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Preferential Accumulation In Vivo of 24R,25-Dihydroxyvitamin D_3 in Growth Plate Cartilage of Rats

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Vitamin D_3 is metabolized in vivo through 25-(OH)D_3 (25D) to both 1α,25-$(OH)_2D_3$ (1,25D) and 24R,25-$(OH)_2D_3$ (24,25D). Whereas it is assumed that this metabolism occurs primarily in the kidney, recent studies show that there are extrarenal 1α- and 24R-hydroxylase activities as well, and in chondrocytes, these enzymes are regulated by hormones and growth factors. Furthermore, chondrocytes from the resting zone of growth plate cartilage are a target cell population for 24,25D action, suggesting that this vitamin D metabolite may be targeted to this tissue in vivo. To test this hypothesis, 30 normal male Sprague Dawley rats (120 ± 20 g) were divided into three groups of eight animals each, and a control group of six animals, and fed ad libitum for 2 wk, a standard rat chow (Teklad LM-485), which contained 3 IU vitamin D_3/g. The rats were then injected im daily at 9:00 AM, for 4 consecutive d, with 0.1 mL of either [^{3}H]-25D, [^{3}H]-1,25D or [^{3}H]-24,25D. Each dose contained 13 pmol of hormone (0.36 μCi/dose). The distribution of these metabolites was assessed in tibial bone (B) following ablation of the bone marrow, articular cartilage from the tibia (AC), costochondral growth plate cartilage (GC), serum (S), small intestine (I), and kidney (K). The use of high specific activity tritiated vitamin D metabolites facilitated determining tissue localization and further metabolism without perturbation of the body pools of each major metabolite. Accumulation of [^{3}H]-1,25D or [^{3}H]-24,25D in each tissue was compared to circulating serum levels. In rats dosed with [^{3}H]-25D, the tissue:serum ratios for 1,25D were 4.1 (AC), 35.4 (GC), 1.3 (B), 0.7 (K), and 3.0 (I); and tissue:serum ratios for 24,25D were 1.6 (AC), 9.9 (GC), 0.04 (B), 0.2 (K), and 0.4 (I). In rats dosed with [^{3}H]-24,25D alone, GC was the only tissue to accumulate the administered metabolite at a concentration significantly higher than that of serum. Similarly, in rats dosed with [^{3}H]-1,25D alone, GC was the only tissue to accumulate 1,25D at a concentration higher than that of serum. These results demonstrate, for the first time, that under in vivo conditions, GC specifically accumulates 24,25D and 1,25D. This suggests that growth plate may be a target organ for these two hormones.

Key Words: Vitamin D_3 metabolites; in vivo; distribution; 1,25-$(OH)_2D_3$; 24,25-$(OH)_2D_3$.

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Introduction

It is well-established that the renal metabolite of vitamin D, 1α,25-$(OH)_2$ vitamin D_3 [1α,25-$(OH)_2D_3$], is a major mediator of the biological actions of the vitamin D endocrine system, particularly in terms of its classical actions in maintaining calcium homeostasis (Henry and Norman, 1984; Reichel et al., 1989). A second major dihydroxylated metabolite of vitamin D, which is found in significant concentrations in the serum of humans (Nguyen et al., 1979; Jongen et al., 1989; Castro-Errecaborde et al., 1991), rats (Jarnagin et al., 1985), and chicks (Goff and Horst, 1995), is 24R,25-$(OH)_2$ vitamin D_3 [24R,25-$(OH)_2D_3$] (Boyle et al., 1973). Although the production of 24R,25-$(OH)_2D_3$ by the kidney is tightly regulated (Henry, 1992; Henry and Norman, 1984; Iida et al., 1995), the biological importance of 24R,25-$(OH)_2D_3$ is still the subject of uncertainty and question (Norman et al., 1982).

Many possible biological roles and sites of action have been suggested for 24R-25$(OH)_2D_3$. Although regulation of parathyroid hormone release from the parathyroid gland (Rudberg et al., 1984; Canterbury and Reiss, 1988) and modulating the action of 1α,25-$(OH)_2D_3$ on chick intesti-

Table 1
Tissue:Serum Ratios of [^{3}H]-Vitamin D_3 Metabolite Uptake In Vivo[a]

Vitamin D_3 Metabolite injected	24,25-$(OH)_2D_3$	1,25-$(OH)_2D_3$	25-(OH)D_3	25-(OH)D_3
Vitamin D_3 Metabolite measured	24,25-$(OH)_2D_3$	1,25-$(OH)_2D_3$	24,25-$(OH)_2D_3$	1,25-$(OH)_2D_3$
Articular cartilage	0.5	1.1	1.6	4.1
Growth plate cartilage	2.6	6.9	9.9	35.4
Bone	0.2	0.4	0.04	1.3
Kidney	0.2	0.2	0.2	0.7
Intestine	0.1	1.1	0.4	3.0

[a]Rats were injected on 4 consecutive d with [^{3}H]-24,25-$(OH)_2D_3$, [^{3}H]-1,25-$(OH)_2D_3$, or [^{3}H]-25-(OH)D_3, as described in the Materials and Methods. At harvest, tissues and serum were collected and then analyzed for their content of [^{3}H]-1,25-$(OH)_2D_3$, and [^{3}H]-24,25-$(OH)_2D_3$. The values shown in the table represent the calculated tissue:serum ratios of picomolar uptake/gram tissue for the indicated vitamin D_3 metabolite.

