The influence of vitamin D metabolites on collagen synthesis by chick cartilage in organ culture

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ABSTRACT

When growth cartilage from rachitic chicks was cultured in the presence of the calcium-regulating hormone 1,25-dihydroxyvitamin D$_3$ (1,25-(OH)$_2$D$_3$), collagen resorption was increased and collagen synthesis decreased compared to control cultures containing no hormone. The minimum concentration of the hormone that caused a statistically significant inhibition of collagen synthesis was $10^{-8}$mol/l. Collagen synthesis by growth cartilage from normal chicks was also reduced by 1,25-(OH)$_2$D$_3$, showing that it was not an abnormal response of vitamin D-depleted tissue. 25-Hydroxyvitamin D$_3$ and 24,25-dihydroxyvitamin D$_3$ also inhibited collagen synthesis by cultures of growth cartilage but only at higher metabolite concentrations. 1,25-Dihydroxyvitamin D$_3$ ($10^{-7}$mol/l) did not significantly inhibit collagen synthesis by cultures of articular fibrocartilage and of sternal cartilage, tissues that do not calcify physiologically. The minimum concentration of 1,25-(OH)$_2$D$_3$ ($10^{-9}$mol/l) necessary to cause decreased collagen synthesis by embryonic chick calvaria was lower than the value obtained with growth cartilage; this suggests that bone cells may be more sensitive to the hormone in this respect than are growth cartilage chondrocytes. These findings provide evidence of a direct role of 1,25-(OH)$_2$D$_3$ in the control of endochondral bone formation which is consistent with its primary role in the maintenance of plasma calcium homeostasis.

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INTRODUCTION

The mechanism by which the vitamin D system acts on skeletal tissues is not clearly understood. The presence of 1,25-dihydroxyvitamin D$_3$ (1,25-(OH)$_2$D$_3$) appears to be necessary for normal bone formation (McNutt & Haussler, 1973) and a specific receptor for this metabolite has been found in bone (Kream, Jose, Yamada & DeLuca, 1977). There is also evidence of the existence of specific receptors for 24,25-dihydroxyvitamin D$_3$ (24,25-(OH)$_2$D$_3$) in bone (Sömjén, Sömjén, Weissman & Binderman, 1982) and of a requirement for 24,25-(OH)$_2$D$_3$ for normal bone formation (Ornoiny, Goodwin, Nof & Edelstein, 1978; Kraft, Offerman, Stedlinger & Gawlik, 1979; Norman, Henry & Malluche, 1980) although other evidence does not appear consistent with such a requirement (Halloran, DeLuca, Barthell et al. 1981; Miller, Halloran, DeLuca et al. 1981; Ameenuddin, Sunde, DeLuca et al. 1982; Dickson, Hall & Jande, 1984).

In-vitro studies have provided unequivocal evidence that vitamin D metabolites can directly influence the metabolism of skeletal tissue cells but the physiological relevance of these findings is more difficult to establish. Concentrations of the two dihydroxylated vitamin D metabolites well below those present in plasma have been shown to stimulate sulphate incorporation into proteoglycan by cultured growth plate chondrocytes (Corvol, Dumontier, Garabedian & Rappaport, 1978). 1,25-Dihydroxyvitamin D$_3$ also inhibits collagen synthesis in cultures of fetal rat (Raisz, Maina, Gworek et al. 1978; Raisz, Kream, Smith & Simmons, 1980) and neonatal mouse (Brinhurst & Potts, 1982; Écarot-Charrrier & Glorieux, 1983)
calvaría as well as in cultures of osteoblast-like cells from mouse calvaria (Wong, Luben & Cohn, 1977). Inhibition of collagen synthesis probably occurs at the level of transcription or of degradation of procollagen messenger RNA (mRNA) since the decrease in collagen synthesis correlates well with levels of functional procollagen mRNA in fetal rat calvaria (Rowe & Kream, 1982).

In the present study we have looked at the effects of vitamin D metabolites on synthesis of collagen by chick growth cartilage in culture. We have compared the effects of 1,25-(OH)2D3 on growth cartilage with those produced by 24,25-(OH)2D3 and 25-hydroxy-vitamin D3 (25-OH-D3), the major circulating metabolites of vitamin D under normal conditions. We have also compared the effect of 1,25-(OH)2D3 on growth cartilage, which calcifies physiologically, with that on articular and on sternal cartilage tissues which do not calcify physiologically.

