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Effect of 1α,25-Dihydroxyvitamin D_3 and 24R,25-Dihydroxyvitamin D_3 on Metalloproteinase Activity and Cell Maturation in Growth Plate Cartilage In Vivo

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Recent studies indicate that 1α,25-dihydroxyvitamin D_3 (1α,25[OH]$_2$$D_3$) and 24R,25-dihydroxyvitamim D_3 (24R,25[OH]$_2$$D_3$) differentially regulate proliferation, differentiation, and matrix synthesis of growth plate chondrocytes. To determine whether both metabolites play the same or different roles in vivo, we used the vitamin D–deficient rat as a model. Rickets was induced and then reversed by administering a single dose of ergocalciferol, 1α,25(OH)$_2$$D_3$, or 24R,25(OH)$_2$$D_3$ and euthanizing the animals after 4, 24, 48, or 72 h. Growth plates were either processed for histology and histomorphometry or extracted with buffered guanidine-HCl. Neutral metalloproteinase activity in the extracts was measured by use of aggrecan-containing beads, and collagenase activity was determined by use of radioactive type I collagen. The levels of tissue inhibitor of metalloproteinases (TIMP) and plasminogen activator were also determined. The morphology of the growth plate varied as a function of treatment. While 24R,25(OH)$_2$$D_3$ appeared to affect cell maturation and 1α,25(OH)$_2$$D_3$ appeared to affect terminal differentiation and calcification, response to ergocalciferol was indicative of the combined responses to the individual metabolites. Enzyme activity was regulated in a differential manner. Treatment with ergocalciferol produced a rapid decline in both neutral metalloproteinase and collagenase activities that was statistically significant by 4 h. By contrast, 1α,25(OH)$_2$$D_3$ had no effect on neutral metalloproteinase activity but caused a significant decrease in both active and total collagenase activity by 4 h, while 24R,25(OH)$_2$$D_3$ decreased neutral metalloproteinase activity by 48 h and had no effect on collagenase activity. Ergocalciferol had no effect on TIMP levels at any time examined, whereas 1α,25(OH)$_2$$D_3$ caused an increase at 48 and 72 h and 24R,25(OH)$_2$$D_3$ completely blocked TIMP production at 4 and 24 h. By contrast, plasminogen activator activity by ergocalciferol was decreased at 4 h, increased by 1α,25(OH)$_2$$D_3$ at 4 and 24 h, and decreased by 24R,25(OH)$_2$$D_3$ at all time points examined. These in vivo results confirm our previous cell culture observations showing that growth plate chondrocytes are differentially regulated by 1α,25(OH)$_2$$D_3$ and 24R,25(OH)$_2$$D_3$. Moreover, they show definitively that these two vitamin D metabolites play distinct roles not only in regulating neutral metalloproteinase and collagenase activities in growth plate cartilage but in cell maturation and calcification of this tissue in vivo.

Key Words: Endochondral ossification; 1α,25-dihydroxyvitamin D_3; 24R,25-dihydroxyvitamin D_3; metalloproteinase; tissue inhibitor of metalloproteinases.

Introduction

In growth plate cartilage, chondrocytes traverse through several well-defined maturation states. As these cells progressively mature, both the cells and surrounding matrix are remodeled *(1,2)*. Chondrocytes in the growth zone begin to enlarge and become hypertrophic. As these cells approach terminal differentiation, mineral deposition in the extracellular matrix is initiated.

It is generally agreed that during the maturation of the chondrocytes, a significant amount of extracellular matrix remodeling must occur. When compared to the immature resting zone, the hypertrophic zone matrix is characterized by a relative reduction in types II and IX collagen *(3,4)*, an increase in type X collagen *(3)*, an increase in pericellular collagen degradation *(5)*, and an increase in the abundance of the C-propeptide of type II collagen *(6)*. The large aggre-

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gating proteoglycan, aggrecan, is also remodeled in preparation for calcification. Many in vitro studies have shown that aggrecan is a potent inhibitor of mineralization *(7)*, suggesting that these macromolecules must be removed for calcification to occur in vivo *(8–10)*. However, others have shown that aggrecan is not reduced in size and state of ag-gregation at the time calcification is initiated. In fact, high concentrations of aggrecan may be required to initiate focal sites of matrix calcification *(11–13)*.

The sequence of events leading up to mineralization in healing rachitic rats closely duplicates the normal process, except that the hypertrophic cell zone is greatly expanded as a consequence of the rickets *(5,14,15)*. Thus, the rachitic rat model is useful for studying specific characteristics of this zone that are involved in calcification, as well as for identifying characteristics specific to each zone of endochondral maturation. By using this model, it has been shown that collagenase activity is present in the growth plate and that this activity is significantly elevated in the hypertrophic cell zone compared with the proliferative cell zone *(5,14, 16,17)*. Based on these results, we suggested that increased amounts of active collagenase are essential for chondrocyte hypertrophy and preparation of the collagenous matrix for calcification. In contrast to our view, an alternative proposal has been advanced based on elongation of the chondrocytes in the absence of matrix degradation *(18)*.

In parallel studies using the same rachitic rat model, proteoglycan aggregation has been shown to vary with cell zone, demonstrating an overall decrease in aggregate size with calcification *(19,20)*. Related studies using an in vitro model *(21)* have shown that growth plate chondrocytes produce matrix vesicles that contain metalloproteinases capable of digesting proteoglycans, and that are regulated by vitamin D metabolites *(22)*. These results suggest that matrix metalloproteinases in vivo might be involved in the remodeling of proteoglycans necessary for calcification to begin.