nal mucosa have been reported (Wilhelm et al., 1986), a major focus of many laboratories with respect to 24R,25-$(OH)_2D_3$ has been in the area of skeletal (Tam et al., 1986) and bone biology (Malluche et al., 1980; Matsumoto et al., 1992; Ornoy et al., 1978). Lidor et al. (1987a) showed that local administration of this metabolite resulted in the healing of rachitic lesions in chick epiphyseal growth plates. In addition, the consequences of systemic administration of 24R,25-$(OH)_2D_3$ to normal rabbits on bone volume and turnover (Nakamura et al., 1992), as well as improvement in mechanical strength of canine bone (Yamaura et al., 1993), have been described.

In the skeletal system, it has been the suggested that 24,25-$(OH)_2D_3$ targets growth plate cartilage. More than a decade ago, evidence for specific nuclear localization of [^{3}H]-24R,25-$(OH)_2D_3$ by cartilage tissue was presented (Fine et al., 1985; Corvol et al., 1985) and preliminary evidence for a nuclear receptor for 24R,25-$(OH)_2D_3$ had been published (Somjen et al., 1982). In addition, several reports have emphasized the involvement of 24R,25-$(OH)_2D_3$ in callus formation associated with fracture-healing (Lidor et al., 1987b,c).

Using a rat costochondral chondrocyte culture model, Boyan et al. (Schwartz and Boyan, 1988; Boyan et al., 1992) have demonstrated that cells derived from resting zone cartilage respond primarily to 24R,25-$(OH)_2D_3$, while cells derived from growth zone cartilage (prehypertrophic and upper hypertrophic zones) respond primarily to 1α,25-$(OH)_2D_3$. Based on this model, it has been proposed that the response to vitamin D metabolites involves rapid membrane effects, some of which are nongenomic, including activation of alkaline phosphatase and phospholipase A_2 activities in matrix vesicles (Schwartz et al., 1988a,b) and metabolite-specific changes in matrix vesicle and plasma membrane fluidity (Swain et al., 1993). Recently, it was found that chondrocytes in vitro not only respond to 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$, but also have the ability to produce these metabolites (Schwartz et al., 1992a), suggesting that in cartilage, endogenous hormone may be more important than exogenous hormone.

Despite the many studies, to date, concerning the effects of 24R,25-$(OH)_2D_3$ on chondrocytes and cartilage, no reports have described the in vivo distribution of [^{3}H]-24R,25-$(OH)_2D_3$ or its further metabolism in the tissue. It is essential to obtain some information on these points so as to gain insight into the possible mechanism of action of 24R,25-$(OH)_2D_3$. In the present study, groups of normal vitamin D-replete rats were given, separately, either [^{3}H]-25-(OH)D_3, [^{3}H]-1α,25-$(OH)_2D_3$, or [^{3}H]-24R,25-$(OH)_2D_3$ for 4 d and the tissue localization of vitamin D metabolites in serum, kidney, intestine, growth plate cartilage, articular cartilage, and long bone determined. Evidence is presented showing the preferential accumulation of vitamin D metabolites in growth plate cartilage.

Results

Circulating Levels of Vitamin D_3 Metabolites in Control Rats

The circulating levels of 25-(OH)D_3, 24R,25-$(OH)_2D_3$, and 1α,25-$(OH)_2D_3$ were found to be in the normal range: 94.9 ± 16.5 n*M* 25-(OH)D_3; 5.6 ± 0.8 n*M* 24R,25-$(OH)_2D_3$; and 0.36 ± 0.09 n*M* 1α,25-$(OH)_2D_3$. All values are the mean ± SEM for an $n = 5$.

Injection of [^{3}H]-1α,25-$(OH)_2D_3$

When rats were injected with [^{3}H]-1α,25-$(OH)_2D_3$, there was more than a six-fold enrichment of [^{3}H]-1α,25-$(OH)_2D_3$ per gram of tissue in the growth plate cartilage when compared to serum (Table 1, Fig. 1). The amount of radiolabeled 1,25-$(OH)_2D_3$ in serum was comparable to that measured in articular cartilage and intestine, but higher than that detected in bone and kidney. There was also some metabolism of the injected metabolite over the course of the

