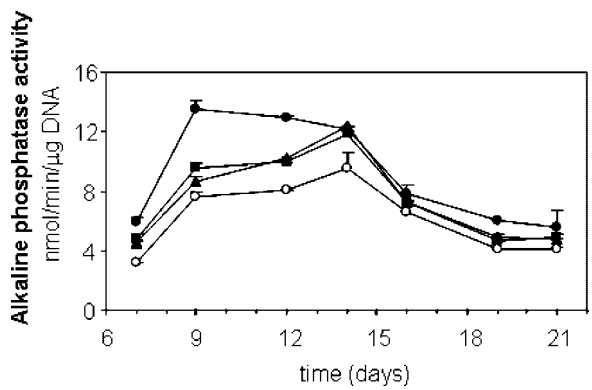

Fig. 1. Alkaline phosphatase (ALP) activity during culture in basal condition (vehicle, ○) and after continuous treatment with 10^{-8} M 1α,25-$(OH)_2D_3$ (▲), 10^{-7} M 24R,25-$(OH)_2D_3$ (■), and 10^{-6} M 1α,24R,25-$(OH)_3D_3$ (●). Data shown are means ± SD and graphs are representative of data obtained from three independent experiments of two cultures each.

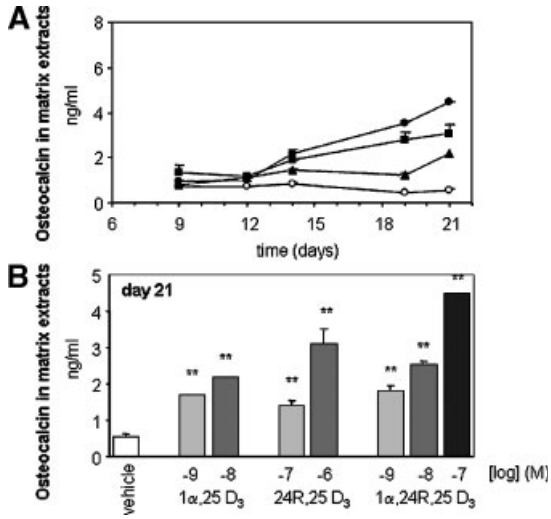

Fig. 2. **A**: Osteocalcin (OC) in matrix throughout culture in basal condition (vehicle, ○) and after continuous treatment with 10^{-8} M 1α,25-$(OH)_2D_3$ (▲), 10^{-7} M 24R,25-$(OH)_2D_3$ (■), and 10^{-7} M 1α,24R,25-$(OH)_3D_3$ (●). **B**: Dose-response effects of the different vitamin D metabolites on matrix OC are shown at day 21 of culture. $^{**}P < 0.01$, versus vehicle on the corresponding day. Data shown are means ± SD and graphs are representative of data obtained from three independent experiments of two cultures each.

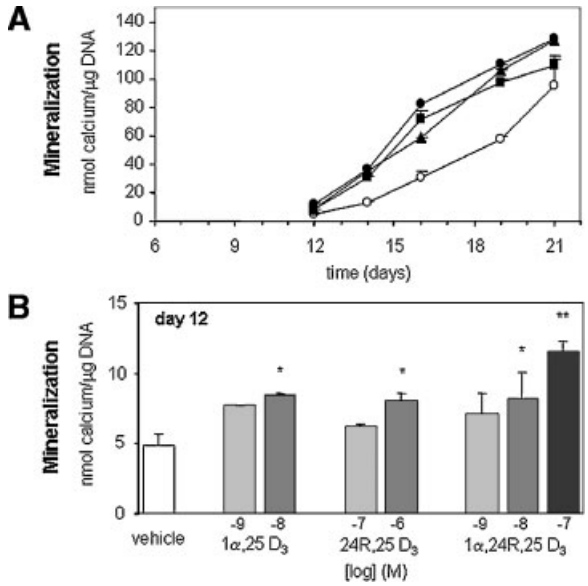

Fig. 3. **A**: Mineralization of the extracellular matrix formed during culture at basal conditions (vehicle) and after continuous treatment with 10^{-8} M 1α, 25-$(OH)_2D_3$ (▲), 10^{-7} M 24R,25-$(OH)_2D_3$ (■), and 10^{-7} M 1α,24R,25-$(OH)_3D_3$ (●). **B**: Dose-response effects of the different vitamin D metabolites are shown at day 12 of culture. $^{*}P < 0.05$, $^{**}P < 0.01$, versus vehicle at the corresponding day. Data shown are means ± SD and graphs are representative of data obtained from three independent experiments of two cultures each.

gradually increased during time in culture. Continuous treatment with 1α,25-$(OH)_2D_3$, 24R,25-$(OH)_2D_3$, and 1α,24R,25-$(OH)_3D_3$, did not change the amount of DNA of SV-HFO cells during differentiation (data not shown).