It has been known for some time that vitamin D is essential for the proper regulation of endochondral ossification. Boyan et al. *(23)* have demonstrated that chondrocyte response to vitamin D_3 metabolites is both vitamin D metabolite specific and cell maturation dependent, with growth zone chondrocytes responding primarily to 1α,25-dihydroxyvitamin D_3 (1α,25$[OH]_2D_3$), and resting zone chondrocytes responding primarily to 24R,25-dihydroxyvitamin D_3 (24R,25$[OH]_2D_3$). Interestingly, growth zone and resting zone chondrocytes have also been found to produce vitamin D metabolites when presented with exogenous 25-hydroxyvitamin D_3, and they express both 1α- and 24-hydroxylases *(24)*. These observations invoke a possible autocrine function for these secosteroids. Further, the addition of dexamethasone or transforming growth factor-β1 (TGF-β1) to the culture medium augments production of vitamin D metabolites by these cells *(24,25)*.

These data suggest that both vitamin D_3 metabolites play a role in endochondral ossification in vivo and that 24R,25$(OH)_2D_3$ has some specificity for inducing cell maturation and chondrogenesis, while 1α,25$(OH)_2D_3$ probably regulates terminal differentiation and calcification. This hypothesis is supported by studies showing that 24R,25$(OH)_2D_3$ regulates synthesis of DNA, proteoglycan, and type II collagen production *(26)*; it is concentrated in growth plate cartilage when either [^{3}H]-25$(OH)D_3$ or [^{3}H]-24,25$(OH)_2D_3$ is injected into vitamin D–replete rats *(27)*, and cartilage cells have receptors for this metabolite *(28–31)*. In addition, 24R,25$(OH)_2D_3$ can induce the maturation of resting zone cells in culture, causing them to become responsive to 1α,25$(OH)_2D_3$, the metabolite active in growth zone cells *(32)*. Atkin et al. *(33,34)* also showed that 24R,25$(OH)_2D_3$ can promote healing of rickets when injected into rachitic rats; similarly, 24R,25$(OH)_2D_3$ enhances healing of fractures when it is injected into fracture callus *(35)*.

The recent progress on elucidating the physiologic roles of vitamin D_3 metabolites we just described, coupled with our interest in matrix remodeling and our earlier observations on the effect of 1α,25$(OH)_2D_3$ and 24R,25$(OH)_2D_3$ in reversing the rachitic lesions in our rat model *(33,34)*, led to the current study. We tested the hypothesis that part of the effect of vitamin D_3 metabolites is mediated by regulation of metalloproteinase and plasminogen activator activities. We used the rachitic rat model and examined the effect of ergocalciferol, 1α,25$(OH)_2D_3$, and 24R,25$(OH)_2D_3$ on the levels of collagenase, neutral metalloproteinase, plasminogen activator, and tissue inhibitor of metalloproteinases (TIMP) in the growth plate during reversal of rickets. We then correlated these enzymatic changes with changes in morphometric parameters of the growth plate.

Results

Morphologic Observations

The height of the growth plate in –VDP animals was greatly expanded over that found with normal animals (Fig. 1A). The hypertrophic cell zone enlarged to approx 66% of the height of the entire growth plate, and no calcification was present in the lower hypertrophic cell zone, as evidenced by the absence of von Kossa staining. When –VDP animals were treated for 24 h with 24R,25$(OH)_2D_3$ or 1α,25$(OH)_2D_3$ before they were euthanized, a number of changes in the morphology/histology of the growth plate could be seen, and those changes were related to the vitamin D metabolite given to reverse the –VDP rickets.

In animals receiving 24R,25$(OH)_2D_3$, the cellularity of the resting and proliferating cell zones was decreased compared with that of the –VDP animals, and the organization of the cells became more columnar (Fig. 1B). The cells in the hypertrophic cell zone appeared to retain the same degree of hypertrophy and interterritorial matrix seen in the –VDP animals, but the organization of the cells was more columnar, as seen in the resting and proliferative cell zones. In addition, partially expanded cells could be seen

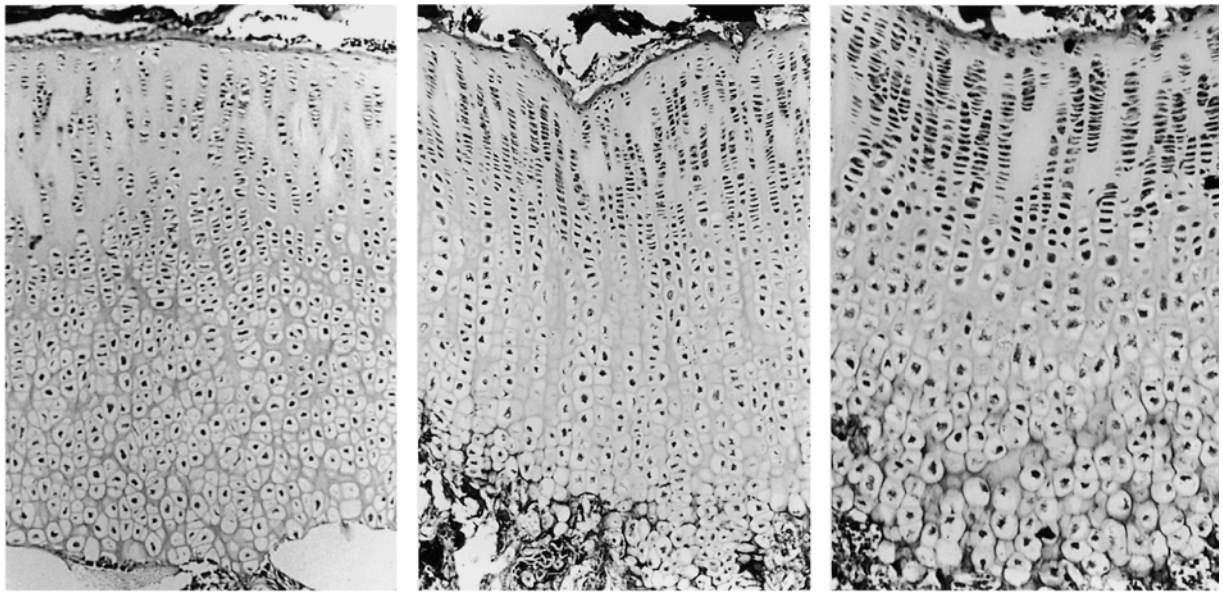

Fig. 1. Light photomicrographs of growth plates from –VDP rats and –VDP rats treated with vitamin D_3 metabolites for 24 h prior to euthanasia. At euthanasia, the proximal tibial growth plate cartilage was removed from the animal and then fixed, sectioned, and stained with von Kossa and counterstained with hematoxylin & eosin (H&E). (**A**) Growth plate of –VDP rat; (**B**) growth plate of –VDP rat treated with 24R,25$(OH)_2D_3$ for 24 h prior to euthanasia; (**C**) growth plate of –VDP rat treated with 1α,25$(OH)_2D_3$ for 24 h prior to euthanasia. Original magnification: ×100.

in the upper hypertrophic cell zone, but only those cells bordering on the metaphysis were fully hypertrophied. In contrast to the –VDP animals, von Kossa staining in these growth plates could be seen extending from the metaphyseal border into the lower hypertrophic cell zone.