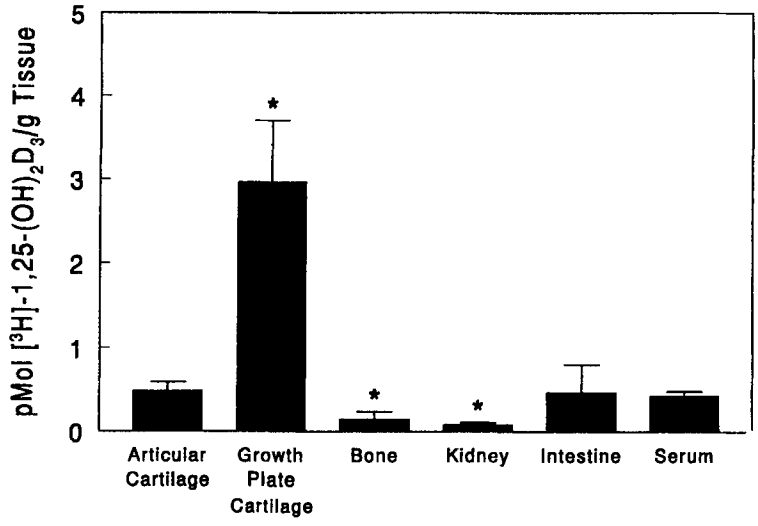

Fig. 1. Tissue distribution of $[^3H]$-1,25-$(OH)_2D_3$ after the injection of 13 pmol of $[^3H]$-1,25-$(OH)_2D_3$ daily for 4 consecutive d. Each point is the mean ± SEM of either two or four samples as described in the Materials and Methods section. $*P < 0.05$, tissue vs serum.

experiment. The relative percent distribution of $[^3H]$-1,25-$(OH)_2D_3$ in growth plate cartilage, articular cartilage, bone, intestine, and serum was consistently greater than that of $[^3H]$-1,24,25-$(OH)_3D_3$ (Fig. 2). Approximately 40% of the $[^3H]$ was associated with 1,25-$(OH)_2D_3$, whereas between 10–20% was associated with 1,24,25-$(OH)_3D_3$. In kidney, the relative percentage of each of these two metabolites was comparable.

Injection of $[^3H]$-24R,25-$(OH)_2D_3$

When rats were injected with $[^3H]$-24R,25-$(OH)_2D_3$, there was a significant two-fold enrichment of $[^3H]$-24,25-$(OH)_2D_3$ in the growth plate cartilage over that in the serum (Table 1, Fig. 3). All other tissues had less radiolabeled 24,25-$(OH)_2D_3$ than found in the serum; of these, articular cartilage contained the highest levels of isotope and intestine the lowest. Further, it was observed that $[^3H]$-24,25-$(OH)_2D_3$ was hydroxylated, yielding $[^3H]$-1,24,25-$(OH)_3D_3$ (Fig. 4). The relative percent distribution of $[^3H]$-24,25-$(OH)_2D_3$ was greater than that of $[^3H]$-1,24,25-$(OH)_3D_3$ in growth plate cartilage, kidney, and serum. In these tissues, $[^3H]$-24,25-$(OH)_2D_3$ accounted for more than 40% of the total vitamin D metabolites present.

Injection of $[^3H]$-25-$(OH)D_3$

Similarly, when $[^3H]$-25-$(OH)D_3$ was injected, there was a significant enrichment of $[^3H]$-1,25-$(OH)_2D_3$ (Table 1, Fig. 5) and $[^3H]$-24,25-$(OH)_2D_3$ (Table 1, Fig. 6) in the growth plate cartilage. The level of $[^3H]$-1,25-$(OH)_2D_3$ in articular cartilage and intestine was higher than that in serum (Fig. 5), while that of $[^3H]$-24,25-$(OH)_2D_3$ in these tissues was similar to the level found in serum (Fig. 6). The level of $[^3H]$-1,25-$(OH)_2D_3$ in bone and kidney was comparable to that in serum (Fig. 5), while the level of $[^3H]$-24,25-$(OH)_2D_3$ in bone and kidney was significantly less than that found in serum (Fig. 6). Every tissue type examined contained more $[^3H]$-24,25-$(OH)_2D_3$ than either of the other two metabolites (Fig. 7). Relative amounts of $[^3H]$-1,25-$(OH)_2D_3$ and $[^3H]$-1,24,25-$(OH)_3D_3$ were comparable and generally found at one-half the level of $[^3H]$-24,25-$(OH)_2D_3$. In serum, the largest proportion of the vitamin D metabolites was present as $[^3H]$-24,25-$(OH)_2D_3$; relatively small amounts of the other two metabolites were present.

Analysis of tissue:serum ratios of pico-molar uptake of $[^3H]$-1α,25-$(OH)_2D_3$ and $[^3H]$-24,25-$(OH)_2D_3$ confirmed that these metabolites of $[^3H]$-25-$(OH)D_3$ were concentrated in growth plate cartilage (Table 1). The accumulation, relative to serum, for 1α,25-$(OH)_2D_3$ was 4.1 in articular cartilage, 35.4 in growth plate cartilage, 1.3 in bone, 0.7 in kidney, and 3.0 in intestine. For 24R,25-$(OH)_2D_3$, the tissue:serum ratios were 1.6 in articular cartilage, 9.9 in growth plate cartilage, 0.04 in bone, 0.2 in kidney, and 0.4 in intestine.

Discussion

This study has shown that there is a differential distribution of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ in tissues identified as target organs for one or both of these vitamin D metabolites. Whether rats were injected with $[^3H]$-1,25-$(OH)_2D_3$, $[^3H]$-24,25-$(OH)_2D_3$, or the prohormone, $[^3H]$-25-$(OH)D_3$, both dihydroxylated metabolites were concentrated in growth plate cartilage. The other target tissues for vitamin D_3 metabolites, bone, intestine, and kidney, contained radiolabeled 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ at levels either comparable to those found in serum or at reduced levels. The targeting of the metabolites to the growth plate was confirmed by analysis of the ratio of picomolar uptake in the stated tissue divided by the uptake in the serum. These data showed that there was a 35-fold accumulation of 1,25-$(OH)_2D_3$ and a 10-fold accumulation of 24,25-$(OH)_2D_3$ in the growth plate after injection of $[^3H]$-25-$(OH)D_3$.