Osteoblast Mineralization

1α,25-$(OH)_2D_3$ has a direct positive effect on the mineralization process exerted by human osteoblasts ($P < 0.001$, Fig. 3A). 24R,25-$(OH)_2D_3$ (10^{-6} M) and 1α,24R,25-$(OH)_3D_3$ (10^{-7} M) also significantly (both $P < 0.001$) enhanced mineralization to a similar level as 1α,25-$(OH)_2D_3$ (Fig. 3A).) There was clear dose-dependency at the early stages of mineralization (day 12) (Fig. 3B).

Figure 4 shows the comparison of equimolar concentrations of 1α,25-$(OH)_2D_3$ and 1α,24R,25-$(OH)_3D_3$ (Fig. 4A,B; 10^{-8} M) and of 24R,25-$(OH)_2D_3$ and 1α,24R,25-$(OH)_3D_3$ (Fig. 4C,D; 10^{-7} M). 1α,24R,25-$(OH)_3D_3$ and 1α,25-$(OH)_2D_3$ stimulated OC expression equipotent (Fig. 4A), ALP activity (data not shown), and mineralization (Fig. 4B). Whereas 1α,24R,25-$(OH)_3D_3$ was significantly more potent than 24R,25-$(OH)_2D_3$ in stimulating OC expression in matrix ($P < 0.001$, Fig. 4C), ALP activity (data not shown) and mineralization ($P < 0.05$, Fig. 4D).

Induction of mRNA Expression in Human Osteoblast Cell Lines

In accordance with the positive effects seen on SV-HFO cells, 1α,25-$(OH)_2D_3$ and 1α,24R,25-$(OH)_3D_3$ dose-dependently increased OC and 24-hydroxylase mRNA expression in the human osteoblast like cell lines MG 63 (Fig. 5A,B) and SaOS-2 (data not shown). Also in these osteoblast cell lines 24R,25-$(OH)_2D_3$ was active but less potent than the 1α-hydroxylated compounds.

Nuclear Vitamin D Receptor

Figures 6 and 7 show the effects of the nuclear VDR antagonist ZK159222 (10^{-6} M) on OC mRNA expression, OC production, and mineralization, respectively. ZK159222 alone did not affect either one of these responses. The effects of 1α,25-$(OH)_2D_3$ and 24R,25-$(OH)_2D_3$ on OC mRNA expression were totally blocked by 10^{-6} M ZK159222 while the effect of 1α,24R,25-$(OH)_3D_3$, was partially inhibited at all time points during differentiation (Fig. 6A–C).

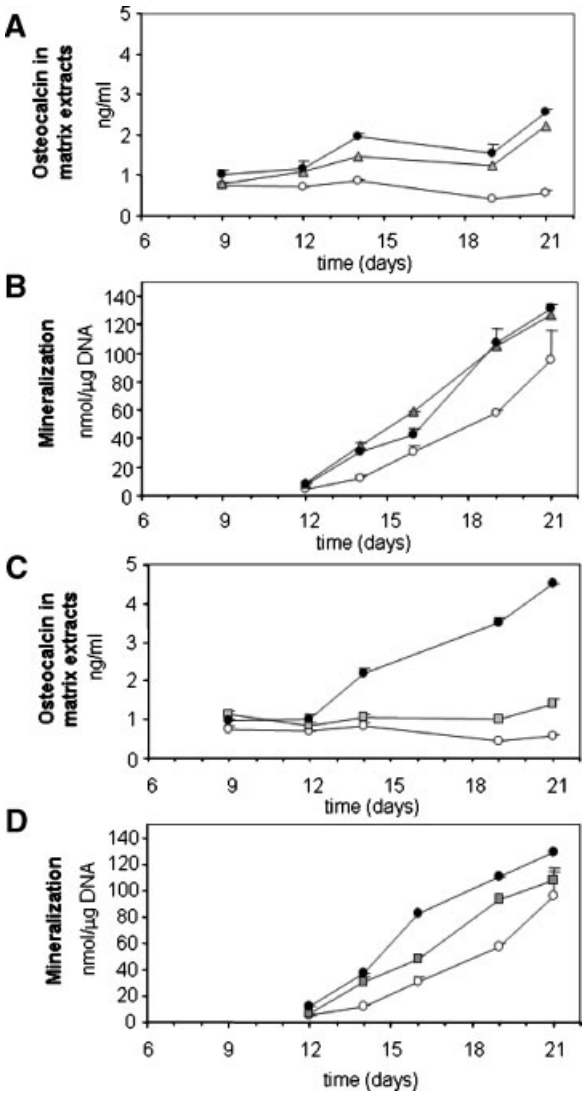

Fig. 4. Comparison of equimolar concentrations (**A**) and (**B**) of 10^{-8} M 1α,25-$(OH)_2D_3$ (▲) and 1α,24R,25-$(OH)_3D_3$ (●), and (**C**) and (**D**) of 10^{-7} M 24R,25-$(OH)_2D_3$ (■) and 1α,24R,25-$(OH)_3D_3$ (10^{-7} M) (●) for matrix OC (A) and (C) and mineralization (B) and (D) throughout culture. Vehicle is indicated as (○). Data shown are means ± SD and graphs are representative of data obtained from three independent experiments of two cultures each.

