Vitamin D metabolites regulate osteocalcin synthesis and proliferation of human bone cells *in vitro*


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ABSTRACT

The effects of six natural vitamin D metabolites of potential biological and therapeutic interest, 1,25-dihydroxyvitamin D$_3$ (1,25-(OH)$_2$D$_3$), 25-hydroxyvitamin D$_3$ (25-OH-D$_3$), 25R,25-dihydroxyvitamin D$_3$ (24R,25-(OH)$_2$D$_3$), 1,25,26-trihydroxyvitamin D$_3$ (1,24R,25-(OH)$_3$D$_3$), 25S,26-dihydroxyvitamin D$_3$ (25S,26-(OH)$_2$D$_3$) and 1,25S,26-trihydroxyvitamin D$_3$ (1,25S,26-(OH)$_3$D$_3$) on cell replication and expression of the osteoblastic phenotype in terms of osteocalcin production were examined in cultured human bone cells. At a dose of 5 x 10$^{-12}$ mol/l, 1,25-(OH)$_2$D$_3$ stimulated cell proliferation, whereas at higher doses (5 x 10$^{-9}$-5 x 10$^{-6}$ mol/l) cell growth was inhibited in a dose-dependent manner. The same pattern of effects was seen for the other metabolites in a rank order of potency: 1,25-(OH)$_2$D$_3$ > 1,25S,26-(OH)$_2$D$_3$ > 1,24R,25-(OH)$_3$D$_3$ > 25S,26-(OH)$_2$D$_3$ = 24R,25-(OH)$_2$D$_3$ = 25-OH-D$_3$. Synthesis of osteocalcin was induced by 1,25-(OH)$_2$D$_3$ in doses similar to those required to inhibit cell proliferation. Biphasic responses were observed for some of the metabolites in terms of osteocalcin synthesis, inhibitory effects becoming apparent at 5 x 10$^{-6}$ mol/l. The cells did not secrete osteocalcin spontaneously. These results indicate that vitamin D metabolites may regulate growth and expression of differentiated functions of normal human osteoblasts.

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INTRODUCTION

It is generally accepted that bone is a target tissue for vitamin D metabolites. Thus, physiological levels of 1,25-dihydroxyvitamin D$_3$ (1,25-(OH)$_2$D$_3$) stimulate bone resorption *in vitro* (Raisz, Trummel, Holick & DeLuca, 1972) and 1,25-(OH)$_3$D$_3$ may play a role in bone growth and repair (Brumbaugh, Speer & Pitt, 1982). This latter effect may require another vitamin D metabolite, 24,25-dihydroxyvitamin D$_3$ (Ornord, Goodwin, Noff & Edelstein, 1978). Although the physiological function of metabolites other than 1,25-(OH)$_2$D$_3$ remains controversial, effects of vitamin D metabolites in pharmacological doses may be of therapeutic importance. Specific cytoplasmic 1,25-(OH)$_2$D$_3$ receptors have been demonstrated in a variety of cultured osteoblast-like cells (Chen, Hirsh & Feldman, 1979; Manolagas, Haussler & Deftos, 1980; Partridge, Frampton, Eisman et al. 1980). Other vitamin D metabolites bind to this receptor with lower affinity (Mellon & DeLuca, 1980; Manolagas & Deftos, 1981). The ability of the metabolites to stimulate bone resorption *in vitro* is well correlated with their affinity for the osteoblastic 1,25-(OH)$_2$D$_3$ receptor (Stern, 1981).