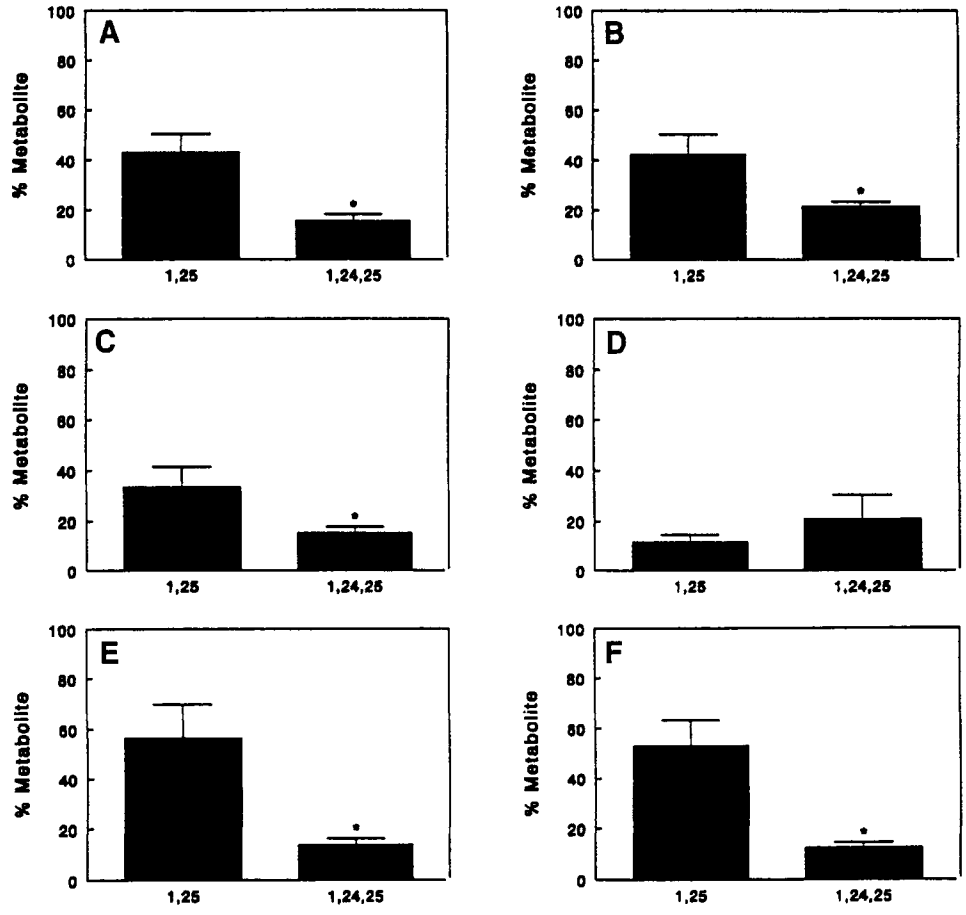

Fig. 2. Relative percent of [^{3}H]-1,25-$(OH)_2D_3$ (1,25) and [^{3}H]-1,24,25-$(OH)_3D_3$ (1,24,25) of the total radiolabel found in **(A)** growth plate cartilage, **(B)** articular cartilage, **(C)** bone, **(D)** kidney, **(E)** intestine, and **(F)** serum after daily injection of 13 pmol [^{3}H]-1,25-$(OH)_2D_3$ for 4 consecutive d. *$P < 0.05$, 1,25 vs 1,24,25.

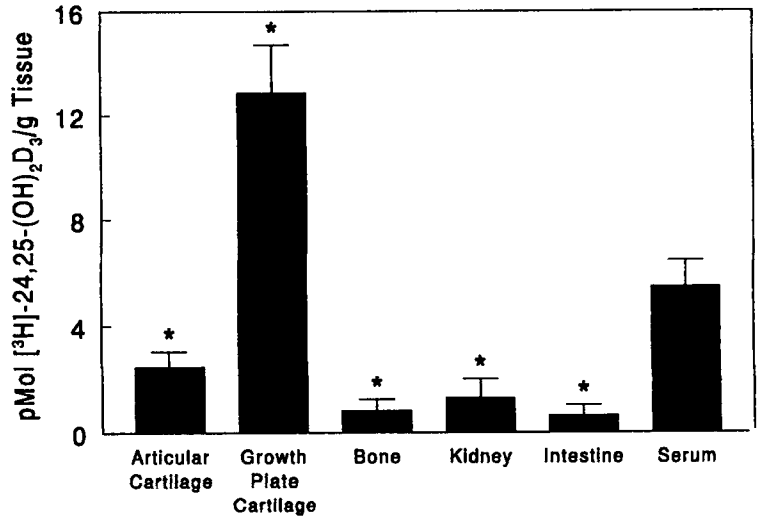

Fig. 3. Tissue distribution of [^{3}H]-24,25-$(OH)_2D_3$ after the daily injection of 13 pmol of [^{3}H]-24,25-$(OH)_2D_3$ for 4 consecutive d. Each point is the mean ± SEM of either two or four samples as described in the Materials and Methods section. *$P < 0.05$, tissue vs serum.