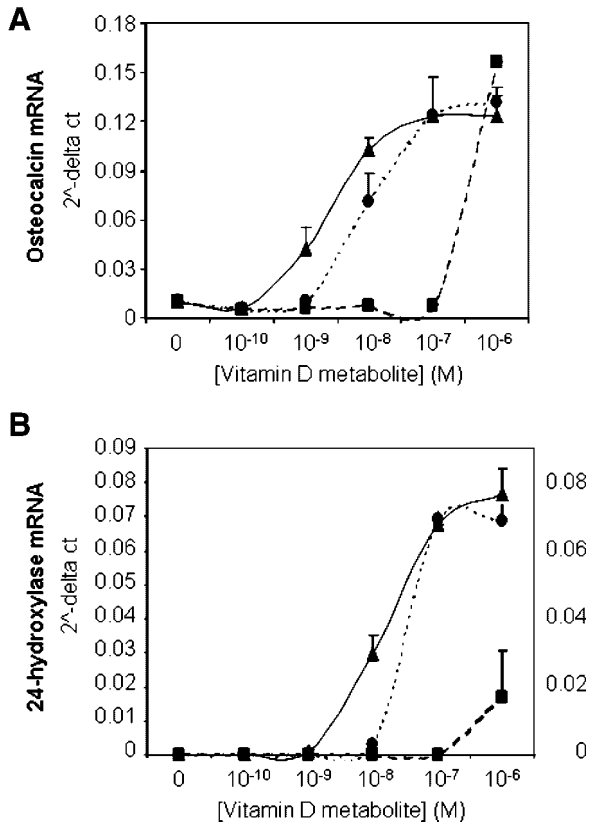

Fig. 5. Dose-response curves of 1α,25-$(OH)_2D_3$ (▲), 24R,25-$(OH)_2D_3$ (■) and 1,24R,25-$(OH)_2D_3$ (●) on (**A**) OC mRNA expression, and (**B**) 24-hydroxylase mRNA expression in MG-63 cells. Data are corrected for GAPDH mRNA expression (2^-delta ct (delta ct = ct of target gene minus ct of GAPDH)) and are means ± SD.

1α,25-$(OH)_2D_3$ as well as 24R,25-$(OH)_2D_3$ stimulated OC protein production was significantly blocked by ZK159222 (Fig. 7A). A partial but significant inhibition was found for 1α,24R,25-$(OH)_3D_3$. The same was observed for ALP activity (data not shown). As is seen for OC mRNA expression, the inhibition of vitamin D metabolite effects on OC protein and ALP activity by ZK159222 was observed at all time-points during SV-HFO differentiation (data not shown). Both 1α,25-$(OH)_2D_3$- and 1α,24R,25-$(OH)_3D_3$-stimulated mineralization was significantly blocked by ZK159222 (Fig. 7B). Continuous treatment with ZK159222 had no effect on cell growth as measured by DNA content of SV-HFO cells (data not shown).

Membrane Bound Vitamin D Receptor

The membrane receptor antagonist 1β,25-$(OH)_2D_3$ was not effective in the concentrations 10^{-7} M and 10^{-8} M (10^{-8} M data not shown), while at 10^{-6} M 1β,25-$(OH)_2D_3$ appeared to act as an agonist (Fig. 8). Co-incubation with 10^{-6} M 1β,25-$(OH)_2D_3$ did not inhibit the stimulation of OC by either one of the vitamin D metabolites. At 10^{-6} M 1β,25-$(OH)_2D_3$ even an enhancement of the effect of the various metabolites occurred. The effects shown for day 14 were also observed at all other time-points measured (not shown). For ALP activity and mineralization similar observations as for OC were made: no inhibition of