In animals receiving 1α,25$(OH)_2D_3$, the cellularity of the resting and proliferating cell zones was similar to that of animals receiving 24R,25$(OH)_2D_3$, including the arrangement of the cells in columns (Fig. 1C). However, the organization and morphology of the cells in the hypertrophic cell zone in these animals was strikingly different from those in the 24R,25$(OH)_2D_3$-treated animals. The cells in the upper hypertrophic cell zone were arranged in columns and partially expanded. As the maturation of the cells increased (closer to the calcification front), the cells became larger, and their arrangement was less organized in columns, approximating their organizational habit in normal growth plate. In addition, the number of cells near the metaphyseal border that was fully hypertrophied was greater than that seen with 24R,25$(OH)_2D_3$ treatment. Von Kossa staining revealed deposits extending halfway up into the hypertrophic zone, in parallel with the fully expanded cell morphology typical of hypertrophic cells.

When vitamin D metabolite treatment was extended to 48 h, additional changes in growth plate morphology could be discerned (Fig. 2). In growth plates from –VDP animals treated with 24R,25$(OH)_2D_3$, the number of cells in the resting and proliferating cell zones increased, but the cells retained their columnar arrangement (Fig. 2A). In the hypertrophic cell zone, the number of cells also increased, but the cells were not arranged in well-organized columns, as seen in sections from the 24-hour animals. Further, the number and arrangement of the cells in this zone suggested that, although cells were "recruited" ("pushed") into this region, there was a blockade of terminal differentiation that caused the cells in the hypertrophic zone to accumulate. Von Kossa staining was evident in the lower 20% of the hypertrophic cell zone (data not shown).

In animals treated with 1α,25$(OH)_2D_3$ for 48 h, the number of cells in the resting and proliferating cell zones was increased from that of the animals treated for 24 h (Fig. 2B). The morphology and arrangement of cells in the hypertrophic cell zone after 24 and 48 h of treatment was quite different. In cartilage from animals treated for 48 h, the cells in the upper hypertrophic cell zone were less columnar, with more interterritorial matrix present. The region of fully expanded cells was decreased, even though there were more cells. In addition, the amount of von Kossa staining was less than seen after 24 h of treatment (data not shown).

In –VDP rats treated with ergocalciferol for 48 h, the morphology of the growth plate displayed signs of returning to normal (Fig. 2C). The disk-shaped resting zone cells were arranged in columns that gave rise to proliferative cells. The upper hypertrophic cell zone contained cells that were partially expanded, and as they matured, they continued to expand. At the metaphyseal border, they were fully hypertrophied. Von Kossa staining extended almost halfway up into the hypertrophic cell zone.

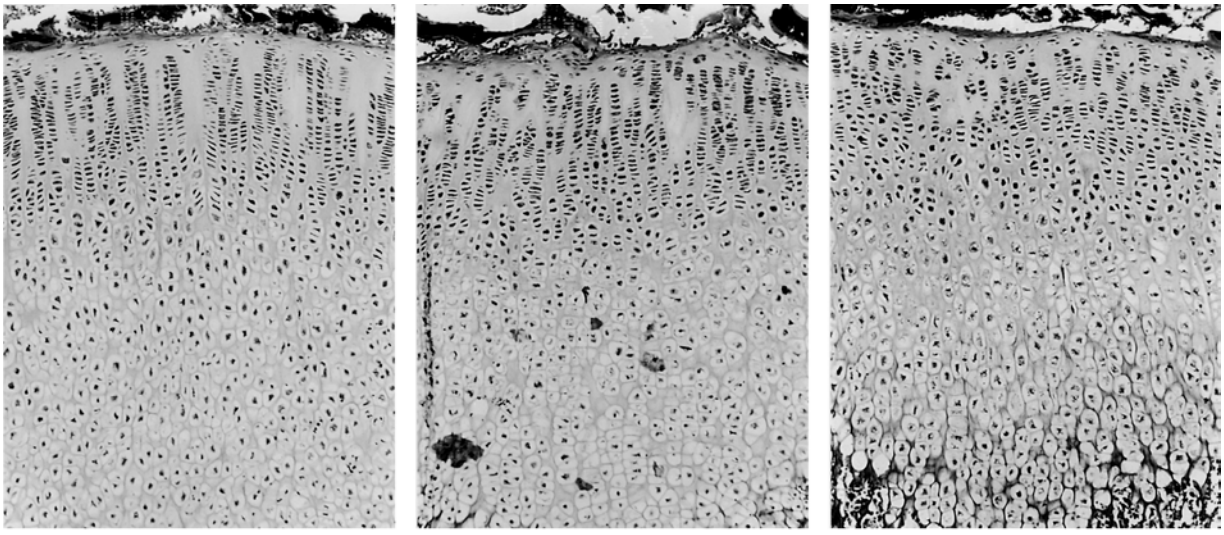

Fig. 2. Light photomicrographs of growth plates from –VDP rats treated with vitamin D_3 metabolites or ergocalciferol for 48 h prior to euthanasia. At euthanasia, the proximal tibial growth plate cartilage was removed from the animal and then fixed, sectioned, and stained with von Kossa and counterstained with H&E. (**A**) growth plate of –VDP rat treated with 24R,25$(OH)_2D_3$ for 48 h prior to euthanasia; (**B**) growth plate of –VDP rat treated with 1α,25$(OH)_2D_3$ for 48 h prior to euthanasia; (**C**) growth plate of –VDP rat treated with ergocalciferol for 48 h prior to euthanasia. Original magnification: ×100.