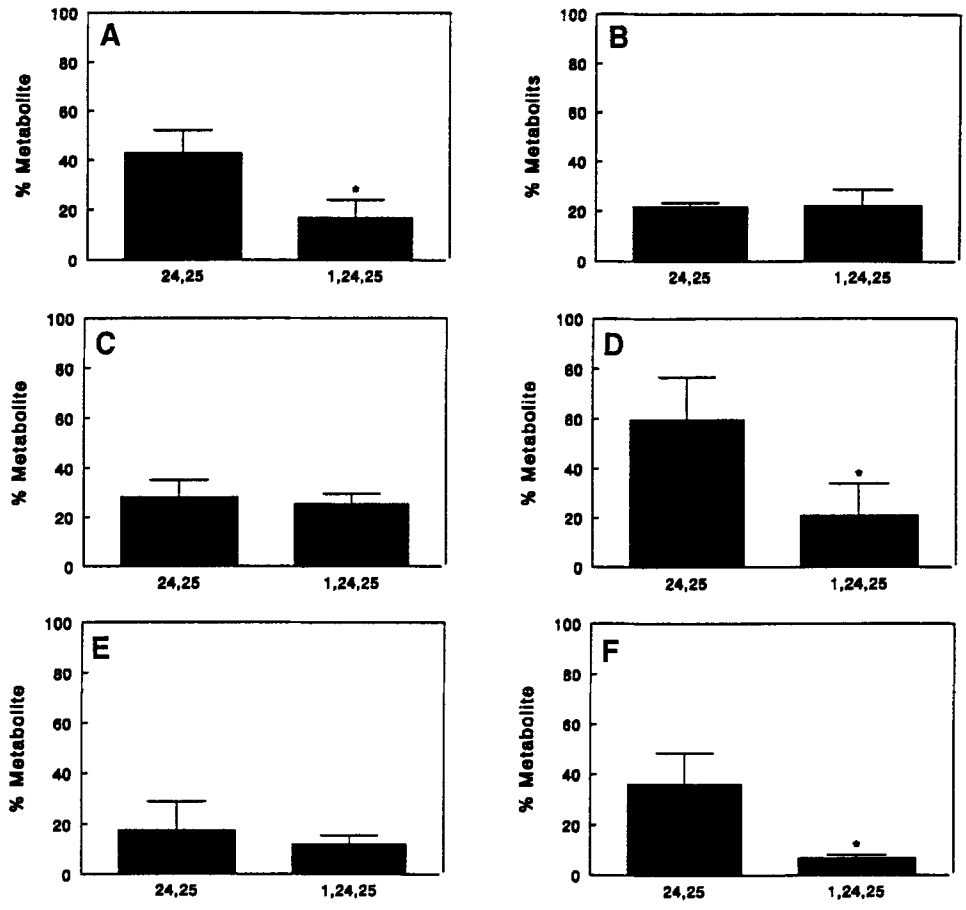

Fig. 4. Relative percent of [^{3}H]-24,25-$(OH)_2D_3$ (24,25) and [^{3}H]-1,24,25-$(OH)_3D_3$ (1,24,25) of the total radiolabel found in **(A)** growth plate cartilage, **(B)** articular cartilage, **(C)** bone, **(D)** kidney, **(E)** intestine, and **(F)** serum after daily injection of 13 pmol [^{3}H]-24,25-$(OH)_2D_3$ for 4 consecutive d. *$P < 0.05$, 24,25 vs 1,24,25.

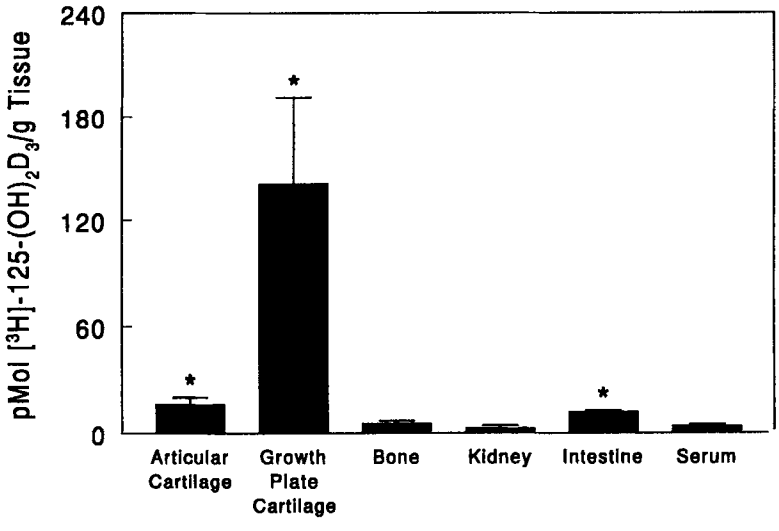

Fig. 5. Tissue distribution of [^{3}H]-1,25-$(OH)_2D_3$ after the injection of 13 pmol of [^{3}H]-25-$(OH)D_3$ daily for 4 consecutive d. Each point is the mean ± SEM of either two or four samples as described in the Materials and Methods section. *$P < 0.05$, tissue vs serum.