To explain the apparent paradox that the potent bone-resorbing hormone 1,25-(OH)$_2$D$_3$ influences the activity of the bone-forming osteoblasts, Rodan & Martin (1981) hypothesized that osteoblasts may play a pivotal role in hormone-regulated bone resorption. Theoretically, inhibition of the proliferation of osteoblasts could lead to defects in the putative contiguous layer of osteoblasts covering bone matrix (Jones & Boyde, 1976) exposing matrix constituents to resorbing osteoclasts. Interestingly, the major non-collagenous protein of bone matrix, osteocalcin (bone Gla protein), has been shown to be chemotactic to monocytes (Malone, Teitelbaum, Griffin et al. 1982), the presumptive osteoclast precursors. This protein
has also been implicated as an inhibitor of mineralization (Price, 1983). 1,25-Dihydroxyvitamin D$_3$ stimulates the synthesis of osteocalcin by cultured osteoblast-like cells (Price & Baukol, 1980; Beresford, Gallagher, Poser & Russell, 1984) suggesting that osteocalcin may mediate some of the effects of 1,25-(OH)$_2$D$_3$ on bone. Thus, vitamin D-induced reduction of osteoblastic cell number uncovering matrix osteocalcin and other chemotactic constituents combined with de-novo synthesis of osteocalcin may lead to the known in-vitro effect of vitamin D metabolites, activation of bone-resorbing cells.

The present study was designed to establish the effects of vitamin D metabolites of possible therapeutic importance on cell proliferation and synthesis of osteocalcin in a recently developed system of cultured human bone cells exhibiting certain phenotypic features of osteoblasts (Gallagher, Beresford, McGuire et al. 1984). The actions of 1,25-(OH)$_2$D$_3$, 25-hydroxyvitamin D$_3$ (25-OH-D$_3$), 24R,25-dihydroxyvitamin D$_3$ (24R,25-(OH)$_2$D$_3$), 25S,26-dihydroxyvitamin D$_3$ (25S,26-(OH)$_2$D$_3$), 1,24R,25-trihydroxyvitamin D$_3$ (1,24R,25-(OH)$_3$D$_3$) and 1,25S,26-trihydroxyvitamin D$_3$ (1,25S,26-(OH)$_3$D$_3$) were compared to define a possible rank order of potency. Furthermore, possible qualitative differences between metabolites were considered. This is the first comparative study of effects of physiological vitamin D metabolites on human bone-derived cells in vitro.

**Isolation and culture of human bone cells**

This recently developed technique has been reported previously (Beresford et al. 1984; Gallagher et al. 1984). In this system cells are present which express a stable osteoblast-like phenotype in terms of responsiveness to parathyroid hormone and vitamin D metabolites and capacity to produce collagen, alkaline phosphatase and osteocalcin.

Specimens of trabecular bone obtained at surgery were seeded onto 9 cm culture dishes containing medium with 10% (v/v) fetal calf serum, 2 mm-L-glutamine, 50 units penicillin/ml and 15 μg streptomycin/ml. Cell outgrowth was seen by day 7 and the cells reached confluence by day 21–28. Cells were passaged by detachment with 0.5 g trypsin/l and 0.2 g EDTA/l into 3.5 cm multiwells at a density of 5 x 10$^4$ cells/well. After 3 days the cells were washed with serum-free medium after which medium supplemented with either 5% (v/v) fetal calf serum or 5% charcoal-extracted human serum, 50 μg/ml ascorbate and 10$^{-3}$ m-vitamin K (menadione), and containing various concentrations of vitamin D metabolites was added. The experimental incubation period was 48 h.

**Extraction of 1,25-(OH)$_2$D$_3$ from serum**

Human serum was incubated overnight with 0.1 μCi [$_3$H]1,25-(OH)$_2$D$_3$ per ml. Extraction of the sterol was accomplished by adding dry Norit-A charcoal to a final concentration of 10 mg/ml serum. This was followed by shaking at room temperature for 2 h and recovery of treated serum by centrifugation (50,000 g for 15 min). This treatment removed >99% of the radioactivity from the serum.

**Measurement of cell numbers**

Cells were plated out into 3.5 cm multiwells at a density of 1.25 x 10$^4$ cells/well. On culture day 1 the medium was removed and unattached cells were washed away with medium. Subsequently, the medium containing metabolites was changed every other day. Cells were harvested by detachment with trypsin–EDTA and counted after 10 days using a Coulter counter. In one experiment the cells were reincubated for 3 days before being incubated with 1,25-(OH)$_2$D$_3$ in 1,25-(OH)$_2$D$_3$-stripped fetal calf serum for 48 h.