Histomorphometric Observations

Throughout the entire 48 to 72-h treatment period, growth plate height, calcification distance, cartilage cellularity, and all biochemical parameters were unchanged in the –VDP animals.

The height of the growth plate in the rachitic rats was sensitive to treatment with ergocalciferol as well as with the vitamin D metabolites (Fig. 3). The effect was the same regardless of treatment, but it was time dependent. Treatment of –VDP animals with ergocalciferol (Fig. 3A), 1α, 25$(OH)_2D_3$ (Fig. 3B), or 24R,25$(OH)_2D_3$ (Fig. 3C) caused a decrease in growth plate height within 4 h of treatment that was significant only for ergocalciferol and 24R,25$(OH)_2D_3$. After 24 h of treatment, no change in growth plate height was found when compared with the rachitic animals. After 48 h, the height of the growth plate was significantly increased.

The distance the calcification front extended into the hypertrophic cell zone was dependent on the treatment used (Fig. 4). Ergocalciferol caused an increase in calcified cartilage only after 48 h (Fig. 4A). 1α,25$(OH)_2D_3$ (Fig. 4B) and 24R,25$(OH)_2D_3$ (Fig. 4C) both stimulated calcification at 24 h, but the effect was greater in response to 1α, 25$(OH)_2D_3$. In rachitic animals treated with either metabolite for 48 h, calcification of the hypertrophic cartilage was also increased. Although the extent of mineralized cartilage at 48 h was reduced in comparison to 24 h in response to 1α,25$(OH)_2D_3$, it was comparable with the effect at 24 h in response to 24R,25$(OH)_2D_3$. Neither metabolite alone caused mineral deposition to the same extent as seen in animals treated 48 h with ergocalciferol.

Ergocalciferol, 1α,25$(OH)_2D_3$, and 24R,25$(OH)_2D_3$ caused time-dependent changes in cellularity that varied with the cartilage zone (Table 1). Cell number in the proliferative cell zone was reduced at 4 and 24 h in response to all three agents, but this effect was not evident at 48 h. 1α,25$(OH)_2D_3$ caused an increase in cell number in the zone of maturation at 24 and 48 h, whereas 24R,25$(OH)_2D_3$ caused a decrease in cell number at 4 and 24 h. All agents caused a decrease in the zone of elongation at 4 h. All agents caused an increase in cell number in the hypertrophic cell zone at 48 h, but this effect was statistically significant only in rachitic animals treated with ergocalciferol or 24R,25$(OH)_2D_3$.

Proteinase Activities and TIMP

Specific activities of neutral metalloproteinase, collagenase, and TIMP were differentially expressed as a function of vitamin D status. The specific activity of both neutral metalloproteinase and collagenase (active or total) was significantly higher in –VDP animals compared with normal control animals (Fig. 5). By contrast, the activity of TIMP was significantly less in the –VDP animals. Whereas most of the collagenase activity in normal growth plates was in latent form (i.e., total minus active), in the –VDP growth plates, the collagenase was almost exclusively active. Similarly, neutral metalloproteinase activity showed the same trends.

Regulation of neutral metalloproteinase activity was dependent on the vitamin D metabolite used (Fig. 6). Treating –VDP animals with ergocalciferol significantly reduced the activity in a time-dependent manner. The effect

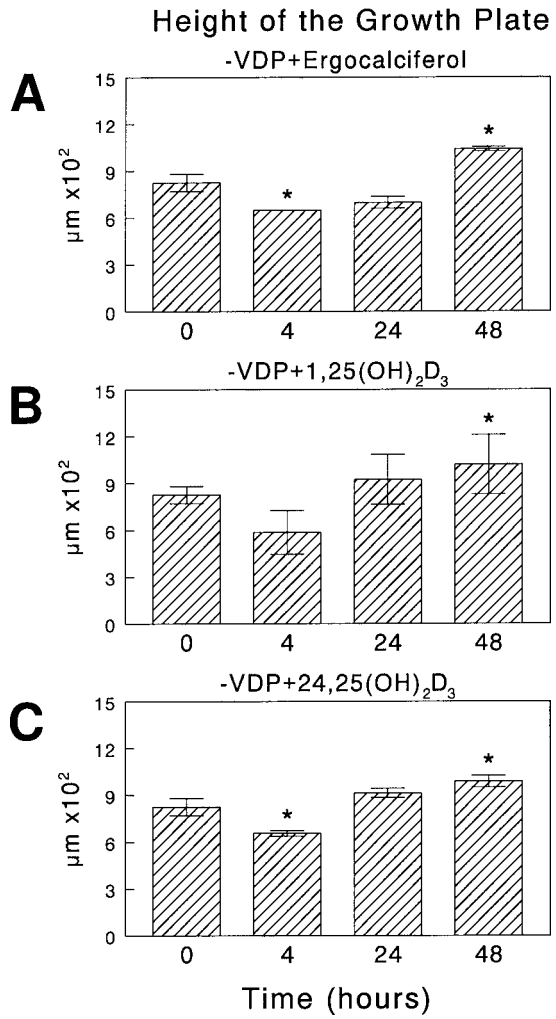

Fig. 3. Height of the growth plate from –VDP rats and –VDP rats treated with ergocalciferol (**A**), 1α,25$(OH)_2D_3$ (**B**), or 24R,25$(OH)_2D_3$ (**C**) for 4, 24, or 48 h prior to euthanasia. At euthanasia, the proximal tibial growth plate cartilage was removed from the animal and then fixed, sectioned, and stained with H&E. Growth plate height was measured on six sections from each of three to four rats as described in Materials and Methods. Data are the mean ± SEM for three or four rats. *$p < 0.05$, treatment vs –VDP.

