

## Role of 1,25-dihydroxycholecalciferol in growth-plate cartilage: Inhibition of terminal differentiation of chondrocytes *in vitro* and *in vivo*

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**ABSTRACT** The effects of vitamin D metabolites on alkaline phosphatase [ALPase; orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] activity, a marker of terminal differentiation, in chondrocyte cultures and growth plates *in vivo* were examined. In cultures of pelleted rabbit growth-plate chondrocytes, 1,25-dihydroxycholecalciferol (1,25-dihydroxyvitamin D<sub>3</sub>) increased the contents of DNA and macromolecules containing uronic acid (proteoglycans). It also decreased ALPase activity with an ED<sub>50</sub> of <1 nM. Other vitamin D<sub>3</sub> metabolites, such as 24,25-dihydroxycholecalciferol and 25-hydroxycholecalciferol, had little effect on these biochemical parameters. In rachitic growth plates, the uronic acid content was half that in normal growth plates, whereas ALPase activity was 2.5 times that in normal growth plates. Administration of 1,25-dihydroxycholecalciferol at a low dose (0.1 μg per kg of body weight) to rachitic rats increased the uronic acid content 1.4-fold and decreased ALPase activity by 40%. This compound, like 24,25-dihydroxycholecalciferol (10 μg per kg of body weight), increased the calcium level of the blood. However, administration of 24,25-dihydroxycholecalciferol had little effect on the uronic acid and ALPase contents in growth plates. These observations suggest that 1,25-dihydroxycholecalciferol is a bioactive form of vitamin D that plays an important role in the control of chondrocyte terminal differentiation.

In growth plates, chondrocytes undergo cell changes including proliferation, formation of a proteoglycan matrix, terminal differentiation to hypertrophic cells, and calcification until puberty. Hypertrophic chondrocytes and the calcified matrix are invaded by capillaries and chondroclasts and eventually replaced by bone. In the process of endochondral bone formation, vitamin D seems essential; vitamin D deficiency results in abnormal expansion of the growth plate, mainly due to widening of the zone of hypertrophic chondrocytes (1, 2). In general, the enlargement of the hypertrophic zone is thought to be a consequence of retardation of hypertrophic chondrocyte death due to lack of calcification, because vitamin D deficiency causes a decrease in blood calcium and phosphate levels. However, it could be due to acceleration of differentiation of maturing chondrocytes into hypertrophic cells. To test this possibility, we examined the effects of vitamin D metabolites on alkaline phosphatase [ALPase; orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] activity, a marker of hypertrophic chondrocytes, in a well characterized chondrocyte culture system and in growth plates *in vivo*.

Previous studies have shown that certain vitamin D<sub>3</sub> (cholecalciferol) metabolites, such as 1,25-dihydroxychole-

calciferol [1,25(OH)<sub>2</sub>D<sub>3</sub>] and 24,25(OH)<sub>2</sub>D<sub>3</sub>, increase or decrease ALPase activity in hypertrophic chondrocytes (3) or matrix vesicle fractions (4, 5) in monolayer cultures. However, results *in vitro* have been conflicting, perhaps because of partial loss of chondrocyte phenotypic expression in monolayer cultures. Previous studies have shown that chondrocytes readily lose the differentiated characteristics after proliferating for several generations on plastic culture dishes (6, 7).

In the present study, rabbit growth-plate chondrocytes were maintained as a packed mass in a centrifuge tube. These cells became reorganized into a cartilage-like tissue and retained the ability to produce ALPase at a very high level similar to that of growth plates *in vivo* (8, 9). The chondrocyte system was therefore useful in studying direct actions of vitamin D in hypertrophic chondrocytes. Results showed that 1,25(OH)<sub>2</sub>D<sub>3</sub>, but not other vitamin D metabolites, suppressed the induction of ALPase but increased the uronic acid content of chondrocyte cultures. Furthermore, administration of 1,25(OH)<sub>2</sub>D<sub>3</sub>, but not 24,25(OH)<sub>2</sub>D<sub>3</sub>, to rachitic rats decreased the ALPase activity and increased the uronic acid content in growth plates. These results suggest that 1,25(OH)<sub>2</sub>D<sub>3</sub> is involved physiologically in inhibiting precocious or excess hypertrophy and that its deficiency destroys the balance of inhibition and stimulation of chondrocyte hypertrophy, resulting in widening of the hypertrophic zone in rickets.