MATERIALS AND METHODS

Animals

One-day-old cockerels (Ranger cockerels; Ross Poultry, Woodhall Spa, Lincs) were raised on a rachitogenic diet (Dickson & Kodicek, 1979) containing 1-2%, Ca and 0-7% P, in a room lit by tungsten lights. After 4 weeks the chicks exhibited the characteristic signs of rickets, including hypocalcaemia, raised levels of plasma alkaline phosphatase and widened epiphysial cartilage growth plates. For studies with vitamin D-replete chicks either a diet supplemented with vitamin D3 (50 µg/kg) was used or chicks were given vitamin D3 orally (5 µg in arachis oil) twice each week.

Isolation of cartilage

Chicks aged 4–5 weeks were anaesthetized with chloroform, bled by cardiac puncture and then killed with chloroform. The legs were dislocated at the proximal end of the femur and the distal end of the tibiotarsus. Skin was removed and the surface of the muscle swabbed with 70% (v/v) ethanol in a laminar flow cabinet. The proximal end of the tibiotarsus was dissected free of muscle tissue and split longitudinally to expose the growth plate. Pieces of articular fibrocartilage or of epiphysial cartilage from the middle of the growth plate (see Fig. 1), approximately 2–3 mm2 and 1 mm deep, were quickly dissected, tissue for experimental and control cultures being cut from adjacent areas, and placed in medium as described below. Cartilage pieces from the keel (xiphoid process) of the sternum were dissected and treated in a similar fashion.

![FIGURE 1. Cross-section of the proximal epiphysis of the tibiotarsus of (a) normal and (b) rachitic chicks. The age of the chicks was 4 weeks. Articular fibrocartilage was dissected for culture from the areas marked X and growth cartilage from the areas marked Y.](image_url)

Cartilage culture

Tissue was cultured on grids made of stainless-steel mesh (ref. 978 MM; Exapet, Stanton Works, Hartlepool, Teesside), either in 3 cm Petri dishes or in Petri dishes containing multicompartments of 2 cm2. Incubation was at 37°C with a gas phase of 5% CO2:95% air. The chemically defined medium used was similar to the BGJb medium of Biggers, Gwatkin & Heyner (1961) except with respect to the following components (concentrations, in mg/l, are given in parentheses): KCl (350); KH2PO4 (35); NaCl (6200); Na acetate (83); NaHCO3 (2200); NaH2PO4 (140); m-inositol (1); Ca pantothenate (0-5); thiamine HCl (2); ascorbic acid (150). The medium was changed daily.

Collagen synthesis

Collagen synthesis was estimated by measuring the rate of formation of [3H]hydroxyproline from [3H]proline, the amino acids being separated by ion-exchange column chromatography. For the last 4 h of culture the medium was replaced with fresh medium containing 1-[5-3H]proline (5 µCi/ml; Amersham International plc, Bucks). The tissue was afterwards washed briefly twice with cold 0-15 M NaCl, lightly blotted on filter paper and hydrolysed with 5-9 mol HCl for 18 h at 105°C in tubes having a screw cap with a Teflon liner. The acid was then removed by evaporation in a vacuum desiccator containing NaOH pellets.

The tissue hydrolysate was redissolved in 1 ml citrate buffer (pH 3-1 at 20°C, 0-2 mol/l with respect to Na+).

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A portion of this solution was taken for analysis for hydroxyproline (Woessner, 1961). A further portion (0.5 ml) was applied to a column of cation-exchange resin (1 × 10 cm; Dowex 50X8 resin, 200–400 mesh; Sigma Chemicals Co., Poole, Dorset) equilibrated with citrate buffer, pH 3.1, and eluted with the same buffer at a flow rate of 30 ml/h at room temperature. Fractions (2 ml) were collected and portions of these were taken for liquid scintillation counting. The elution positions of hydroxyproline and proline were first determined by chromatography of a mixture of [3H]proline and hydroxyproline and analysis of fractions for 3H activity and for hydroxyproline. In the chromatography of test specimens, the total 3H activity in fractions containing the hydroxyproline peak was calculated and results were expressed as d.p.m. [3H]hydroxyproline/μg hydroxyproline in the hydrolysate.