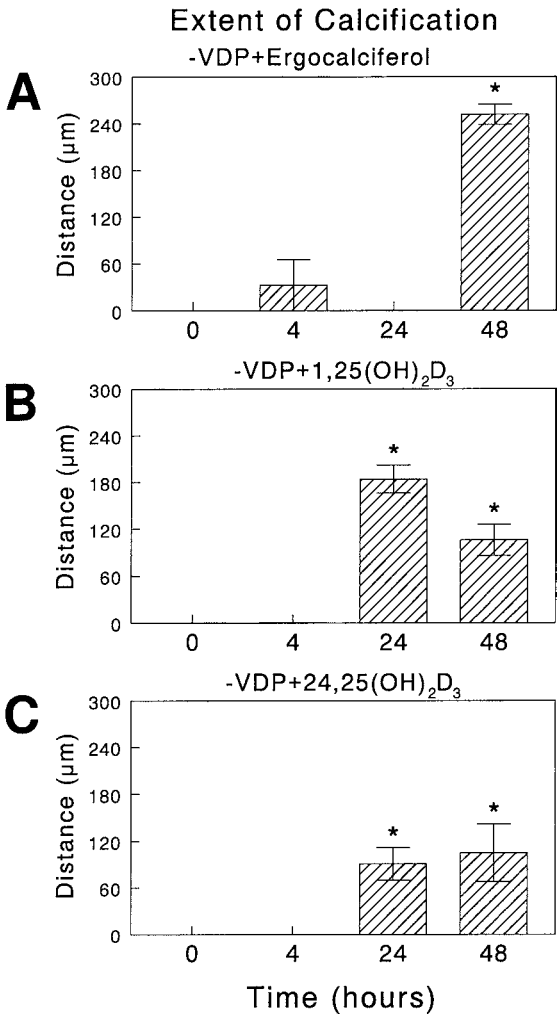

Fig. 4. Distance calcification extends into the hypertrophic cell zone in growth plates from –VDP rats and –VDP rats treated with ergocalciferol (**A**), 1α,25$(OH)_2D_3$ (**B**), or 24R,25$(OH)_2D_3$ (**C**) for 4, 24, or 48 h prior to euthanasia. At euthanasia, the proximal tibial growth plate cartilage was removed from the animal and then fixed, sectioned, and stained with von Kossa and counterstained with H&E. Extension of calcification into the hypertrophic cell zone was measured on six sections from each of three to four rats as described in Materials and Methods. Data are the mean ± SEM for three or four rats. Where no bar can be seen, the value was zero. *$p < 0.05$, treatment vs –VDP.

on the active form of the enzyme was noted within 4 h, whereas significant reductions in total activity were not observed until after 24 h of treatment (Fig. 6A). Treatment with 24R,25$(OH)_2D_3$ produced a similar inhibitory effect on both active and total neutral metalloproteinase activity, although it was found only after 48 h of treatment (Fig. 6C). By contrast, 1α,25$(OH)_2D_3$ had no effect on enzyme activity at any of the times examined (Fig. 6B).

Regulation of collagenase activity was also dependent on the vitamin D metabolite used (Fig. 7). Ergocalciferol caused a rapid decrease in active and total collagenase activity that was evident by 4 h and that remained constant through 48 h (Fig. 7A). In animals treated for 72 h, there was a further decrease such that active enzyme was virtually undetectable. 1α,25$(OH)_2D_3$ also caused a time-dependent decrease in collagenase activity (Fig. 7B), but the effect was less rapid than that seen in the ergocalciferol-treated animals. The amount of active enzyme was reduced by approx 33% at 4 h and by 66% at 24 h. As with ergocalciferol, active enzyme was almost absent by 72 h. Total enzyme activity was not significantly affected until 48 h of treatment; however, even at 72 h, it was still almost 80% of the enzyme present in extracts from the –VDP growth plates. 24R,25$(OH)_2D_3$ had no effect on collagenase levels at any time, whether active or total enzyme activity was being measured (Fig. 7C).

Table 1
Number of Cells in Each Major Zones of –VDP Growth Plate Before and After Vitamin D Metabolite Treatment[a]

	No. of cells in each zone			
	Proliferative cell zone	Zone of maturation	Zone of elongation	Hypertrophic cell zone
–VDP (n = 5)	24.2 ± 2.3	11.6 ± 0.5	13.0 ± 1.7	11.6 ± 2.0
–VDP + ergocalciferol				
4 h (n = 2)	16.0 ± 1.0[b]	11.5 ± 0.5	7.5 ± 0.5[b]	9.5 ± 1.5
24 h (n = 3)	17.2 ± 1.9[b]	11.3 ± 1.4	9.0 ± 1.2	11.3 ± 1.7
48 h (n = 5)	18.7 ± 4.1	12.6 ± 1.4	9.2 ± 1.3	18.8 ± 2.5[b]
–VDP + 1a,25$(OH)_2D_3$				
4 h (n = 2)	16.0 ± 2.0[b]	14.0 ± 1.0	6.5 ± 1.5[b]	8.5 ± 0.5
24 h (n = 5)	15.0 ± 1.4[b]	15.6 ± 1.5[b]	9.0 ± 1.5	19.2 ± 3.3
48 h (n = 6)	23.0 ± 1.3	18.7 ± 1.1[b]	9.6 ± 1.1	17.1 ± 2.8
–VDP + 24R,25$(OH)_2D_3$				
4 h (n = 4)	16.8 ± 0.5[b]	9.0 ± 0.4[b]	6.8 ± 0.5[b]	8.5 ± 1.0
24 h (n = 6)	18.0 ± 1.0[b]	9.0 ± 0.4[b]	10.3 ± 1.4	16.5 ± 1.9
48 h (n = 6)	24.7 ± 0.7	12.3 ± 0.9	11.2 ± 1.5	22.8 ± 3.8[b]

[a]All values are the mean ± SEM for the indicated number of rats.
[b]$p < 0.05$, treatment vs –VDP.