### MATERIALS AND METHODS

**Chondrocyte Cultures.** Growth-plate chondrocytes were isolated from rib cartilage of 3-week-old male New Zealand rabbits as described by Shimomura *et al.* (10). Freshly isolated chondrocytes were suspended in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 50 μg of ascorbic acid, and 60 μg of kanamycin per ml (medium A). The cell suspension (8 × 10<sup>4</sup> cells in 1 ml of medium A) was transferred to a 15-ml plastic centrifuge tube (Corning 25319) and centrifuged at 500 × g (1500 rpm in a clinical centrifuge) for 5 min (8, 9). The resulting cell pellet was incubated at 37°C in 5% CO<sub>2</sub>/95% air. Cultures were fed with fresh medium A (1 ml) 6 days after seeding, and thereafter the medium was changed every other day. In some experiments, 10-day-old cultures were transferred to 1 ml of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium supplemented with 0.3% fetal bovine serum, 1 mg of bovine serum albumin, 50 μg of ascorbic acid, 32 units of penicillin, and 40 μg of streptomycin per ml

Abbreviations: ALPase, alkaline phosphatase; TrACPase, tartrate-resistant acid phosphatase; pNP, 2-(p-nitrophenyl) phosphate; 1,25(OH)<sub>2</sub>D<sub>3</sub>, 1,25-dihydroxyvitamin D<sub>3</sub>; 25(OH)D<sub>3</sub>, 25-hydroxyvitamin D<sub>3</sub>.

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(medium B). In other experiments, 14-day-old cultures were transferred to 1 ml of MEM supplemented with 50  $\mu\text{g}$  of ascorbic acid and 60  $\mu\text{g}$  of kanamycin per ml in the presence of 10% charcoal-treated serum.

**Tissue Preparation.** Samples of growth-plate cartilage and permanent cartilage from the ribs were obtained from rachitic or normal rats. For induction of rickets, 21-day-old Sprague-Dawley rats were housed in the absence of UV light (using a UV-cut filter) and given a vitamin D-deficient diet (Diet-11, Oriental Yeast Co., Tokyo) and distilled water ad libitum for 6 weeks.  $1,25(\text{OH})_2\text{D}_3$  or  $24,25(\text{OH})_2\text{D}_3$  was dissolved in triglyceride of medium chain length, and some rachitic rats received oral supplements of these metabolites or solvent alone 1, 3, 5, and 7 days before sacrifice.

**Treatment of Serum with Charcoal.** Fetal bovine serum was incubated with 10% volumes (wt/vol) of charcoal at 4°C for 24 hr (11), and then the charcoal was removed by centrifugation at  $15,000 \times g$  for 30 min. This treatment reduced the  $1,25(\text{OH})_2\text{D}_3$  concentration from 47 pg/ml to an undetectable level and the  $24,25(\text{OH})_2\text{D}_3$  concentration from 1.4 ng/ml to 0.7 ng/ml (12).

**Measurement of ALPase Activity.** ALPase activity was measured by a modification of the method of Bessey *et al.* (13) with 2-(*p*-nitrophenyl) phosphate (*p*NP) as substrate. Cell pellets or cartilage segments were homogenized with a glass homogenizer in 0.9% NaCl/0.2% Triton X-100 at 0°C and were centrifuged for 15 min at  $12,000 \times g$ . The activity of the supernatant, which contained 95% of the total activity, was assayed in 0.5 M Tris-HCl buffer (pH 9.0) containing 0.5 mM *p*NP and 0.5 mM  $\text{MgCl}_2$ . The reaction mixture was incubated at 37°C for 15 or 30 min, and the reaction was stopped by addition of 0.25 vol of 1 M NaOH. Hydrolysis of *p*NP was monitored as change in  $A_{410}$  in a Hitachi spectrometer. Nitrophenol was used as a standard. One unit of ALPase was defined as the amount of enzyme required to hydrolyze 1 nmol of *p*NP per 30 min at pH 9.0.