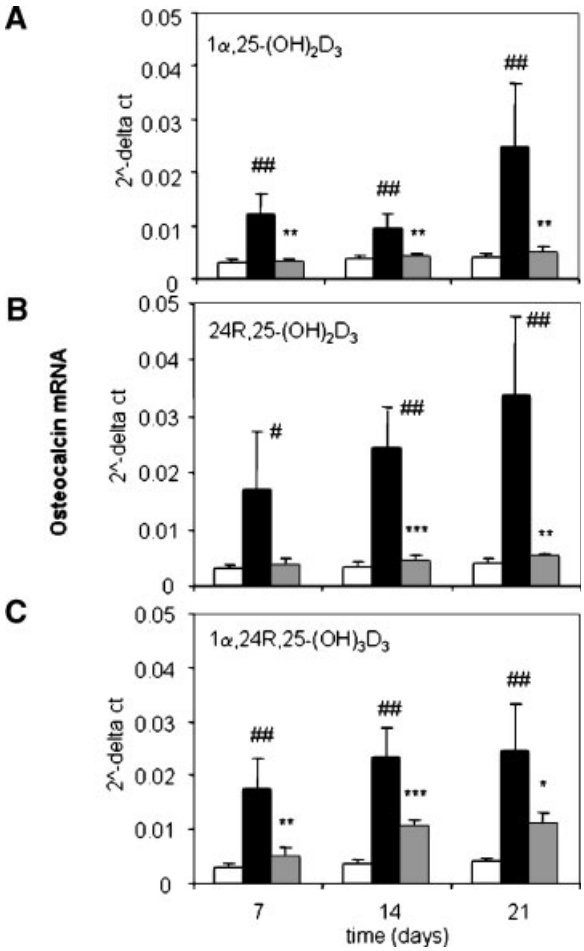

Fig. 6. Effect of nuclear vitamin D receptor antagonist ZK159222 (10^{-6} M) (▩) on OC mRNA expression after co-treatment with (**A**) 10^{-8} M 1α,25-$(OH)_2D_3$ (■), (**B**) 10^{-7} M 24R,25-$(OH)_2D_3$ (■), and (**C**) 10^{-7} M 1α,24R,25-$(OH)_3D_3$ (■). Data are corrected for GAPDH mRNA expression (2^-delta ct (delta ct = ct of target gene minus ct of GAPDH)) and are means ± SD and graphs are representative of data obtained from three independent experiments of two cultures each. #$P < 0.05$, ##$P < 0.01$, versus vehicle at the corresponding day. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ for inhibition of the various vitamin D metabolites at the corresponding day.

10^{-8} M 1α,25-$(OH)_2D_3$, 10^{-6} M 24R,25-$(OH)_2D_3$ or 10^{-7} M 1α,24R,25-$(OH)_3D_3$ effects (data not shown). Agonistic action of 1β,25-$(OH)_2D_3$ is further demonstrated in Figure 9. Figure 9A,B show the dose-dependent effect of 1β,25-$(OH)_2D_3$ on ALP activity and mineralization during osteoblast culture. 10^{-6} M 1β,25-$(OH)_2D_3$ also increased OC and 24-hydroxylase mRNA expression in MG-63 and SaOS-2 cells (Fig. 9C).

The stimulation of OC production by 10^{-6} M 1β,25-$(OH)_2D_3$ was significantly inhibited by the nuclear receptor antagonist ZK15922 at

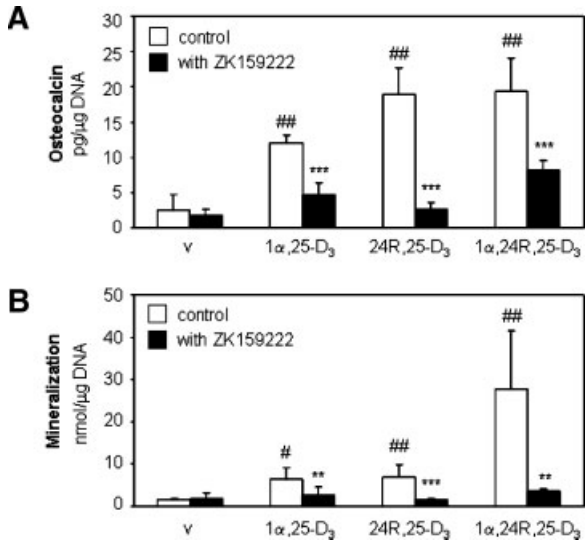

Fig. 7. Effect of the nuclear VDR antagonist ZK159222 (10^{-6} M) on (**A**) OC production at day 14 and (**B**) mineralization at day 16 in basal condition (vehicle) and after co-treatment with 10^{-8} M 1α,25-$(OH)_2D_3$, 10^{-7} M 24R,25-$(OH)_2D_3$ and 10^{-7} M 1α,24R,25-$(OH)_3D_3$. #$P < 0.01$, ##$P < 0.001$ versus vehicle. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ for inhibition of the various vitamin D metabolites. Data shown are means ± SD and graphs are representative of data obtained from three independent experiments of two cultures each.

days 7, 14, and 21 of culture (Fig. 10A). This was also found for ALP activity (data not shown). The positive effect of 10^{-6} M 1β,25-$(OH)_2D$ on mineralization at day 14 was significantly inhibited by ZK159222 (Fig. 10B). 1β,25-$(OH)_2D_3$ did not change the DNA amount of SV-HFO cells during culture (data not shown).

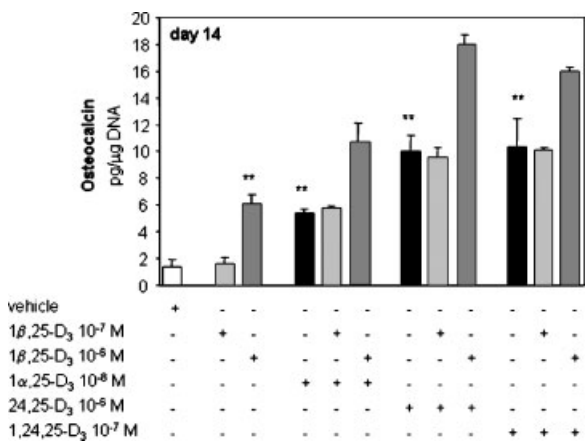

Fig. 8. Effect of 1β,25-$(OH)_2D_3$ on basal and vitamin D metabolite induced OC production. *$P < 0.01$, **$P < 0.001$ versus vehicle at the corresponding day. Data shown are means ± SD and graphs are representative of data obtained from three independent experiments of two cultures each.

