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EFFECT OF 1,25-DIHYDROXYVITAMIN D_3 ON ALKALINE PHOSPHATASE ACTIVITY AND COLLAGEN SYNTHESIS IN OSTEOBLASTIC CELLS, CLONE MC3T3-E1[1]

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SUMMARY: A stimulative effect of 1,25-dihydroxyvitamin D_3 was tested on osteoblastic cells, clone MC3T3-E1, cultured in serum-free medium with 0.1% bovine serum albumin. This steroid increased alkaline phosphatase activity in a dose-related fashion. The steroid also stimulated dose-dependently collagen and non-collagen protein syntheses, their maximal effects being observed at 12 and 24 h, respectively. The incorporation of [^{3}H]-proline into collagen or non-collagen protein in cells exposed to this steroid for 12 h was 2.9 or 1.9-fold over that of control cultures, respectively. These results strongly indicate the stimulative effects of 1,25-dihydroxyvitamin D_3 on the differentiation of osteoblasts *in vitro*.

The effects of vitamin D_3 on bone metabolism have been viewed from the perspective of its potent effect on bone resorption, which has been demonstrated by *in vivo* and *in vitro* studies (1,2). On the other hand, other evidence has emerged in support of an anabolic role of vitamin D_3 in the skeleton (3,4). However, it remains unclear whether these anabolic effects on the skeleton are due to normalization of serum ion concentrations by the stimulatory effect of this steroid on intestinal absorption, or whether this steroid has a direct anabolic effect on bone collagen synthesis or mineralization, or both.

We have previously reported that physiological concentrations of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] stimulated alkaline phosphatase (ALP) activity in osteoblastic cells, clone MC3T3-E1 (5,6), in the presence of serum (7). However, in supplemented media, a detailed analysis of the mechanism underlying vitamin D_3 action on osteoblast differentiation is difficult

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because undefined factors in the medium may interfere. Therefore, cultivation of osteoblastic cells in serum-free medium should clarify how vitamin D_3 affects the cells. Moreover, to know the effect of $1,25(OH)_2D_3$ on osteoblasts, it is indispensable to assay collagen synthesis.

In this study we investigated the effect of $1,25(OH)_2D_3$ on clone MC3T3-E1 cells cultured in serum-free medium, judged by assays of ALP activity and collagen synthesis.

MATERIALS AND METHODS

Materials: $1,25(OH)_2D_3$ was generously provided by Dr. M. Kiyoki (Teijin Inst. for Bio-Medical Reserch, Tokyo). Bovine serum albumin (BSA) was from Sigma Chemical Co., St. Louis. α-MEM was purchased from Flow Laboratories, Rockville. Fetal bovine serum was from Irvine Scientific, Santa Ana. Tritiated proline (L-[3,4-^{3}H], 20-30 Ci/m mol) was obtained from New England Nuclear Corp., Boston.

Cell culture: Clone MC3T3-E1 cells were isolated from the MC3T3-E cell line which had been derived from newborn C57BL/6 mouse calvaria (5). 5×10^4 cells were plated in 35-mm plastic dishes in 2 ml of α-MEM containing 10% fetal bovine serum, incubated for 3 days, and were then cultured in serum-free medium supplemented with 0.1% BSA. After one day, the cells were transfered to media containing 0.1% BSA plus various concentrations of $1,25(OH)_2D_3$, and then were cultured for appropriate periods.

Assay of ALP activity and DNA and protein contents: After appropriate periods of cultivation, cells were washed 3 times with PBS(-), scraped into 2 ml of 0.2% Nonidet P-40 containing 1 mM $MgCl_2$, and sonicated for 5 min with a sonifier cell disruptor (Model UCD-100, Tosho, Yokohama). The sonicates were centrifuged for 10 min at 3,000 rpm, and the supernatants were used for the enzyme assay. ALP activity was assayed by the method of Lowry et al. (8), with p-nitrophenyl phosphate as substrate. DNA content was measured by a fluorometric method (9). Protein content was estimated by the method of Bradford (10).

Collagen synthesis assay: Cells in culture were treated with various concentrations of $1,25(OH)_2D_3$ for the appropriate time and then media were replaced with 1 ml of α-MEM containing 50 μg each of ascorbic acid and β-aminopropionotrile, and labeling with 10 μCi of [^{3}H]-proline was conducted for 3 h. At the end of the labeling period, the media were removed and the cells taken from the dishes by scraping. Proteins in both the medium and cell homogenate were precipitated with 10% trichloroacetic acid and 0.5% tannic acid (final concentrations). After centrifugation, the precipitates were washed 3 times with the same solution and twice ice-cold acetone. Collagenase-digestible protein and non-collagen protein were determined according to the procedures of Peterkofsky and Diegelmann (11).