**Determinations of DNA and Uronic Acid.** Cartilage segments or cell pellets were washed with phosphate-buffered saline and then homogenized at 0°C–4°C in 1 ml of 0.9% NaCl/0.2% Triton X-100. The homogenates were then incubated at 37°C for 16 hr with 3 mg of Pronase E (protease type XIV; Sigma) in 3 ml of 0.05 M Tris-HCl buffer (pH 8.0) containing 1 mM  $\text{CaCl}_2$ , 0.9% NaCl, and 0.2% Triton X-100. Digests of tissues or cell pellets were used for assays of DNA (14) and uronic acid (15).

**Determination of Levels of Vitamin D Metabolites in Plasma.** Vitamin D metabolites in rat plasma were determined by combinations of Sephadex LH-20 chromatography, HPLC, competitive protein binding assays, and radioreceptor assays (16).

**Determinations of Calcium, Phosphate, ALPase, and Tartrate-Resistant (Tr) Acid Phosphatase [ACPase; Orthophosphoric-Monoester Phosphohydrolase (Acid Optimum), EC 3.1.32] Levels in Plasma.** Reported methods were used for determinations of calcium (17), inorganic phosphate (18), ALPase (13), and TrACPase (19) in plasma. One unit of TrACPase was defined as the amount of enzyme required to hydrolyze 1 nmol of *p*NP per 30 min at 37°C in the presence of 40 mmol of sodium L-(+)-tartrate per liter at pH 5.5.

## RESULTS

### Effects of Vitamin D Metabolites in Chondrocyte Cultures.

In cultures of pelleted growth-plate chondrocytes, the addition of  $1,25(\text{OH})_2\text{D}_3$  (1–10 nM) resulted in 1.5-fold increases in the contents of DNA and macromolecules containing uronic acid (proteoglycans) (Fig. 1A). Other vitamin D metabolites,  $24,25(\text{OH})_2\text{D}_3$  (Fig. 1A),  $1(\text{OH})\text{D}_3$ , and  $25(\text{OH})\text{D}_3$  (data not shown), had little effect on the DNA or uronic acid content.