Regulation of TIMP was more complex (Fig. 8). Ergocalciferol had no effect on inhibitor levels (Fig. 8A), whereas 1α,25$(OH)_2D_3$ caused a greater than twofold increase in TIMP levels at 48 and 72 h (Fig. 8B). By contrast, 24R,25$(OH)_2D_3$ treatment resulted in complete inhibition of TIMP at 4 and 24 h, and a partial inhibition at 72 h (Fig. 8C).

Regulation of plasminogen activator by ergocalciferol reflected the combined, but opposing, effects of 1α,25$(OH)_2D_3$ and 24R,25$(OH)_2D_3$ (Fig. 9). Ergocalciferol caused a decrease in plasminogen activator activity at 4 h (Fig. 9A). By 24 h, activity had returned to levels comparable with those seen in the rachitic rat growth plates. 1α,25$(OH)_2D_3$ stimulated plasminogen activator activity at 4 h (Fig. 9B). Levels were still elevated at 24 h, but for 48 h, enzyme activity was returned to levels seen in rachitic tissue. By contrast, 24R,25$(OH)_2D_3$ inhibited plasminogen activator at all times examined; the effect was greatest at 4 h and was gradually reduced over time (Fig. 9C).

Discussion

We showed that ergocalciferol and the vitamin D metabolites, 1α,25$(OH)_2D_3$ and 24R,25$(OH)_2D_3$, are not only capable of reversing –VDP rickets, but they modulate matrix remodeling and cell maturation in the growth plate in a vitamin D metabolite–specific manner. The methods used for assessing metalloproteinase activity have the advantage of demonstrating the amount of enzyme activity present in the matrix and whether it is in active or latent form. In addition, these methods give insight into the extent the two major components of the extracellular matrix are being degraded, since the substrates used for the enzyme assays are the endogenous substrates for these enzymes in vivo (e.g., aggrecan and collagen).

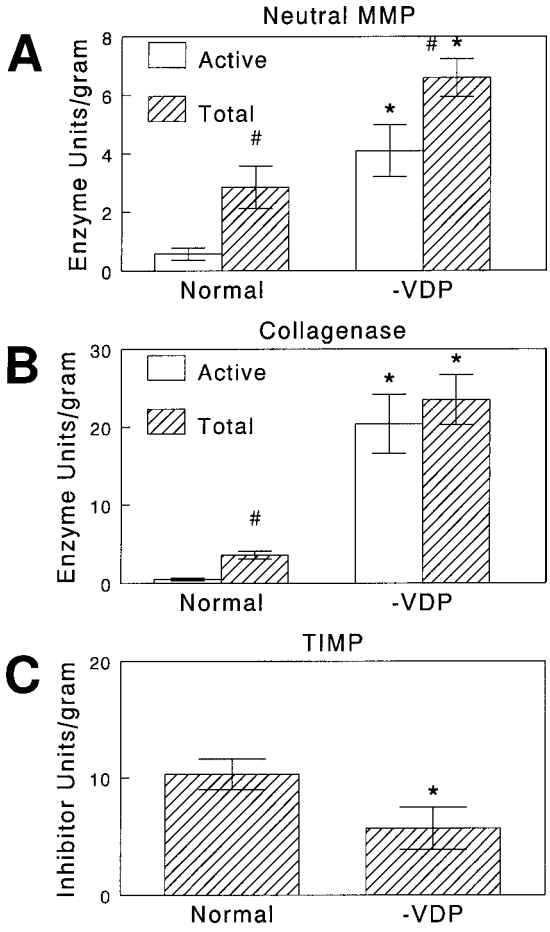

Fig. 5. Neutral metalloproteinase (**A**), collagenase (**B**), and TIMP (**C**) content of growth plate cartilage from normal and –VDP rats. At euthanasia, the proximal tibial growth plate cartilage was removed from the animal and extracted in buffered guanidine as described in Materials and Methods. Extracts were assayed for both active (□) and total (▨) neutral metalloproteinase and collagenase activities as well as TIMP content, as described in Materials and Methods. All values are the mean ± SEM in enzyme or inhibitor units/gram of wet wt tissue for $n \geq 7$ samples. *$p < 0.05$, normal vs –VDP; #$p < 0.05$, active vs total.

The exact identity of the metalloproteinase(s) responsible for degrading the aggrecan substrate in the present study was not determined. The aggrecan molecule contains two N-terminal globular domains, G1 and G2, that are separated by a proteolytically sensitive interglobular domain. Many MMPs, including MMP-1, -2, -3, -7, -8, -9, -13, and -14, have been shown to cleave aggrecan in this region *(36–41)*. Another group of metalloproteinases, the so-called aggrecanases, have been found to cleave in this domain as well *(42,43)*. The identification of the metalloproteinases responsible for degrading aggrecan in our growth plate extracts remains to be accomplished.

It is likely that the collagenase activity measured in the present study is MMP-13, the predominant collagenase in the rat *(44)*. Like the human homolog (MMP-1), MMP-13 has the ability to specifically cleave triple helical collagen, resulting in the characteristic three-quarter and one-quar-