Chick calvarial cultures

Fertile eggs (Winter Egg Farm, Tringlowl, Cambs) were incubated for 16 days. The skin of the head was cut back and the calvarium exposed. At this stage the frontal bone could be readily excised as two halves, which were immediately placed in medium. The composition of the medium was similar to that used for the cartilage culture except that it contained the following additional ingredients (concentration, in mg/l, in parentheses): alanine (250); aspartic acid (150); glycine (800); serine (200); bovine serum albumin (5000). The prefrontal bone and a ridge of the frontal bone adjacent to the eye were removed with ophthalmic surgical scissors with curved blades (J. Weiss & Son Ltd, London). The calvaria were cultured on grids of stainless-steel mesh as described for cartilage culture.

Vitamin D metabolites

1,25-Dihydroxyvitamin D₃ and 24,25-(OH)₂D₃ were generously donated by Roche Products Ltd, Welwyn Garden City, Herts and 25-OH-D₃ was donated by Roussel Products (UK) Ltd, Wembley, Middx. Homogeneity of metabolites was determined by chromatography on a column (1 × 60 cm) of Sephadex LH-20 using as eluant a mixture of chloroform/petroleum ether (40–60°C distillate fraction)/methanol (75:23:2, by vol.). The concentration of the metabolites in ethanolic solution was estimated by making the assumption that the value of A₄_{1cm} 265 for vitamin D₃ was 470. The spectrum of the metabolites in the region 240–270 nm was routinely checked and solutions in which the absorption maximum had shifted to 260 nm or below were not used for experiments.

To prepare solutions of metabolites in chemically defined medium, one volume of an ethanolic solution of a metabolite, usually approximately 10 μg/ml, was mixed with five volumes of medium and to this was added one volume of heat-inactivated (30 min, 60°C) rachitic chick serum to bind the sterol. This mixture was then diluted further with medium to appropriate concentrations of the metabolite; the concentration of ethanol in medium used for culture was always less than 1%. A similar amount of ethanol was added to the medium used for control cultures except in two experiments, in which the effect on collagen synthesis of varying the concentrations of the metabolites was studied in cartilage cultures. The addition of 1% ethanol to cultures had no effect on collagen synthesis (results not shown).

RESULTS

When growth cartilage from rachitic chicks was incubated in medium containing 1,25-(OH)₂D₃ (Table 1), the proportion of total hydroxyproline present in the medium after 24 h was significantly (P < 0.01) greater than in control cultures containing no metabolite. In cultures of articular cartilage from rachitic chicks, the proportion of hydroxyproline present in the medium was much less than in cultures of growth cartilage, and the presence of 1,25-(OH)₂D₃ in the medium had no apparent effect. The increased release of hydroxyproline-containing material into the medium probably results from a stimulus to growth cartilage resorption. An alternative explanation is that a marked stimulus to collagen synthesis has caused larger numbers of newly synthesized collagen molecules to be released into the culture medium. To distinguish between these two possibilities the influence of 1,25-(OH)₂D₃ on collagen synthesis by rachitic growth cartilage was studied, using the rate of hydroxylation

<table>
<thead>
<tr>
<th>Hydroxyproline in medium</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Growth cartilage</strong></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10.39 ± 0.81 (20)</td>
</tr>
<tr>
<td>1,25-(OH)₂D₃</td>
<td>14.21 ± 0.97 (20)*</td>
</tr>
<tr>
<td><strong>Articular cartilage</strong></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.07 ± 0.07 (6)</td>
</tr>
<tr>
<td>1,25-(OH)₂D₃</td>
<td>1.09 ± 0.06 (6)</td>
</tr>
</tbody>
</table>

* P < 0.01 compared with control (t-test).

The hydroxyproline content of the medium and of the cartilage was estimated after a 24-h incubation and the former expressed as a percentage of total hydroxyproline.

TABLE 1. Effect of 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃; 10⁻⁷ mol/l) on release of hydroxyproline into the medium by rachitic chick cartilage. Values are means ± S.E.M.; numbers of observations are shown in parentheses.
of $[^{3}H]$proline as an indicator of collagen synthesis. Formation of $[^{3}H]$hydroxyproline, related to total tissue hydroxyproline, was lower in cultures containing this metabolite than in control cultures (Table 2); the difference was statistically significant ($P<0.05$) after 48 h of culture and was greater after 72 h. In other studies (results not shown) significant differences were observed at 24 h or earlier.