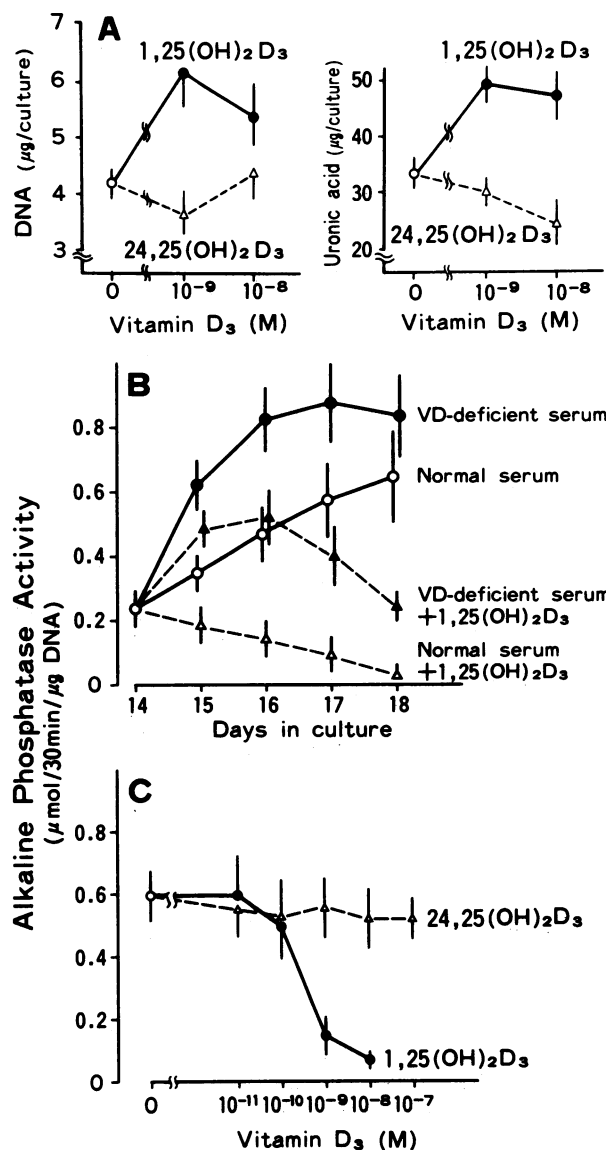


FIG. 1. Effects of  $1,25(\text{OH})_2\text{D}_3$  and  $24,25(\text{OH})_2\text{D}_3$  on DNA (A, Left), uronic acid (A, Right), and ALPase (B and C) contents in chondrocyte cultures. Rabbit growth plate chondrocytes were seeded and maintained as a pelleted mass in a centrifuge tube. (A) After 10 days in culture, cells were transferred into 1 ml of medium B in the presence or absence of vitamin D metabolites. On day 20, the DNA and uronic acid contents of cultures were determined. Results are averages  $\pm$  SD for five or six cultures. (B and C) After 14 days in culture, cells were transferred for 1–4 (B) or 4 (C) days to 1 ml of MEM supplemented with 50  $\mu\text{g}$  of ascorbic acid and 60  $\mu\text{g}$  of kanamycin per ml and 10% fetal bovine serum (B and C) or MEM supplemented with 50  $\mu\text{g}$  of ascorbic acid and 60  $\mu\text{g}$  of kanamycin per ml and 10% charcoal-treated serum (C) in the presence of 10 nM (B) or various concentrations (C) of  $1,25(\text{OH})_2\text{D}_3$  or  $24,25(\text{OH})_2\text{D}_3$ . Results are averages  $\pm$  SD for four to six cultures. VD, vitamin D.

Next we examined the effects of vitamin D metabolites on ALPase activity in chondrocytes in medium with 10% charcoal-treated or normal serum. Treatment of fetal bovine serum with charcoal reduced the  $1,25(\text{OH})_2\text{D}_3$  and  $24,25(\text{OH})_2\text{D}_3$  concentrations by 95% and 50%, respectively. When 14-day-old chondrocyte cultures were exposed to 10% charcoal-treated serum, ALPase activity increased 1.5 to 2 times more in 24 hr than in cultures with 10% normal serum (Fig. 1B). ALPase activity was consistently higher in vitamin D-deficient serum than in normal serum during a 4-day incubation period. However, addition of  $1,25(\text{OH})_2\text{D}_3$  to

Table 1. Blood vitamin D metabolite levels in animals with rickets

Dietary vitamin D	Administration	n	Blood level, ng/ml		
			25(OH)D <sub>3</sub>	1,25(OH) <sub>2</sub> D <sub>3</sub>	24,25(OH) <sub>2</sub> D <sub>3</sub>
Experiment 1					
+	None	8	16.8 ± 6.1	0.209 ± 0.023	3.16 ± 0.96
-	None	8	3.9 ± 0.5	0.080 ± 0.028	0.16 ± 0.02
-	1,25(OH) <sub>2</sub> D <sub>3</sub>	8	3.6 ± 0.6	0.149 ± 0.024	0.17 ± 0.03
-	24,25(OH) <sub>2</sub> D <sub>3</sub>	8	24.2 ± 4.4	0.008 ± 0.001	10.69 ± 1.16
Experiment 2					
+	None	5	19.6 ± 3.8	0.199 ± 0.051	3.00 ± 0.61
-	None	5	5.8 ± 1.2	0.056 ± 0.007	0.57 ± 0.31
-	1,25(OH) <sub>2</sub> D <sub>3</sub>	5	5.5 ± 0.8	0.204 ± 0.008	0.31 ± 0.08
-	24,25(OH) <sub>2</sub> D <sub>3</sub>	5	15.4 ± 2.6	0.011 ± 0.007	13.13 ± 4.31

Rats were given a normal or a vitamin D-deficient diet for 6 weeks. Rachitic rats received oral supplements of 1,25(OH)<sub>2</sub>D<sub>3</sub> (0.1 μg per kg of body weight) or 24,25(OH)<sub>2</sub>D<sub>3</sub> (10 μg per kg of body weight) 1, 3, 5, and 7 days before sacrifice. Results are expressed as averages ± SD. n, Number of animals.

vitamin D-deficient cultures decreased ALPase activity within 24 hr (Fig. 1B). The dose dependence of the effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on ALPase is shown in Fig. 1C. This metabolite decreased the enzyme activity in chondrocytes with an ED<sub>50</sub> of <1 nM. In this system, 24,25(OH)<sub>2</sub>D<sub>3</sub> and 25(OH)D<sub>3</sub> (data not shown) had little effect on ALPase activity.