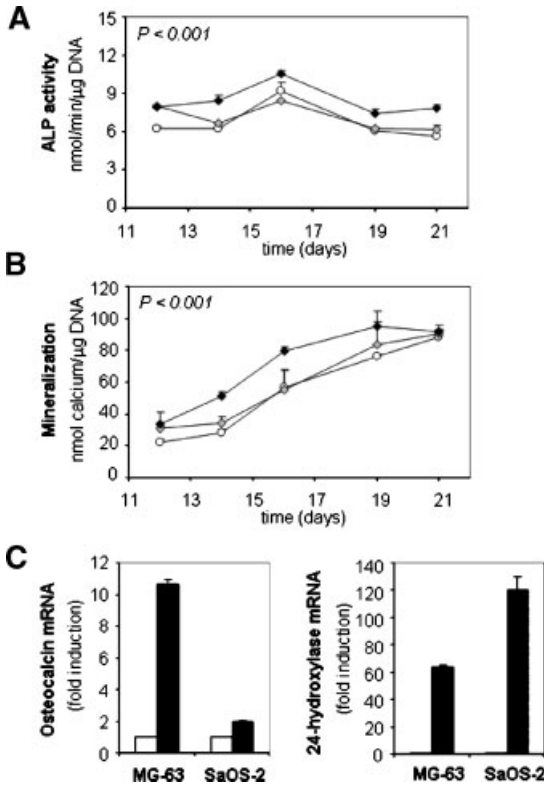

Fig. 9. Effect of 10^{-7} M (●) and 10^{-6} M (◆) 1β,25-$(OH)_2D_3$ on (**A**) ALP activity and (**B**) mineralization in SV-HFO cells. Effect of 10^{-6} M 1β,25-$(OH)_2D_3$ (black bars) versus vehicle (open bars) on OC and 24-hydroxylase mRNA expression in MG-63 and SaOS-2 osteoblast-like cells. *P*-values in the figure indicate significant differences between the vitamin D metabolite and the vehicle (○) treated cultures. Data shown are means ± SD and graphs are representative of data obtained from three independent experiments of two cultures each.

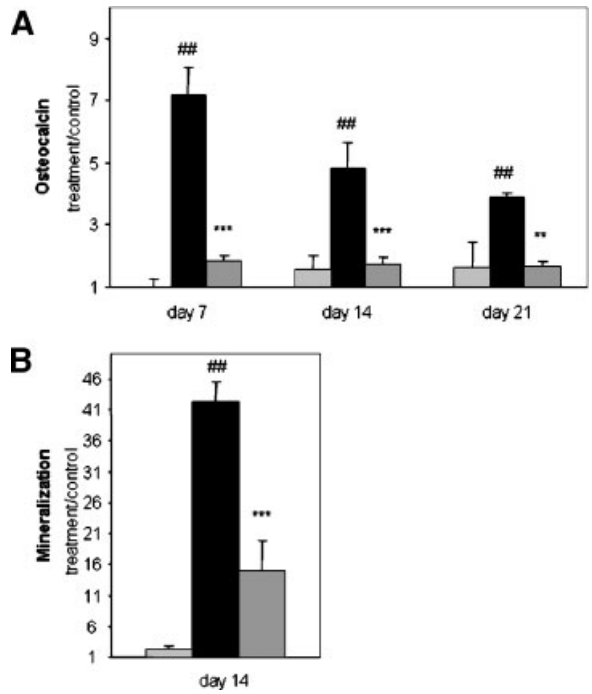

Fig. 10. Effect of nuclear vitamin D receptor antagonist ZK159222 (10^{-6} M) on 10^{-6} M 1β,25-$(OH)_2D_3$ stimulated (**A**) OC production and (**B**) mineralization (ZK159222 ▒, 1β,25-$(OH)_2D_3$ ■, 1β,25-$(OH)_2D_3$ + ZK159222 ▒). Data are expressed as treatment over control and shown as means ± SD of three independent experiments of two cultures each. $^{\#\#}P < 0.001$ versus vehicle at the corresponding day. $^{**}P < 0.01$, $^{***}P < 0.001$ for inhibition of 1β,25-$(OH)_2D_3$ at the corresponding day.