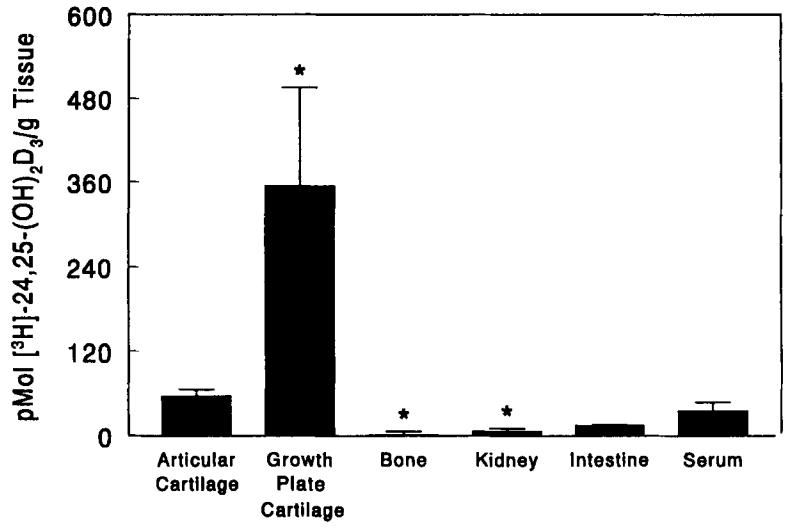

Fig. 6. Tissue distribution of [^{3}H]-24,25-$(OH)_2D_3$ after the injection of 13 pmol of [^{3}H]-25-$(OH)D_3$ daily for 4 consecutive d. Each point is the mean ± SEM of either two or four samples as described in the Materials and Methods section. *$P < 0.05$, tissue vs serum.

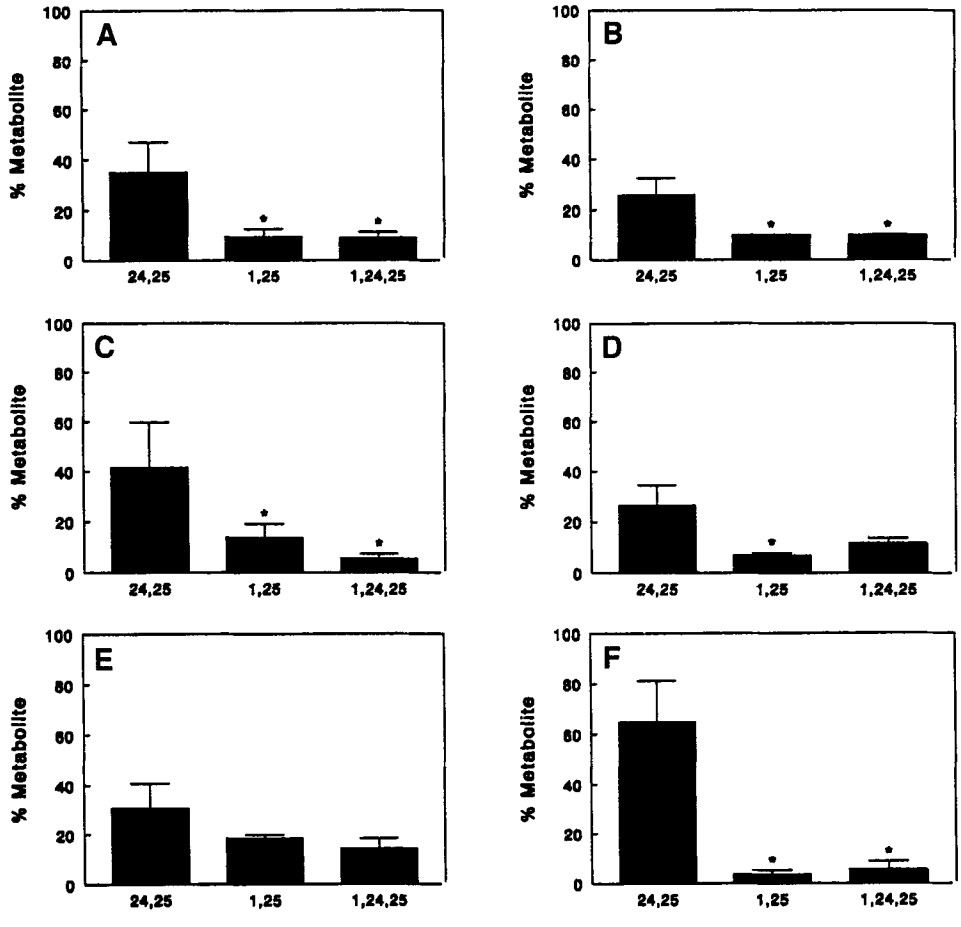

Fig. 7. Relative percent of [^{3}H]-1,25-$(OH)_2D_3$ (1,25), [^{3}H]-24,25-$(OH)_2D_3$ (24,25), and [^{3}H]-1,24,25-$(OH)_3D_3$ (1,24,25) of the total radiolabel found in **(A)** growth plate cartilage, **(B)** articular cartilage, **(C)** bone, **(D)** kidney, **(E)** intestine, and **(F)** serum after daily injection of 13 pmol [^{3}H]-25-$(OH)D_3$ for 4 consecutive d. *$P < 0.05$ vs 24,25-$(OH)_2D_3$.