**[$_3$H]Thymidine incorporation**

At the start of the incubation period 1-0 μCi [$_3$H]thymidine was added to each well. After 48 h the medium was removed and the cells were washed extensively with cold phosphate-buffered saline. The cells were detached from the wells with trypsin–EDTA and transferred to tubes. Precipitation was carried out by addition of trichloroacetic acid (TCA) to a final

**MATERIALS AND METHODS**

**Materials**

Crystalline 1,25-(OH)$_2$D$_3$, 25-OH-D$_3$, 24R,25-(OH)$_2$D$_3$, 25S,26-(OH)$_2$D$_3$, 1,24R,25-(OH)$_3$D$_3$ and 1,25S,26-(OH)$_3$D$_3$ were generous gifts from Dr M. R. Uskovic, Dr J. J. Partridge and Dr E. G. Baggioni of Hoffman LaRoche Co. (Nutley, NJ, U.S.A.). The metabolites were dissolved in absolute ethanol and added to the cultures so that the concentration of ethanol in the medium was below 0.01%. Equal amounts of ethanol were added to control media.

Tissue-culture medium (Eagle's modified essential medium), fetal calf serum, L-glutamine, l-ascorbic acid, vitamin K, trypsin–EDTA and penicillin–streptomycin were purchased from Gibco-Europe Ltd, Paisley, Strathclyde. [6-$_3$H]Thymidine (30 Ci/mmol) and [26,27(n)-$_3$H]1,25-(OH)$_2$D$_3$ (120 Ci/mmol) were supplied by Amersham International plc, Bucks. Goat anti-rabbit gammaglobulin was obtained from Calbiochem, Bishop's Stortford, Herts. Tissue-culture dishes were from Falcon, Oxford. All other chemicals and reagents were of analytical grade.

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volume of 7.5% and left overnight at 4°C, followed by centrifugation and a wash with 7.5% TCA. The material was digested in 1M-KOH at 60°C for 2 h. The radioactivity of the TCA-insoluble material was counted in a liquid scintillation counter (LKB 1218 Rack beta) after neutralization with HCl.

Assay of osteocalcin
Osteocalcin was measured by a specific radioimmunoassay using an antibody raised in rabbits to purified osteocalcin isolated from calf bone. This antibody cross-reacts completely with osteocalcin extracted from human bone. Bovine osteocalcin was used for the preparation of the standard and the 125I-labelled tracer. Antibody/osteocalcin complexes were separated from free iodinated tracer by a second-antibody method using goat anti-rabbit gammaglobulin. The assay buffer consisted of 0.14-M NaCl, 0.025-M EDTA, 0.01-M Tris, pH 7.4. The radioimmunoassay procedures have been described in detail by Price & Nishimoto (1980).

Statistical analysis
Statistical analysis was by analysis of variance. Significant differences between groups were calculated using the standard error of differences between means derived from the residual variation.

RESULTS
Effects of vitamin D metabolites on human bone cell proliferation in vitro
In preliminary experiments cells were incubated with and without 1,25-(OH)2D3 (10^{-11}–10^{-8} mol/l for 10 days) (Fig. 1). Inhibition of cell proliferation by 1,25-(OH)2D3 was seen in doses above 10^{-10} mol/l.

As thymidine incorporation may be assumed to reflect DNA synthesis in S phase, the method of measuring [3H]thymidine incorporation was chosen to determine effects of vitamin D metabolites on cell replication over a wide dose range (Fig. 2). 1,25-Dihydroxyvitamin D3 progressively inhibited [3H]thymidine incorporation in doses above 5 x 10^{-10} mol/l. At the highest concentration tested (5 x 10^{-7} mol/l), [3H]thymidine incorporation was reduced to 40% of control. Similar dose–response curves were seen for the other metabolites in a rank order of potency: 1,25-(OH)2D3 > 1,25S,26-(OH)2D3 = 1,24R, 25-(OH)3D3 > 25S,26-(OH)2D3 = 24R, 25-(OH)2D3 = 25-OH-D3. This order of potency was confirmed in successive experiments using incomplete dose–response curves (see Table 1). The relative potency of 25-OH-D3 and 24R,25-(OH)2D3 with
Table 1. Effect of vitamin D metabolites on incorporation of [3H]thymidine in cultured human osteoblast-like cells. Values are means ± S.E.M.; n = 4