<table>
<thead>
<tr>
<th>Time in culture (h)</th>
<th>$^{[3]}H$Hydroxyproline (d.p.m./µg hydroxyproline)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>$^{1,25}(OH)<em>{2}D</em>{3}$</td>
</tr>
<tr>
<td>4</td>
<td>2580 ± 748</td>
</tr>
<tr>
<td>24</td>
<td>3987 ± 325</td>
</tr>
<tr>
<td>48</td>
<td>3287 ± 286</td>
</tr>
<tr>
<td>72</td>
<td>3217 ± 501</td>
</tr>
</tbody>
</table>

* $P<0.05$, ** $P<0.02$ compared with control ($t$-test).
For the last 4 h of culture fresh medium containing 5 µCi $[^{3}H]$proline/ml was used. $[^{3}H]$Hydroxyproline was separated by an ion-exchange chromatography method.
For details see Methods section.

The lowest concentration of $^{1,25}(OH)_{2}D_{3}$ at which significant and reproducible effects on collagen synthesis could be observed was $10^{-7}$ mol/l (Fig. 2). 24,25-Dihydroxyvitamin $D_{3}$ and $25$-$OH$-$D_{3}$ could also inhibit production of $[^{3}H]$hydroxyproline but the minimum concentrations necessary were higher: $10^{-7}$ and $10^{-6}$ mol/l respectively (Fig. 2).

Growth cartilage differs from most other cartilage tissues in that it calcifies physiologically. To see whether $^{1,25}(OH)_{2}D_{3}$ had a comparable effect on non-calcifying cartilage, studies were made of articular fibrocartilage, adjacent to the proximal tibial growth cartilage, and of sternal cartilage from rachitic chicks. The rate of collagen synthesis in cultures of articular fibrocartilage or sternal cartilage was less than in cultures of growth cartilage (Table 3). In the presence of $^{1,25}(OH)_{2}D_{3}$, collagen synthesis was consistently slightly reduced in cultures of the former two tissues (Table 3) but these decreases were not statistically significant even after culture for 72 h (see Table 3).

Initially the investigations were confined to cartilage tissue from rachitic chicks. Further experiments were carried out using cartilage from normal chicks of comparable age to determine whether or not the responses observed with rachitic cartilage were a peculiarity of vitamin D-depleted tissues. 1,25-Dihydroxyvitamin $D_{3}$ decreased collagen synthesis in cultures of growth cartilage from these normal chicks (Fig. 3) but the slight, inhibitory effects on collagen synthesis observed with cultures of articular fibrocartilage and sternal cartilage were not statistically significant.

TABLE 2. Influence of $^{1,25}$-dihydroxyvitamin $D_{3}$ ($^{1,25}-(OH)_{2}D_{3}$; $10^{-7}$ mol/l) on incorporation of $[^{3}H]$hydroxyproline into growth cartilage collagen of rachitic chicks. Values are means ± S.E.M. of four observations in each case

![Figure 2](image)

TABLE 3. Influence of $^{1,25}$-dihydroxyvitamin $D_{3}$ ($^{1,25}-(OH)_{2}D_{3}$; $10^{-7}$ mol/l) on incorporation of $[^{3}H]$hydroxyproline into cartilage from rachitic chicks. Values are means ± S.E.M. of eight observations in each case

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The effects of 1,25-(OH)\(_2\)D\(_3\) on collagen synthesis by embryonic chick calvaria in culture were studied to obtain information about the relative sensitivity of bone and cartilage cells to this hormone. 1,25-Dihydroxyvitamin D\(_3\) was found to inhibit collagen synthesis in calvarial cultures (Fig. 4), the minimum concentration (10\(^{-9}\) mol/l) of the metabolite necessary to produce a statistically significant effect being lower than the corresponding value for cultures of growth cartilage (10\(^{-8}\) mol/l).