RESULTS AND DISCUSSION

Clone MC3T3-E1 cells, plated at a initial density of 5×10^4 cells in 35-mm plastic dishes containing α-MEM with 10% fetal bovine serum, were cultured for 3 days until nearly confluent and then were cultured in serum-free medium with 0.1% BSA for one more day. In order to study the effect of $1,25(OH)_2D_3$ on ALP activity in the cells, they were further

Table 1. Effect of $1,25(OH)_2D_3$ on DNA and protein contents and ALP activity in clone MC3T3-E1 cells

		DNA (μg/dish)	protein (mg/dish)	ALP activity (units/μg DNA)	(units/mg protein)
Control		6.974±0.346	0.337±0.035	1.587±0.063	32.84±2.08
$1,25(OH)_2D_3$ (pg/ml)	0.2	6.662±0.350	0.329±0.048	1.798±0.099	36.41±1.98
	1.0	6.404±0.306	0.349±0.019	2.448±0.107*	44.95±6.43*
	5.0	6.256±0.414	0.383±0.022	4.455±0.245**	72.77±0.97**
	10.0	6.124±0.154	0.401±0.042	3.748±0.336**	57.23±3.52**

Values are means±SE for 5 dishes. *, $P<0.05$, **, $P<0.01$ compared to control

cultured in medium containing or lacking the steroid (5 pg/ml). The addition of the steroid increased significantly ALP activity as early as 12 h after its addition. The greatest differences between the control and steroid-treated cultures were observed at day one, at which time the activities of both cultures were 35.6±2.08 and 80.5±3.72 units/mg protein ($P<0.01$), respectively. This is 3 days earlier compared with the results obtained in previous work (7). Thus, the effects of various concentrations of $1,25(OH)_2D_3$ on DNA and protein content and ALP activity were investigated at day one after the steroid addition.

$1,25(OH)_2D_3$ had a slight but significant effect on protein content, but not on DNA content (Table 1). This steroid increased dose-dependently ALP activity in the cells up to concentrations of 5 pg/ml which are one-tenth of those observed in the cells cultured in medium containing 2% serum (7). The maximal effect obtained in the cells cultured in medium containing the steroid (5 pg/ml) was 2.2-fold above that of control cultures.

Next, we examined the effect of $1,25(OH)_2D_3$ on collagen and non-collagen protein syntheses in clone MC3T3-E1 cells. The maximal stimulation of collagen synthesis induced by 5 pg/ml of $1,25(OH)_2D_3$ occurred at 12 h, and then its synthesis decreased toward the control value at 48 h as shown in Fig. 1. On the other hand, non-collagen protein synthesis reached a maximum at 24 h. Finally, the effect of various concentrations of $1,25(OH)_2D_3$ increased their syntheses in a dose-related fashion with concentrations up to 5 pg/ml: the steroid led to the maximal increases in the

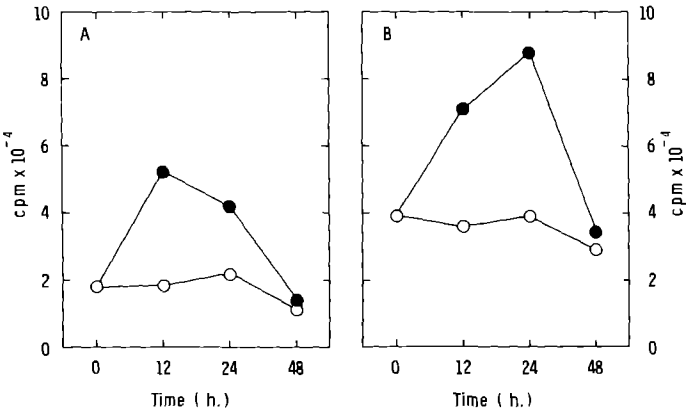

Fig. 1. Time course of the effect of $1,25(OH)_2D_3$ on collagen (A) and non-collagen protein (B) syntheses in clone MC3T3-E1 cells. Results are presented as cpm/dish for [^{3}H]-proline incorporated into both the medium and cell homogenate. Values are means for 5 dishes. ○, no addition; ●, $1,25(OH)_2D_3$ (5 pg/ml).

corporation of [^{3}H]-proline into collagen and non-collagen protein from 1.82±0.04 and 3.60±0.27 cpm 10^{-4}/dish to 5.29±0.40 and 7.10±0.27 cpm 10^{-4}/dish ($p<0.01$), respectively. These results indicate that $1,25(OH)_2D_3$ is closely linked to the differentiation of osteoblasts in vitro.

In combination with PTH and $24R,25(OH)_2D_3$, $1,25(OH)_2D_3$ stimulates chick embryonic bone calcification in organ cultures (12). Recently, Majeska and Rodan (13) have reported that $1,25(OH)_2D_3$ increases ALP activity in a clonal osteoblast-like osteosarcoma line. Also, we demonstrated the inductive effect of $1,25(OH)_2D_3$ on ALP activity in clone MC3T3-E1 cells (7). However, in these experiments other factors contained in chick embryonic extract (12) and serum (7,13) may affect the action of $1,25(OH)_2D_3$, and higher doses of the steroid were necessary for stimulation of an increase in ALP activity (9,13). Thus, to our knowledge, this is the first report that $1,25(OH)_2D_3$ alone stimulates increases in ALP activity and collagen synthesis in osteoblasts in vitro. Moreover, these stimulative effects were observed at one-tenth of the physiological dose of $1,25(OH)_2D_3$ (14). Other hormones, prostaglandins and insulin, also stimulate the differentiation of osteoblastic cells (15,16). Thus, $1,25(OH)_2D_3$ is by itself insufficient and needs other factors for maximal stimulation of osteoblast differen-

tiation, but our in vitro findings strongly suggest that this steroid may be involved in bone formation in vivo as well.

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