<table>
<thead>
<tr>
<th>[3H]Thymidine incorporation (% of control)</th>
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<tr>
<td>5 x 10^{-10} mol/l</td>
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<tr>
<td>-------------------</td>
</tr>
<tr>
<td>1,25-(OH)\textsubscript{2}D\textsubscript{3}</td>
</tr>
<tr>
<td>1,25S,26-(OH)\textsubscript{2}D\textsubscript{3}</td>
</tr>
<tr>
<td>1,24R,25-(OH)\textsubscript{2}D\textsubscript{3}</td>
</tr>
<tr>
<td>25S,26-(OH)\textsubscript{2}D\textsubscript{3}</td>
</tr>
<tr>
<td>24R,25-(OH)\textsubscript{2}D\textsubscript{3}</td>
</tr>
<tr>
<td>25-OH-D\textsubscript{3}</td>
</tr>
<tr>
<td>Control = 100 ± 2.53</td>
</tr>
</tbody>
</table>

*P < 0.001 compared with control (analysis of variance).

Culture conditions were as described in Fig. 2.

1,25-(OH)\textsubscript{2}D\textsubscript{3} = 1.25-dihydroxyvitamin D\textsubscript{3} (1,25-dihydroxycholecalciferol; calcitriol); 24R,25-(OH)\textsubscript{2}D\textsubscript{3} = 24R,25-dihydroxyvitamin D\textsubscript{3}; 25S,26-(OH)\textsubscript{2}D\textsubscript{3} = 25S,26-dihydroxyvitamin D\textsubscript{3}; 1,24R,25-(OH)\textsubscript{2}D\textsubscript{3} = 1,24R,25-trihydroxyvitamin D\textsubscript{3}; 25-OH-D\textsubscript{3} = 25-hydroxyvitamin D\textsubscript{3}.

![Figure 3](image)

**FIGURE 3. Effects of vitamin D metabolites on production of osteocalcin by human bone cells in vitro.** Cell cultures were incubated with vitamin D metabolites for 48 h, after which the media were removed and the content of osteocalcin was determined. Values were corrected for subtraction of the amount of endogenous osteocalcin in medium due to the presence of fetal calf serum. Each point is the mean of duplicate measurements, which differed by less than 10%. The stippled area represents mean (± S.E.M.) control, n = 6 (control = cultures without addition of metabolite). 1,25-Dihydroxyvitamin D\textsubscript{3} (○); 1,24R,25-trihydroxyvitamin D\textsubscript{3} (□); 1,25S,26-trihydroxyvitamin D\textsubscript{3} (△); 25-hydroxyvitamin D\textsubscript{3} (◇); 24R,25-dihydroxyvitamin D\textsubscript{3} (■); 25S,26-dihydroxyvitamin D\textsubscript{3} (●).

Respect to 1,25-(OH)\textsubscript{2}D\textsubscript{3} was between 1:100 and 1:1000. In lower concentration some of the metabolites, but not 1,25-(OH)\textsubscript{2}D\textsubscript{3}, were seen to stimulate cell proliferation significantly (P < 0.01) (Fig. 1 and Table 1). Inasmuch as the serum concentration of 1,25-(OH)\textsubscript{2}D\textsubscript{3} may be as high as 10^{-9} mol/l (Stern, 1980), it seemed possible that high levels of endogenous 1,25-(OH)\textsubscript{2}D\textsubscript{3} in fetal calf serum might obscure stimulatory effects of lower levels of 1,25-(OH)\textsubscript{2}D\textsubscript{3}. Additional experiments were performed in medium containing human serum stripped of 1,25-(OH)\textsubscript{2}D\textsubscript{3} by charcoal extraction.