**DISCUSSION**

It is well established that 1,25-(OH)\(_2\)D\(_3\) can stimulate resorption of bone in organ culture (Raisz, Trummel, Holick & DeLuca, 1972; Reynolds, Holick & DeLuca, 1973). However, the observations we have made with growth cartilage could not be explained by the presence of bone cells within the tissue. Bone cells are present in the growth plate within the zone of degeneration and hypertrophy, where osteoid is deposited and calcified cartilage is resorbed. Although ambiguity might arise in the case of explants of normal growth cartilage, because of the technical difficulty of separating this zone, this would not be the case with rachitic growth cartilage because of the greatly increased size of the growth plate in this condition, mainly due to enlargement of the zone of chondrocyte proliferation (Bisaz, Schenk, Kunin et al. 1975; Jande & Dickson, 1980), and because the zone of hypertrophy and degeneration is small and poorly developed in rickets. The hypothesis that growth cartilage chondrocytes are target cells for 1,25-(OH)\(_2\)D\(_3\) is consistent with our earlier observations (Dickson & Kodicek, 1982), using much higher doses of the metabolite, that it had a greater effect on cartilage from the zone of proliferation and maturation than upon tissue from the zone of hypertrophy and calcification. It is also supported by other studies: Wientroub & Reddi (1982) have shown that the metabolite affects the proliferative activity of mesenchymal cells and Corvol, Dumontier, Tsagris et al. (1981) have observed that the metabolite increases DNA polymerase activity in chondrocytes during the logarithmic phase of division. In studies of the in-vivo effects of 1,25-(OH)\(_2\)D\(_3\) on cartilage growth in mouse condylar cartilage (Silberman, Mirsky, Levitan & Weisman, 1983), the histological changes observed seemed consistent with the view that the metabolite was interfering with the differentiation of prechondroblasts.

The minimum concentrations of 1,25-(OH)\(_2\)D\(_3\) found to cause significant inhibition of collagen synthesis by cultures of chick growth cartilage and embryonic chick calvaria were 10\(^{-8}\) and 10\(^{-9}\) mol/l respectively; these are higher than the corresponding volumes of 10\(^{-11}\) mol/l for fetal rat calvaria (Raisz et al. 1978, 1980) and neonatal murine calvaria.
We major concentrations et tissues bolites calvaria to Glorieux markedly (Bringhurst, Krieger, the Bringhurst al. 1983). It should be noted that our culture system differs markedly from that used by Raisz et al. (1978, 1980), Bringhurst & Potts (1982) and Écarot-Charrier & Glorieux (1983). In a comparison of several in-vitro procedures for studying bone resorption (Steen & Krieger, 1983), a response to 1,25-(OH)_2D_3 could be observed at a concentration of 10^{-11} mol/l with the most sensitive system whereas the least sensitive one failed to respond at a concentration of 10^{-9} mol/l. The values of 10^{-6}-10^{-7} mol/l obtained in this study for the effects of 25-OH-D_3 and 24,25-(OH)_2D_3 on collagen synthesis by chick growth cartilage are similar to those obtained for these metabolites with rat calvaria (Raisz et al. 1980) and murine calvaria (Bringhurst & Potts, 1982).

The normal physiological concentration of 1,25-(OH)_2D_3 is reported to be around 10^{-10} mol/l in the plasma of chicks and rats (Mallon, Boris & Bryce, 1981). Under such conditions in-vivo bone collagen synthesis and bone growth would not be inhibited in the growing animal. Thus the finding that 1,25-(OH)_2D_3, at a concentration of 10^{-11} mol/l or greater, inhibits collagen synthesis by rat calvarial cultures shows that caution must be observed in relating in-vitro data to the situation prevailing in vivo. The cellular response to 1,25-(OH)_2D_3 and other metabolites could be modified by a number of factors including variation in the number or the affinity of receptors, and also by the presence within skeletal tissues of enzyme systems which are capable of hydroxylating vitamin D metabolites in the chick. These have been observed in growth cartilage (Garabedian, du Bois, Corvol et al. 1978) and embryonic calvarium (Turner, Puzas, Forte et al. 1980). There is evidence from in-vivo studies that high doses of 1,25-(OH)_2D_3 can have an inhibitory effect on bone formation (Hock, Kream & Raisz, 1982; Dickson et al. 1984). It would seem logical that abnormally high concentrations of 1,25-(OH)_2D_3, which would occur in vivo when there is a disorder of calcium homeostasis, should be able to inhibit synthesis of collagen by cells of bone and growth cartilage since inhibition of matrix synthesis, and hence of bone formation, could have a major effect in conserving plasma calcium.

ACKNOWLEDGEMENTS

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REFERENCES