**Effects of Administration of Vitamin D<sub>3</sub> Metabolites on Plasma Calcium, Phosphate, ALPase, and TrACPase Levels, and on Uronic Acid and ALPase Contents of Growth Plates.** Young rats were given a vitamin D-deficient diet for 6 weeks and then four administrations of 1,25(OH)<sub>2</sub>D<sub>3</sub> (0.1 μg per kg of body weight) or 24,25(OH)<sub>2</sub>D<sub>3</sub> (10 μg per kg of body weight) in 1 week. Vitamin D deficiency was confirmed by determining vitamin D<sub>3</sub> metabolites in the plasma: the levels of 25(OH)D<sub>3</sub>, 1,25(OH)<sub>2</sub>D<sub>3</sub>, and 24,25(OH)<sub>2</sub>D<sub>3</sub> were 30%, 30%, and 5–20%, respectively, of those in age-matched normal rats (Table 1). Administrations of 1,25(OH)<sub>2</sub>D<sub>3</sub> and 24,25(OH)<sub>2</sub>D<sub>3</sub> restored the respective plasma levels. Administration of 24,25(OH)<sub>2</sub>D<sub>3</sub> also caused a further decrease in the 1,25(OH)<sub>2</sub>D<sub>3</sub> level (Table 1), probably by suppressing 25(OH)D<sub>3</sub>-1-hydroxylase activity.

Furthermore, treatment with either 1,25(OH)<sub>2</sub>D<sub>3</sub> or 24,25(OH)<sub>2</sub>D<sub>3</sub> increased the plasma calcium and phosphate concentrations to nearly the control levels (Table 2). 24,25(OH)<sub>2</sub>D<sub>3</sub> had a greater effect than 1,25(OH)<sub>2</sub>D<sub>3</sub> on the calcium level. The increase in the calcium level in 24,25(OH)<sub>2</sub>D<sub>3</sub>-treated rats may have been due to formation of 1,24,25(OH)<sub>3</sub>D<sub>3</sub>, because 24,25(OH)<sub>2</sub>D<sub>3</sub> is known to be converted to 1,24,25(OH)<sub>3</sub>D<sub>3</sub> in animals with rickets (20).

In experiment 1, but not in experiment 2, the ALPase activity in rachitic plasma was 1.8 times that in normal plasma

Table 2. Blood calcium and phosphate levels in animals with rickets

Dietary vitamin D	Administration	Blood level, mg/dl	
		Calcium	Phosphate
Experiment 1			
+	None	10.20 ± 0.43	12.1 ± 1.5
-	None	5.48 ± 0.54	9.8 ± 1.4
-	1,25(OH) <sub>2</sub> D <sub>3</sub>	7.46 ± 1.82	11.3 ± 1.1
-	24,25(OH) <sub>2</sub> D <sub>3</sub>	10.46 ± 0.43	10.9 ± 0.9
Experiment 2			
+	None	9.84 ± 0.32	*
-	None	5.16 ± 0.94	*
-	1,25(OH) <sub>2</sub> D <sub>3</sub>	6.55 ± 0.48	*
-	24,25(OH) <sub>2</sub> D <sub>3</sub>	8.64 ± 1.14	*

Vitamin D metabolites were administered as described in Table 1. Results are expressed as averages ± SD for eight rats. \*, Not determined.

(Table 3). The increase in plasma ALPase activity is one of the features of rickets. Furthermore, vitamin D deficiency resulted in a 40–50% decrease in TrACPase activity in the plasma in experiments 1 and 2 (Table 3). The changes in plasma ALPase and TrACPase levels were reversed by administration of 24,25(OH)<sub>2</sub>D<sub>3</sub>. However, administration of 1,25(OH)<sub>2</sub>D<sub>3</sub> at a dose of 0.1 μg per kg of body weight was insufficient to decrease the plasma ALPase activity or increase the plasma TrACPase activity. Previous studies have shown that TrACPase activity in the serum from patients with Paget disease, hyperparathyroidism, or metastatic bone cancers is higher than that in normal serum (19). TrACPase seems to be a serum marker of bone resorption.