The specificity of the affinity of growth plate cartilage for the metabolites was demonstrated by the significantly lower levels of both [^{3}H]-1,25-$(OH)_2D_3$ and [^{3}H]-24,25-$(OH)_2D_3$ found in articular cartilage. Growth plate chondrocytes have been shown to be sensitive to vitamin D metabolites (Schwartz and Boyan, 1988; Schwartz et al., 1989; 1992b; Boyan et al., 1992; Sylvia et al., 1993; Schwartz et al., 1995), suggesting that they may contain high levels of specific receptors or binding proteins for vitamin D_3 metabolites or that further metabolism of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ is reduced. Detection of [^{3}H]-1,24,25-$(OH)_3D_3$ in the tissues indicates that metabolism did occur, but whether it occurred in cartilage, as opposed to the kidney, is not known. The results suggest that most of the 1,24,25-$(OH)_3D_3$ was derived from 1,25-$(OH)_2D_3$, since the tissue:serum ratio for 24,25-$(OH)_2D_3$ between growth plate cartilage and the other tissues was of comparable magnitude whether [^{3}H]-24,25-$(OH)_2D_3$ or [^{3}H]-25-$(OH)D_3$ was injected.

Previous studies (Schwartz and Boyan, 1988; Schwartz et al., 1989; 1992b; Boyan et al., 1992; Sylvia et al., 1993; Schwartz et al., 1995) have shown that 24,25-$(OH)_2D_3$ targets primarily the resting zone cells of the growth plate, whereas 1,25-$(OH)_2D_3$ affects chondrocytes in the more mature prehypertrophic and upper hypertrophic zones (growth zone). Others (Kato et al., 1990) have also shown a differential responsiveness to 1α,25-$(OH)_2D_3$ and 24R,25-$(OH)_2D_3$ in micromass cultures of rabbit rib growth plate chondrocytes, although the effect reported was opposite to that seen in the rat costochondral cartilage cells (Schwartz and Boyan, 1988; Schwartz et al., 1989; 1992b; Boyan et al., 1992; Sylvia et al., 1993; Schwartz et al., 1995). The differences in cell response may have been because of the nature of the culture systems used or the state of differentiation of the cells. Whereas the rat chondrocytes were derived from the resting zone or prehypertrophic/upper hypertrophic cell zones, the rabbit chondrocytes were hypertrophic at the time of exposure to 1α,25-$(OH)_2D_3$ and 24R,25-$(OH)_2D_3$. Both of these cell culture models indicate the importance of these vitamin D metabolites to the growth, differentiation, and metabolism of cartilage cells. In the present study, we used the entire growth plate, so whether there was a specific cellular distribution of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ in the growth plate is not known.

Among the vitamin D_3 target tissues, cartilage is unique, since there is only a limited vascular supply. Some regions of the cartilage are sufficiently far from the vasculature that the cells must rely on glycogenolysis for their energy. Nutrients and endocrine factors reach the cells via diffusion through a complex glycosaminoglycan-rich extracellular matrix. A high affinity of the tissue for regulatory factors, as indicated by this study, would enhance the modulation of cartilage growth and development by vitamin D_3 and its metabolites.

It is also possible that local production of 1,25-$(OH)_2D_3$ and/or 24,25-$(OH)_2D_3$ could account for the enrichment of these metabolites in growth plate cartilage. Growth plate chondrocyte cultures exhibit the ability to metabolize [^{3}H]-25-$(OH)D_3$ to [^{3}H]-1,25-$(OH)_2D_3$, [^{3}H]-24,25-$(OH)_2D_3$, and [^{3}H]-1,24,25-$(OH)_3D_3$ (Schwartz et al., 1992a). The relative amount of each of these metabolites depends on the state of maturation of the cartilage from which the chondrocytes were derived and can be regulated by 1,25-$(OH)_2D_3$, 24,25-$(OH)_2D_3$, dexamethasone, and transforming growth factor beta-1. Extra-renal production of 1,25-$(OH)_2D_3$ has also been shown in macrophages, lymphocytes, and bone cells (Turner et al., 1980; Howard et al., 1981; Reichel et al., 1987a,b), suggesting that locally produced secosteroids may be important to the function of the cells that synthesize and secrete them. Other in vitro studies using the growth plate chondrocyte culture model indicate that locally produced vitamin D_3 metabolites may be retained in the extracellular matrix via direct interaction with extracellular membrane-bound organelles termed matrix vesicles (Schwartz et al., 1988a,b; Swain et al., 1993; Boyan et al., 1994), in part explaining the high levels of [^{3}H]-1,25-$(OH)_2D_3$ and [^{3}H]-24,25-$(OH)_2D_3$ noted in growth plate cartilage, but not articular cartilage.