In these studies low concentrations (5 x 10^{-12} mol/l) of 1,25-(OH)\textsubscript{2}D\textsubscript{3} stimulated thymidine incorporation (136 ± 7% (s.e.m.) of control, n = 6, P < 0.001) whereas at higher concentrations (5 x 10^{-9} mol/l) a significant inhibition was observed (76 ± 5% of control, n = 6, P < 0.001). This correlated well with the cell number data which showed a stimulatory effect of 1,25-(OH)\textsubscript{2}D\textsubscript{3} (10^{-11} mol/l) during a 48-h incubation period in 5% 1,25-(OH)\textsubscript{2}D\textsubscript{3}-stripped fetal calf serum (132 ± 8% of control, n = 6; P < 0.001).

**Effects of vitamin D metabolites on osteocalcin synthesis by human bone cells in vitro**

Tissue-culture medium contained immunoreactive osteocalcin derived from supplement. Incubation of medium at 37 °C for 48 h in the absence of cells did not affect the osteocalcin content of the medium.

No significant production of osteocalcin was detected by bone cells under basal conditions. However, when 1,25-(OH)\textsubscript{2}D\textsubscript{3} was added to the cultures there was a dose-dependent increase in immunoreactive osteocalcin over a range of 5 x 10^{-10}–5 x 10^{-7} mol/l (Fig. 3). The other 1-hydroxylated metabolites and 25S,26-(OH)\textsubscript{2}D\textsubscript{3} also induced synthesis of osteocalcin in a dose-dependent manner but with lower potencies than 1,25-(OH)\textsubscript{2}D\textsubscript{3} (Fig. 3).

In addition to extracting 1,25-(OH)\textsubscript{2}D\textsubscript{3}, treatment of human serum with charcoal also removed immunoreactive osteocalcin. Production of osteocalcin by cells incubated in medium supplemented with 5% charcoal-extracted human serum amounted to
TABLE 2. Effect of vitamin D metabolites on production of osteocalcin by human osteoblast-like cells. Values are means ± S.E.M.; n = 4.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Net osteocalcin production (ng/well)</th>
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<tr>
<td></td>
<td>5 × 10⁻¹⁹ mol/l</td>
</tr>
<tr>
<td>1,25-(OH)₂ D₃</td>
<td>—</td>
</tr>
<tr>
<td>1,25S,26-(OH)₂ D₃</td>
<td>9.0 ± 0.62*</td>
</tr>
<tr>
<td>1,24R,25-(OH)₂ D₃</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>25S,26-(OH)₂ D₃</td>
<td>2.1 ± 0.34*</td>
</tr>
<tr>
<td>24R,25-(OH)₂ D₃</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>25-OH-D₃</td>
<td>&lt;0.5</td>
</tr>
</tbody>
</table>

*P < 0.001 compared with controls (analysis of variance).
Culture conditions were as described in Fig. 3. Negative values resulted from cell-mediated destruction of immunoreactive osteocalcin in tissue culture medium due to the presence of fetal calf serum. 1,25-(OH)₂ D₃ = 1,25-dihydroxyvitamin D₃, 25S,26-(OH)₂ D₃ = 25S,26-dihydroxyvitamin D₃; 1,24R,25-(OH)₂ D₃ = 24R,25-dihydroxyvitamin D₃; 1,24R,25-(OH)₂ D₃ = 1,24R,25-trihydroxyvitamin D₃; 1,25S,26-(OH)₂ D₃ = 1,25S,26-trihydroxyvitamin D₃; 25-OH-D₃ = 25-hydroxyvitamin D₃.

18 ng/well per 48 h on treatment with 5 × 10⁻²⁹ M-1,25-(OH)₂ D₃. No synthesis was detected in untreated cultures.

At higher doses of these metabolites (the 1-hydroxylated metabolites and 25S,26-(OH)₂ D₃) the production of osteocalcin was reduced towards control levels (Fig. 3). This may have been due to inhibition of synthesis or, alternatively, stimulation of osteocalcin breakdown might have occurred. The dose range of metabolites inducing synthesis of osteocalcin correlated well with the concentrations shown to inhibit cell proliferation (Fig. 2).