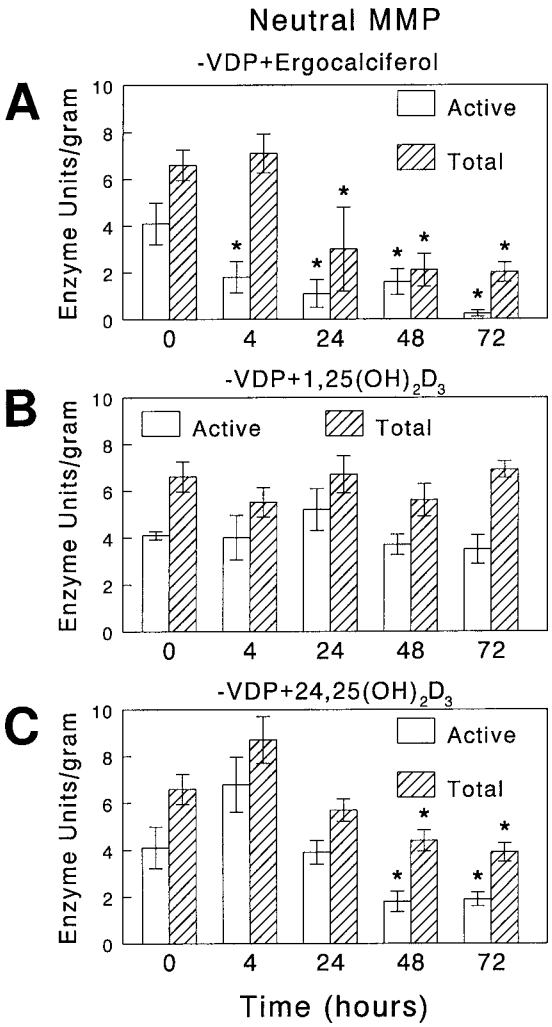

Fig. 6. Neutral metalloproteinase content of growth plate cartilage from –VDP rats and –VDP rats treated with ergocalciferol (**A**), 1α,25(OH)$_2$D$_3$ (**B**), or 24R,25(OH)$_2$D$_3$ (**C**) for 4, 24, 48, or 72 h prior to euthanasia. At euthanasia, the proximal tibial growth plate cartilage was removed from the animal and extracted in buffered guanidine as described in Materials and Methods. Extracts were assayed for both active (☐) and total (▨) neutral metalloproteinase activity on proteoglycan-containing polyacrylamide gel beads as described in Materials and Methods. All values are the mean ± SEM in enzyme units/gram of wet wt tissue for $n \geq 7$ samples. *$p < 0.05$, vs –VDP.

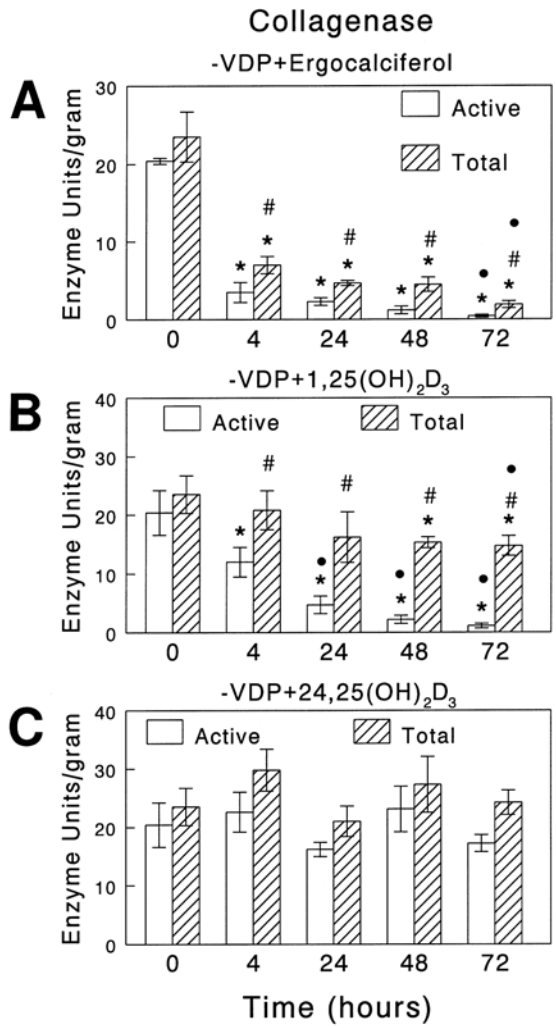

Fig. 7. Collagenase content of growth plate cartilage from –VDP rats and –VDP rats treated with ergocalciferol (**A**), 1α,25(OH)$_2$D$_3$ (**B**), or 24R,25(OH)$_2$D$_3$ (**C**) for 4, 24, 48, or 72 h prior to euthanasia. At euthanasia, the proximal tibial growth plate cartilage was removed from the animal and extracted in buffered guanidine as described in Materials and Methods. Extracts were assayed for both active (☐) and total (▨) collagenase activity on [^{3}H]-telopeptide-free collagen as described in Materials and Methods. All values are the mean ± SEM in enzyme units/gram of wet wt tissue for $n \geq 7$ samples. *$p < 0.05$, vs –VDP; $^{\#}p < 0.05$, active vs total; •$p < 0.05$, vs 4 h.

ter-length products. However, other MMPs, such as MMP-2 and MMP-14, are capable of cleaving collagen *(45,46)*. In addition, MMP-8, previously thought to be exclusively expressed by neutrophil leukocytes, may be involved as well, because normal articular chondrocytes have been shown to produce this MMP *(47)*.

As chondrocytes mature and hypertrophy, degradation of the extracellular matrix is required. Earlier studies showed that neutral "proteoglycanase" activity was produced by growth plate chondrocytes and that peak activity was localized in the hypertrophic cell zone *(48,49)*. In later studies, stromelysin and collagenase were shown by immunohistochemistry to be localized in the upper hypertrophic zone *(50)*. Under vitamin D–deficient conditions, however, there is a change in the content and aggregation state of proteoglycans in the matrix *(19)* as well as in the activity of collagenase *(5)*. The present study confirms that collagenase activity is increased in rachitic growth plates and shows that essentially all the collagenase is in active form. Moreover, there is a marked increase in neutral metalloproteinases capable of digesting proteoglycan, and more than half of this is in the active form as well. One possible reason for this increase in active enzyme is that rachitic growth plates exhibit a corresponding decrease in TIMP.