The uronic acid content in the growth plate of rachitic rats was 30–50% of that in the growth plate of normal rats (Table 4). Nevertheless, the ALPase activity was 2.5 times higher in rachitic growth plates. Administration of 1,25(OH)<sub>2</sub>D<sub>3</sub> to rats with rickets had opposite effects on proteoglycan accumulation and ALPase activity in growth plates: it increased the uronic acid content 1.3- to 1.4-fold but decreased ALPase activity by 30–40% in two independent series of experiments. 24,25(OH)<sub>2</sub>D<sub>3</sub> had less effect on uronic acid content and no significant effect on ALPase content of growth plates (Table 4).

Vitamin D deficiency had little effect on the uronic acid (Table 4) or ALPase (data not shown) content of resting cartilage.

Growth plates of rachitic rats have a widened zone of hypertrophic chondrocytes (Fig. 2), as expected from previous studies on rachitic chickens (1, 2). Treatment with 1,25(OH)<sub>2</sub>D<sub>3</sub> (Fig. 2), but not 24,25(OH)<sub>2</sub>D<sub>3</sub> (data not shown),

Table 3. Blood ALPase and TrACPase levels in animals with rickets

Dietary vitamin D	Administration	n	Blood level, units per 0.05 ml	
			ALPase	TrACPase
Experiment 1				
+	None	8	136 ± 15	103.1 ± 22.7
-	None	8	240 ± 49	62.1 ± 13.4
-	1,25(OH) <sub>2</sub> D <sub>3</sub>	8	221 ± 51	69.9 ± 13.2
-	24,25(OH) <sub>2</sub> D <sub>3</sub>	8	144 ± 51	91.3 ± 28.3
Experiment 2				
+	None	6	172 ± 3	71.1 ± 11.4
-	None	5	184 ± 8	36.8 ± 13.3
-	1,25(OH) <sub>2</sub> D <sub>3</sub>	7	182 ± 10	38.0 ± 5.5
-	24,25(OH) <sub>2</sub> D <sub>3</sub>	7	172 ± 7	63.7 ± 15.0

Vitamin D metabolites were administered as described in Table 1. Results are expressed as averages ± SD. n, Number of animals.

Table 4. Effects of vitamin D deficiency and administration of vitamin D metabolites on uronic acid and ALPase contents of growth plates

Dietary vitamin D	Administration	Uronic acid, $\mu\text{g per } \mu\text{g of DNA}$		ALPase activity, units per $\mu\text{g of DNA}$
		Growth	Resting	Growth
Experiment 1				
+	None	$14.8 \pm 1.3$	$30.5 \pm 4.1$	$1.08 \pm 0.11$
-	None	$7.3 \pm 1.4$	$28.2 \pm 3.5$	$2.35 \pm 0.21$
-	$1,25(\text{OH})_2\text{D}_3$	$10.5 \pm 1.2$	$32.8 \pm 4.6$	$1.40 \pm 0.06$
-	$24,25(\text{OH})_2\text{D}_3$	$8.9 \pm 1.1$	$29.3 \pm 1.7$	$1.98 \pm 0.24$
Experiment 2				
+	None	$13.5 \pm 1.9$	$31.4 \pm 2.2$	$0.92 \pm 0.16$
-	None	$9.1 \pm 0.7$	$28.5 \pm 2.2$	$2.06 \pm 0.15$
-	$1,25(\text{OH})_2\text{D}_3$	$12.7 \pm 1.0$	$30.6 \pm 0.6$	$1.36 \pm 0.80$
-	$24,25(\text{OH})_2\text{D}_3$	$11.1 \pm 0.2$	$29.7 \pm 0.9$	$2.02 \pm 0.10$

Vitamin D metabolites were administered as described in Table 1. Uronic acid content of the growth plate (Growth) and resting (Resting) cartilage and ALPase activity in growth plate cartilage were determined. Results are expressed as averages  $\pm$  SD for four determinations with eight rats.

reduced the width of the hypertrophic zone. Sections from vitamin D-deficient rats treated with  $1,25(\text{OH})_2\text{D}_3$  were indistinguishable from those from normal rats. To our knowledge, this demonstration that  $1,25(\text{OH})_2\text{D}_3$  alleviates histological signs of rickets in mammalian growth plates has not been reported elsewhere.