In contrast to the other metabolites, dose-dependent synthesis of osteocalcin was not observed in response to 24R,25-(OH)₂ D₃ or 25-OH-D₃. At a concentration of 5 × 10⁻⁹ mol/l there was a low level of production in response to these metabolites (Fig. 3 and Table 2). At high doses (5 × 10⁻⁶ mol/l) 25-OH-D₃ and 24R,25-(OH)₂ D₃ significantly reduced the levels of immunoreactive osteocalcin derived from fetal calf serum in the medium (P < 0.01) (Fig. 3 and Table 2). This breakdown appeared to be cell mediated since no such effect was observed in medium incubated in the absence of cells.

DISCUSSION

The results of this investigation demonstrate that isolated human bone cells cultured in the presence of vitamin D metabolites can undergo marked biochemical changes in terms of rate of replication and expression of phenotype. The vitamin D metabolite-induced production of osteocalcin was shown to be associated with a partial inhibition of cell growth. Most striking, however, was the finding that secretion of osteocalcin was not stimulated effectively by 25-OH-D₃ and 24R,25-(OH)₂ D₃. These metabolites have been implicated as specific stimulators of bone growth and mineralization (Ornay et al. 1978). Interestingly, it has been suggested that osteocalcin may regulate mineral deposition in bone (Price, 1983).

In contrast to investigations reported on rodent osteoblast-like osteosarcoma cells, indicating a basal production of osteocalcin, human osteoblast-like cells did not synthesize osteocalcin spontaneously (Beresford et al. 1984 and this report). Thus, it appears that in normal human osteoblast-like cells, production of osteocalcin is absolutely dependent upon vitamin D metabolites. The dose–response curves to the metabolites investigated included the normal range of serum levels of the sterols (Stern, 1980), suggesting that regulation of the production of osteocalcin might partly explain the mechanism of action of vitamin D. Osteocalcin may serve as a local messenger in the process of osteocalc activation. The fact that osteocalcin is chemotactic to monocyte osteoclast precursors (Mundy & Poser, 1983) supports this idea.

As to the regulation of proliferation of human osteoblast-like cells, the rank order of potency of metabolites correlated well with the data on competition for cytosolic 1,25-(OH)₂ D₃ binding sites (Stern, 1981). However, whereas the relative potency of 25-OH-D₃ and 24R,25-(OH)₂ D₃ with respect to 1,25-(OH)₂ D₃ has been reported to be 1:1000 (Stern, 1981), we have found a ratio between 1:100 and 1:1000. There are a number of possible reasons for this disparity. First, the relative potencies of metabolites in a culture system such as this are influenced by their binding to serum proteins including vitamin D-binding protein (Haddad & Chyu, 1971) and possibly by differential rates of transport across the cell membrane. Furthermore, the possibility that human bone cells contain 1- and 24-hydroxylases (Howard, Turner,
Sherrard & Baylink, 1981) has to be considered. Finally, in addition to receptor-mediated events it is possible that there are non-specific effects on the cell due to the sterol structure, which would not reflect the relative affinities of the metabolites for the receptor.

We found a stimulation of osteoblastic cell proliferation at low levels of the physiological dose range of the metabolites, indicating a possible trophic effect on bone formation. In the same line of thought, an inhibition of osteoblastic cell proliferation in hypocalcaemic disease states coupled with a stimulation of the putative osteoclast-chemotactic protein, osteocalcin, might be of importance in initiating resorptive activity.

In conclusion, (i) all metabolites investigated inhibited osteoblastic cell proliferation in vitro at the upper limits of the physiological concentration range, whereas cell replication was stimulated at lower physiological doses. This indicates that vitamin D metabolites over a narrow dose range may modify the metabolism of osteoblasts in opposite directions. This may be important in the subtle regulation of serum calcium in vivo. (ii) Osteocalcin synthesis by human osteoblast-like cells in vitro is completely dependent upon the presence of vitamin D metabolites. However, no effective induction was seen in the presence of 25-OH-D$_3$ and 24R,25-(OH)$_2$D$_3$, suggesting that the possible mechanism of action of these metabolites is not mediated via this protein in vitro. Production of osteocalcin was stimulated by concentrations at the upper end of the physiological levels of 1,25-(OH)$_2$D$_3$. Since osteocalcin attracts osteoclast precursors in vitro, it is possible that induction of bone resorption may be initiated in states of hypocalcaemia in vivo in this way.

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