Induction of Transcription

To examine transcription regulation via nuclear VDR and 1β,25-$(OH)_2D_3$ in more detail, we performed studies on MG-63 cells stably transfected with the *GFP* gene under control of the human OC promoter (OC.12). As shown in Figure 11A the reporter gene was dose-dependently induced by all vitamin D metabolites. The involvement of the nuclear VDR was demonstrated by the inhibition of the GFP expression by ZK19522 (Fig. 11B). The same was shown using an ANF-VDRE luciferase reporter construct transfected in MG-63 cells (data not shown).

DISCUSSION

The major finding of this study is that in this osteoblast differentiation model 1α,25-$(OH)_2D_3$ directly stimulates mineralization, a process in which the nuclear VDR plays a central role. In addition, the current study provides evidence that C24-hydroxylation per se does not lead to an inactive vitamin D metabolite. Hydroxylation of 1α,25-$(OH)_2D_3$ at the C24 position does not impair its activity and also the C24-hydroxylated metabolite of 25-$(OH)D_3$ is active in human osteoblasts. Like 1α,25-$(OH)_2D_3$, 1α,24R,25-$(OH)_3D_3$ and 24R,25-$(OH)_2D_3$ directly stimulate mineralization and osteoblast differentiation via the nuclear VDR.

So far studies towards the direct effects of vitamin D on mineralization of cultured osteoblasts have been ambiguous. Mouse studies showed that 1α,25-$(OH)_2D_3$ could only weakly induce mineralization in primary osteoblast cultures [Shevde et al., 2002] and that only in the presence of dexamethasone 1α,25-$(OH)_2D_3$ treatment resulted in an osteoblastic phenotype in NIH3T3 cells [Shui and Scutt, 2002].

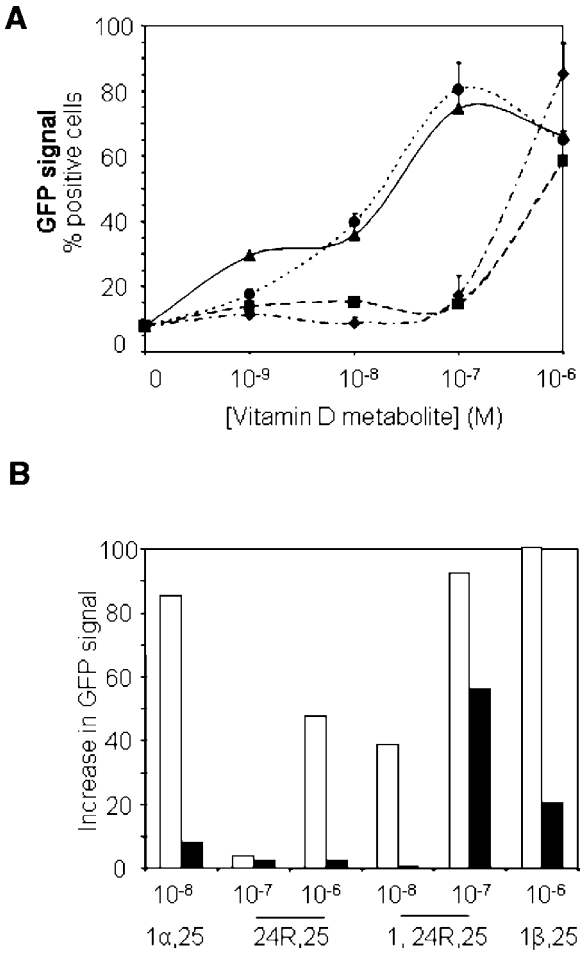

Fig. 11. Effect of vitamin D metabolites on gene transcription. **A**: Dose-response effect of 1α,25-$(OH)_2D_3$ (▲), 24R,25-$(OH)_2D_3$ (■), 1,24R,25-$(OH)_2D_3$ (●), and 1β,25-$(OH)_2D_3$ (◆) and (**B**) inhibition by the nuclear vitamin D receptor antagonist ZK159222 (vehicle □, ZK159222 ■). MG-63 cells were stably transfected with the *GFP* gene under control of the human OC promoter (OC.12).

For human bone marrow stromal cells a single report showed that 1α,25-$(OH)_2D_3$ only in combination with hepatocyte growth factor induced mineralization [D'Ippolito et al., 2002].

In an attempt to resolve the issue of direct regulation of mineralization by 1α,25-$(OH)_2D_3$ in the human context we choose to study the SV-HFO human osteoblast cell line. This is an in vitro differentiating and mineralizing fetal osteoblast cell line and serves as an excellent tool to study hormonal effects, as these might be restricted to particular stages of osteoblast differentiation [Chiba et al., 1993; Arts et al., 1997; Janssen et al., 1999; Weyts et al., 2003]. The current study provides further evidence that osteoblast responsiveness can be differentiation stage dependent. The stimulatory effect of 1α,25-$(OH)_2D_3$ on ALP activity was observed, especially during the period when the extracellular matrix was formed and matured (days 9–14) and just prior or overlapping with the start of the mineralization process. This is in accordance with previous findings of increased ALP activity by 1α,25-$(OH)_2D_3$ at proliferative stages of human primary osteoblasts and not in more confluent and differentiated cells [Siggelkow et al., 1999]. These differentiation-dependent actions of 1α,25-$(OH)_2D_3$ might reflect and explain the differences in effects observed by various osteoblastic cell lines representing different stages of development [Owen et al., 1991; Shalhoub et al., 1998; van Leeuwen et al., 2001b].