## DISCUSSION

In adult animals, the deficiency of vitamin D is characterized by impaired calcification of bone matrix. In growing animals, vitamin D deficiency leads to rickets. Rickets is characterized by abnormal widening of the growth plate with chondrocyte hypertrophy, increase in plasma ALPase activity, and the failure of calcification of the growth plate. The features are reversed at least partially by treatment with  $1,25(\text{OH})_2\text{D}_3$  (1, 2). However, the mechanism of the vitamin D action is not known.

There have been conflicting reports on the effects of vitamin D metabolites on the synthesis of proteoglycan, a main component of the cartilage matrix, probably because its effects have been examined with chondrocytes in monolayer cultures (12, 21, 22), whose phenotypic expression is variable at low levels depending on culture conditions. Using cultures of well differentiated chondrocytes in centrifuge tubes and rachitic growth plates *in vivo*, in the present study we showed that  $1,25(\text{OH})_2\text{D}_3$  is involved physiologically in supporting the formation of a proteoglycan matrix in growth plates.

Administration of  $1,25(\text{OH})_2\text{D}_3$  to rachitic rats increased the uronic acid content of growth plates at a dose that increased the  $1,25(\text{OH})_2\text{D}_3$  level in the blood to the normal level without an increase in the  $24,25(\text{OH})_2\text{D}_3$  or  $25(\text{OH})\text{D}_3$  level. This effect was independent of an increase in the blood calcium level, because  $1,25(\text{OH})_2\text{D}_3$  increased the uronic acid content of chondrocyte cultures. On the other hand,  $24,25(\text{OH})_2\text{D}_3$  had little effect on the uronic acid content of chondrocyte cultures, although its administration to rachitic rats slightly increased the uronic acid content of growth plates *in vivo*. Other metabolites, such as  $25(\text{OH})\text{D}_3$  and  $1(\text{OH})\text{D}_3$ , had no effect on the uronic acid content of chondrocyte cultures. These results provide evidence that of various vitamin D metabolites,  $1,25(\text{OH})_2\text{D}_3$  is the most important for supporting cartilage-matrix formation.

The reason why rachitic growth plates have a wider zone of hypertrophic chondrocytes is unknown. This widening may be a result of retardation of hypertrophic chondrocyte death due to lack of calcification in the hypertrophic zone (1, 2). It is also conceivable that the number of chondroclasts diminishes in rickets, because the formation of osteoclasts has been shown to require  $1,25(\text{OH})_2\text{D}_3$  (23, 24). In the present study, another possibility that vitamin D deficiency results in abnormal acceleration of chondrocyte differentiation from the maturing stage to the hypertrophic stage was examined. If this is the case, vitamin D should be important for maintaining a balance between maturing and hypertrophic chondrocytes in growth plates. Our results showed that

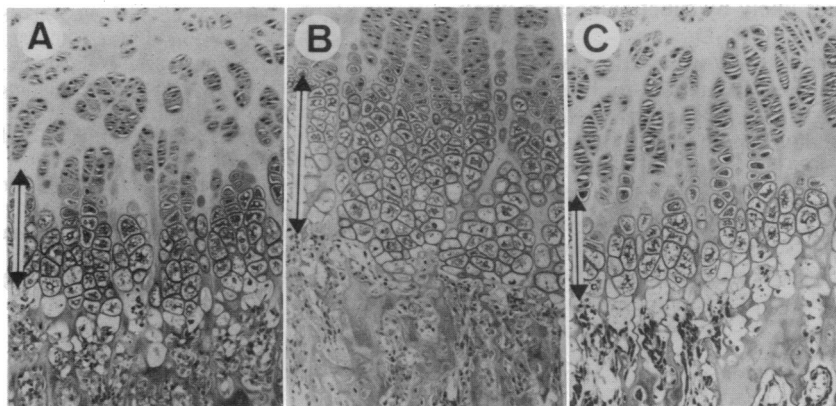


FIG. 2. Photomicrographs of longitudinal sections of growth plates from 9-week-old rats fed for 6 weeks on a normal diet (A) or on a vitamin D-deficient diet (B). (C) Growth plate of a rachitic rat treated with  $1,25(\text{OH})_2\text{D}_3$  ( $0.1 \mu\text{g per kg of body weight}$ ) 1, 3, 5, and 7 days before sacrifice. Typical findings in vitamin D deficiency (B) and after administration of the vitamin D metabolite are shown. The rachitic growth plate has a very wide zone of hypertrophic chondrocytes. Sections were stained with hematoxylin and eosin. Arrows indicate the width of the hypertrophic zone.