To our knowledge, this is the first study to report the distribution of vitamin D metabolites in the target organs of the hormones in vivo. They indicate that the level of the metabolites in tissue does not always correlate with their level in blood (serum) and suggest that there are tissue-specific properties that account for the disparity, including the possibility of binding factors, as well as local metabolism. The study also demonstrates the importance of cartilage, particularly growth plate cartilage, as a target organ for 24,25-$(OH)_2D_3$ in addition to 1,25-$(OH)_2D_3$.

Materials and Methods

Experimental Procedure

Thirty male Sprague-Dawley rats (Harlan, Indianapolis, IN), weighing 120 ± 20 g, were used for this study. The rats were divided into three groups of eight animals each, which were injected with different radioactive vitamin D metabolites, and a control group of six animals. Prior to initiating the experiment, the animals were acclimated to their surroundings for 2 wk. During this time, as well as during the experiment, the rats were fed ad libitum, a standard rat chow (Teklad LM-485, Madison, WI), which contained 3 IU vitamin D_3/gram, resulting in the animals being vitamin D_3-replete. For the experiment, animals were injected im at 9:00 AM every day for 4 d with 0.1 mL of [^{3}H]-25-$(OH)D_3$, [^{3}H]-24R,25-$(OH)_2D_3$, or [^{3}H]-1α,25-$(OH)_2D_3$. Each dose contained 13 pmoles of hormone (0.36 μCi/dose). At the end of the 4 d, the animals were euthanized and their blood (serum), kidneys, and small intestines collected. The rib cage was separated and the costochondral growth plate

removed by sharp-dissection (Schwartz and Boyan, 1988; Schwartz et al., 1988a,b, 1989; Boyan et al., 1992). The tibia was also harvested and the articular cartilage separated from the long bone. The bone marrow was ablated after collection of the articular cartilage. All samples were stored at –20°C until analyzed.

The [^{3}H]-25-$(OH)D_3$ and [^{3}H]-1α,25-$(OH)_2D_3$ metabolites used in this study were purchased from New England Nuclear (Boston, MA). The [^{3}H]-24R,25-$(OH)_2D_3$ was a gift from Kureha Chemical Co. (Tokyo, Japan). All metabolites were dissolved in ethanol:propanol 1:1 (v/v).

Separation of Vitamin D_3 Metabolites by High-Performance Liquid Chromatography (HPLC)

Because the amount of radiolabeled metabolites in each tissue was anticipated to be small, tissues from two or more animals were combined at time of harvest (*see* Statistical Analysis). The intestines, kidneys, bone, or serum from subsets of two animals were pooled. Because of the smaller amount of tissue available, samples of growth plate or articular cartilage were pooled from subsets of four animals. The pooled samples were then weighed and the total lipids extracted with chloroform:methanol 1:1 (v/v) at a ratio of 1 g tissue/mL solvent and dried with nitrogen. The lipids were then redissolved in 0.2 mL isopropanol:hexane 5:95 (v/v) for HPLC. Five samples (0.2 μL) were chromatographed on a Radial Pak 10 μm silica column (8 × 100 mm, Waters, Milford, MA). Vitamin D_3 metabolites were eluted using a gradient of 5 to 20% isopropanol in hexane for 15 min, followed by a 5-min equilibrium delay to convert the gradient to 5% isopropanol in hexane. Twenty-one 2-mL fractions were collected in liquid scintillation vials and 5 mL of counting cocktail (Betafluor, National Diagnostics, Manville, NJ) added to each vial. The radioactive peaks were identified using a Beckman LS-8000 liquid scintillation counter equipped with automatic external standardization.

Determination of Circulating Vitamin D_3 Metabolite Levels

Lipids were extracted from the serum of each of the control rats ($n = 6$). About 9000 dpm each of [26,27-^{3}H]-25-$(OH)D_3$ (15 Ci/mmol), [23,24(n)-^{3}H]-1α,25-$(OH)_2D_3$ (120 Ci/mmol) (Amersham, Arlington Heights, IL), and [6,9,19-^{3}H]-24,25-$(OH)_2D_3$ (51.1 Ci/mmol), were added to the serum sample as tracers to determine the percent recovery of each metabolite before performing the assay of the individual vitamin D_3 metabolite levels in the serum (*see* next paragraph). Extracted lipids were dried under nitrogen and the vitamin D_3 metabolites separated, as described in the prior section, by HPLC.

Steroid competition assays, as described previously (Schwartz et al., 1992a), were used to measure the serum levels of 25$(OH)D_3$ and 24,25-$(OH)_2D_3$ using vitamin D_3-binding protein [DBP], while a hydroxylapatite-binding assay (Bishop et al., 1980) was used to measure 1α,25-$(OH)_2D_3$ using a lyophilized preparation of chick intestinal cytosol receptor.

Statistical Analysis

As described above, it was necessary to pool tissues from subsets of rats in order to have sufficient radiolabeled vitamin D metabolites for detection. Accordingly, data are derived from samples rather than individual rats. The amount of each metabolite in the different tissues was expressed as pmoles/g tissue. The relative distribution of each metabolite in each tissue was expressed as percent of the specific peak over all the other peaks found. Significant differences were determined by using Student's *t*-test with Bonferroni's correction for multiple comparisons using 95% confidence limits ($P < 0.05$).

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