We demonstrate that 1α,25-$(OH)_2D_3$ enhances the mineralization via direct action on human osteoblasts. This proves that, in addition to an indirect way via regulation of calcium (re)absorption, 1α,25-$(OH)_2D_3$ also directly facilitates the mineralization process, which supports an earlier observation [Miyahara et al., 2002b]. Although the exact mechanism is unclear, 1α,25-$(OH)_2D_3$-stimulated ALP activity might play an important role. ALP has been shown to be important for the process of mineralization [Narisawa et al., 1997; Hui and Tenenbaum, 1998; Wennberg et al., 2000]. As expected, 1α,25-$(OH)_2D_3$ stimulated OC production via an increased mRNA expression. A second major observation of the current study concerns the effects of C24-hydroxylated vitamin D metabolites on human osteoblasts. Here we show that 24R,25-$(OH)_2D_3$ has similar effects as 1α,25-$(OH)_2D_3$ on human osteoblasts. 24R,25-$(OH)_2D_3$ stimulated mineralization and ALP activity are suggestive for a similar mechanism as 1α,25-$(OH)_2D_3$. Also OC production is stimulated via increased OC gene expression. This is in line with an OC VDRE reporter study [Uchida et al., 1994] and also it has been found that 24R,25-$(OH)_2D_3$ enhanced the 1α,25-$(OH)_2D_3$ stimulated OC production in human osteoblastic cells [Yamamoto et al., 1998]. The current data show that 24R,25-$(OH)_2D_3$ was active during the same stages of differentiation as 1α,25-$(OH)_2D_3$. Other studies have concluded that the effects of 24R,25-$(OH)_2D_3$ were on less mature cells than the effects of 1α,25-$(OH)_2D_3$ [Boyan et al., 2001, 2002]. However, these studies were either on different cell types, chondrocytes [Boyan et al., 2001], or done with different osteoblastic cell

lines representing restricted osteoblast differentiation stages [Boyan et al., 2002].

Another important C24-hydroxylated metabolite is the direct 1α,25-$(OH)_2D_3$ derivative 1α,24R,25-$(OH)_3D_3$. This metabolite has not been studied thoroughly in human bone research, but beneficial effects on bone have been found in rat studies [Rambeck et al., 1988; Erben et al., 1997]. In the current study we found that 1α,24R,25-$(OH)_3D_3$ stimulates human osteoblast differentiation and mineralization, which was differentiation stage and dose dependent. Compared to the effects of 1α,25-$(OH)_2D_3$ and 24R,25-$(OH)_2D_3$ by using equimolar concentrations, this metabolite was equipotent to 1α,25-$(OH)_2D_3$ and significantly more potent than 24R,25-$(OH)_2D_3$ in stimulating ALP activity, OC expression, and enhancing mineralization. Biological activity of the 24-hydroxylated metabolites was supported by data obtained with two other human osteoblast-like cell lines. Comparison of 24R, 25-$(OH)_2D_3$ and 1α,24R,25-$(OH)_3D_3$ shows that the 1α-hydroxylation is very important for the eventual activity of the vitamin D metabolites, which is conceivable considering the significance of the C-1α position for VDR binding [Bouillon et al., 1995; Muralidharan et al., 1997]. Comparison of 1α,25-$(OH)_2D_3$ and 1α,24R,25-$(OH)_3D_3$ on the other hand shows that hydroxylation at the C24 position per se does not impair the biological activity of 1α,25-$(OH)_2D_3$. An effect of C24 hydroxylation on 1α,25-$(OH)_2D_3$ action is thus dependent on the velocity of the subsequent steps in the degradation pathway following C24-hydroxylation. Not much is known about this and also whether the velocities of these steps might differ between cell types.

It has been demonstrated that osteoblasts show activity of 24-hydroxylase [Armbrecht et al., 1998; Schroeder et al., 2003], and over 20 years ago, it has been shown that osteoblasts also have 1α-hydroxylase enzyme activity [Howard et al., 1981], however so far this has never been confirmed. It is therefore possible that the observed actions of 1α,25-$(OH)_2D_3$ and 24R,25-$(OH)_2D_3$ described in this study can be partly due to the action of 1α,24R,25-$(OH)_3D_3$ to which they might be metabolized. However, it has previously been shown [Miyahara et al., 2002a] and confirmed by our HPLC analyses (data not shown) that SV-HFO cells have a low 24-hydroxylase capacity thereby making the latter less likely.