vitamin D deficiency results in a 2- to 2.5-fold increase in ALPase activity in both rabbit chondrocyte cultures and rat growth plates *in vivo*. This increase in ALPase was reversed by addition of 1,25(OH)<sub>2</sub>D<sub>3</sub>, but not 24,25(OH)<sub>2</sub>D<sub>3</sub>, to rachitic rats or to the medium of rachitic chondrocyte cultures. It is unlikely that changes in ALPase activity are secondary to changes in the rate of cell death, because vitamin D deficiency or the addition of 1,25(OH)<sub>2</sub>D<sub>3</sub> altered the ALPase activity in chondrocyte cultures in the absence of chondroclasts within 24 hr. Thus, 1,25(OH)<sub>2</sub>D<sub>3</sub> has a direct effect on the conversion of maturing chondrocytes to hypertrophic cells.

The results in the present study suggest that 1,25(OH)<sub>2</sub>D<sub>3</sub> plays a role in inhibiting precocious or excess chondrocyte hypertrophy while stimulating the formation of a proteoglycan matrix in growth plates. The suppression of chondrocyte hypertrophy during the matrix-forming stage may be required for enough accumulation of a calcifiable matrix prior to mineral deposition, because chondrocytes lose proteoglycan-synthetic activity once they become hypertrophic (25).

It is unlikely that the effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on terminal differentiation is secondary to its effect on extracellular matrix synthesis. Differentiation of chondrocytes to matrix-forming stage and terminal differentiation to hypertrophic stage can be separated by manipulation of culture conditions (8, 9) or addition of transforming growth factor type  $\beta$  (8), fibroblast growth factor (25), or parathyroid hormone (26). Extracellular matrix synthesis and hypertrophy appear to be regulated by different sets of growth factor and hormones.

The present study also showed that although no calcification occurs in rachitic growth plates, they have a very high level of ALPase activity. This indicates that the decrease in the plasma calcium level predominates over the increase in ALPase activity.

Administration of 1,25(OH)<sub>2</sub>D<sub>3</sub> to rats with rickets had profound effects on the uronic acid content and ALPase activity in growth plates, whereas it had little effect on the biochemical indices in permanent (resting) cartilage. This is in accord with localization of 1,25(OH)<sub>2</sub>D<sub>3</sub> receptors in hypertrophic chondrocytes (9) or growth cartilage (27). Furthermore, in these experiments administration of 1,25(OH)<sub>2</sub>D<sub>3</sub> had little effect on plasma TrACPase or ALPase activity. These results emphasize the specificity of the 1,25(OH)<sub>2</sub>D<sub>3</sub> actions in growth plates.

Treatment of rachitic rats with 24,25(OH)<sub>2</sub>D<sub>3</sub> had little effect on ALPase activity in growth plates. However, it increased the plasma calcium and TrACPase levels and decreased the plasma 1,25(OH)<sub>2</sub>D<sub>3</sub> level. These findings suggest that 24,25(OH)<sub>2</sub>D<sub>3</sub> or its metabolite(s) is involved in calcium homeostasis, most likely by functioning as a weak but long-acting analog of 1,25(OH)<sub>2</sub>D<sub>3</sub>.

Sömjen *et al.* (28, 29) have shown that administration of 24,25(OH)<sub>2</sub>D<sub>3</sub> to rats with rickets causes 2-fold increases in [<sup>3</sup>H]thymidine incorporation into DNA and creatine kinase activity in epiphyses of developing long bones, whereas 1,25(OH)<sub>2</sub>D<sub>3</sub> causes 1.5-fold increases in [<sup>3</sup>H]thymidine incorporation and creatine kinase activity in diaphyses. 24,25(OH)<sub>2</sub>D<sub>3</sub> may have direct actions in chondrocytes in early differentiation stages (30) and in epiphyses (28, 29).

We conclude from the present study that 1,25(OH)<sub>2</sub>D<sub>3</sub> is a bioactive form of vitamin D that plays an important role in the

control of terminal differentiation of chondrocytes in growth plates. This information is important in understanding the role of vitamin D in endochondral bone formation.

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