So far, the action of 1α,25-$(OH)_2D_3$ is considered to take place via binding to the VDR, a member of the nuclear steroid receptor family, and involves direct regulation of gene transcription [Haussler et al., 1995, 1997; MacDonald et al., 2001]. However, reports have come forward that there exists a membrane receptor for 1α,25-$(OH)_2D_3$ as well as a separate one for 24R,25-(OH) [Pedrozo et al., 1999; Nemere et al., 2002; Norman et al., 2002b; Berdal et al., 2003]. Membrane receptors for vitamin D have been reported in osteoblasts and expression of the membrane-associated rapid response steroid-binding protein for 1α,25-$(OH)_2D_3$ has been shown to be associated with biomineralization [Boyan et al., 2002; Mesbah et al., 2002]. For 24R,25-$(OH)_2D_3$ the existence of a membrane receptor has been suggested in membranes of healing fracture callus [Seo et al., 1996; Kato et al., 1998a,b]. We used the nuclear VDR antagonist ZK159222 to test whether the effects of 1α,25-$(OH)_2D_3$ and the 24-hydroxylated metabolites are mediated via the nuclear VDR. It has been described that co-treatment of 1α,25-$(OH)_2D_3$ with a 100-fold higher concentration of ZK159222 resulted in a prominent antagonistic effect in different cell types, including SaOS-2 human osteogenic sarcoma cells. The antagonistic effect is obtained due to a stabilization of an antagonistic conformation of the ligand-binding domain of the VDR within VDR–RXR–VDRE complexes, which then inhibits the interaction of the VDR with co-activator proteins and an induction of transactivation [Herdick et al., 2000a,b; Toell et al., 2001]. All effects of 1α,25-$(OH)_2D_3$ and the C24-hydroxylated metabolites were inhibited by ZK159222 demonstrating the involvement of the nuclear VDR. The effects of 24R,25-$(OH)_2D_3$ were completely blocked by ZK159222 indicating the exclusive involvement of the nuclear VDR. This is interesting as most studies on 24R,25-$(OH)_2D_3$ have reported that the action of 24R,25-$(OH)_2D_3$ is mediated via a membrane-bound receptor, distinct from the one for 1α,25-$(OH)_2D_3$ [Pedrozo et al., 1999; Boyan et al., 2002]. The effects of 1α,25-$(OH)_2D_3$ and 1α,24R,25-$(OH)_3D_3$ were strongly inhibited by ZK159222, though not completely reduced to control levels of ALP activity and OC expression. This might be due to the concentrations

of the compounds used and receptor affinity. However, the concentrations of 24R,25-$(OH)_2D_3$ (10^{-6} M) and 1α,25-$(OH)_2D_3$ (10^{-8} M) as well as 1α,24R,25 (10^{-7} M) used were in accordance to their relative nuclear VDR affinities [Wang et al., 1993; Bouillon et al., 1995], which makes this latter explanation less likely. Alternatively, it might indicate that 1α,25-$(OH)_2D_3$ and 1α,24R,25-$(OH)_3D_3$ use an additional (membrane bound) receptor.

The involvement of a membrane receptor was investigated using 1β,25-$(OH)_2D_3$ which has an inversion of the 1α to the 1β orientation of the hydroxyl on carbon-1. 1β,25-$(OH)_2D_3$ has been reported to function as an antagonist to the 1α,25-$(OH)_2D_3$ rapid actions on stimulation of intestinal Ca^{2+} transport involving opening of voltage-gated Ca^{2+} channels in ROS 17/2.8 cells, insulin release in rat pancreatic islets, and activation of MAP-kinase in human leukemic cells [Baran et al., 1994; Norman et al., 2002a]. It has been reported that 1β,25-$(OH)_2D_3$ could not block genomic 1α,25-$(OH)_2D_3$ induction of chick calbindin-D_{28k}, OC expression by MG-63 cells, and HL-60 cell differentiation [Norman et al., 1993]. Our current data on 1β,25-$(OH)_2D_3$ showing no inhibition of the different vitamin D metabolites does not warrant to conclude about the involvement of a membrane receptor. Besides antagonism, a weak agonistic effect of 1β,25-$(OH)_2D_3$ has been reported [Norman et al., 1993]. We have found that this compound had strong effects on osteoblast differentiation itself, shown by an increase in ALP activity, OC production and mineralization in SV-HFO cells and OC and 24-hydroxylase mRNA expression in the human osteoblasts MG-63 and SaOS-2. Interestingly, these effects were blocked by ZK159222, showing involvement of the nuclear receptor for 1β,25-$(OH)_2D_3$.

In conclusion, the current study shows the direct stimulation of mineralization by 1α,25-$(OH)_2D_3$ via activation of its nuclear VDR in human osteoblasts. These direct effects on mineralization as well as the stimulation of human osteoblasts differentiation may form or strengthen the basis for the development and action of vitamin D analogs for application in bone disorders [Shevde et al., 2002]. Adding to this is the observation that the presumed C-24-hydroxylated "degradation" products of vitamin D also have direct effects on mineralization and the differentiation of human osteoblasts.

ACKNOWLEDGMENTS

The authors thank Mr. W. Klootwijk for expert assistance with HPLC analyses.

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