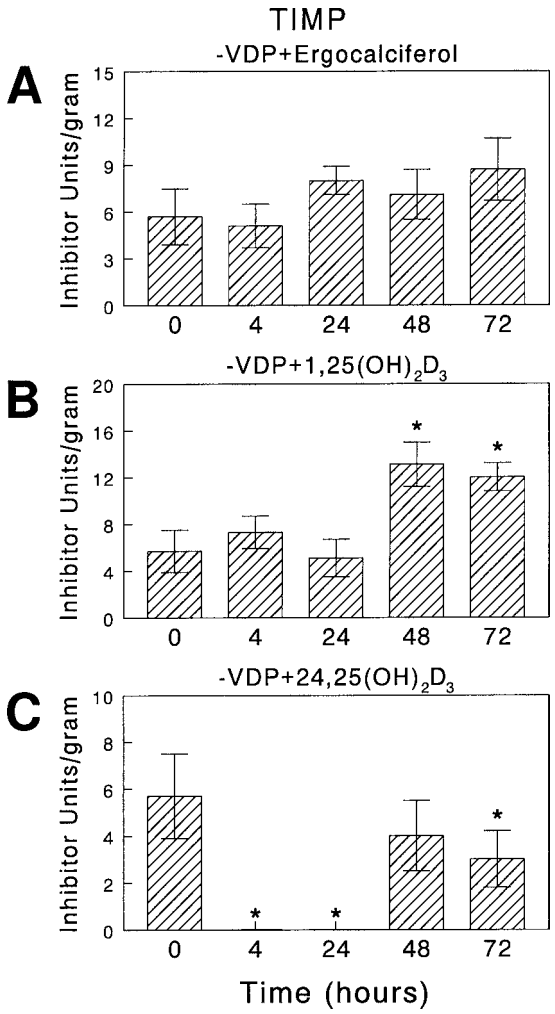

Fig. 8. TIMP content of growth plate cartilage from –VDP rats and –VDP rats treated with ergocalciferol (**A**), 1α,25$(OH)_2D_3$ (**B**), or 24R,25$(OH)_2D_3$ (**C**) for 4, 24, 48, or 72 h prior to euthanasia. At euthanasia, the proximal tibial growth plate cartilage was removed from the animal and extracted in buffered guanidine as described in Materials and Methods. Extracts were assayed for TIMP activity as described in Materials and Methods. All values are the mean ± SEM in inhibitor units/gram of wet wt tissue for $n \geq 7$ samples. *$p < 0.05$, vs –VDP.

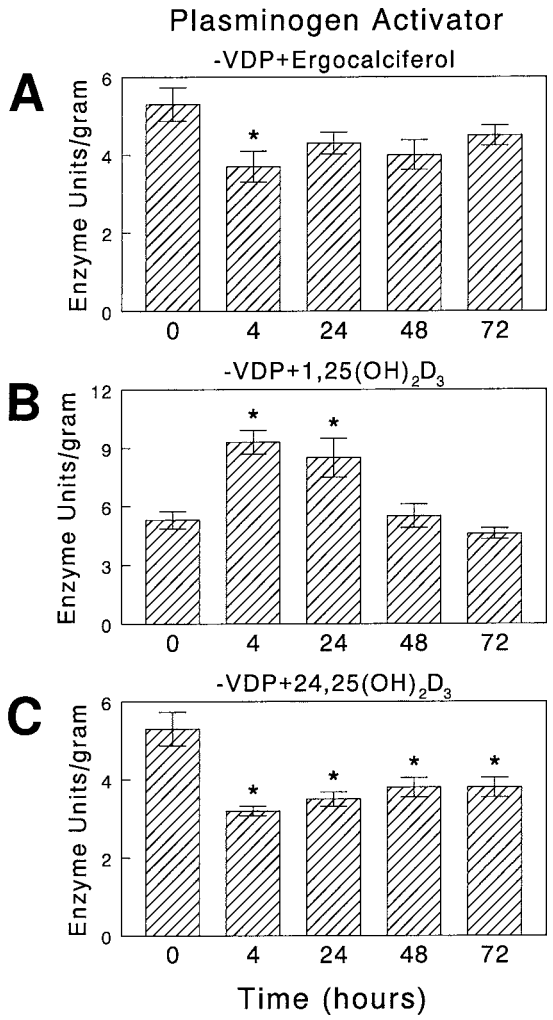

Fig. 9. Plasminogen activator content of growth plate cartilage from –VDP rats and –VDP rats treated with ergocalciferol (**A**), 1α,25$(OH)_2D_3$ (**B**), or 24R,25$(OH)_2D_3$ (**C**) for 4, 24, 48, or 72 h prior to euthanasia. At euthanasia, the proximal tibial growth plate cartilage was removed from the animal and extracted in buffered guanidine as described in Materials and Methods. Extracts were assayed for plasminogen activator activity as described in Materials and Methods. All values are the mean ± SEM in enzyme units/gram of wet wt tissue for $n \geq 7$ samples. *$p < 0.05$, vs –VDP.

These differences between normal and rachitic growth plates suggest that vitamin D regulates the activities of these enzymes. Studies using growth plate chondrocytes derived from vitamin D–replete rats support the hypothesis that metalloproteinases are regulated by vitamin D *(51)*. When resting zone chondrocytes were treated with 24R,25$(OH)_2D_3$, neutral metalloproteinase activity was dose-dependently increased, and collagenase activity was unaffected. By contrast, when cells from the growth zone were treated with 1α,25$(OH)_2D_3$, collagenase activity was dose-dependently inhibited, whereas neutral metalloproteinase activity was unchanged. These studies demonstrate that the effects of the vitamin D metabolites were specific to their target cell and that each metabolite plays a distinct role. The present in vivo study, using vitamin D–deficient rats, confirms our in vitro observations.

Interestingly, the effects observed with the growth zone chondrocyte cultures were reflected in our growth plate extracts. Neutral metalloproteinase activity in growth plate extracts from –VDP rats treated with 1α,25$(OH)_2D_3$ was unchanged, whereas collagenase activity was significantly decreased. This would be expected, because much of the tissue used to prepare the extracts contained predominantly cartilage from prehypertrophic and hypertrophic cell zones, a region similar to that where the growth zone chondrocytes were originally derived.

The effect of the vitamin D metabolites on metalloproteinase levels in the extracts cannot be ascribed entirely to

an effect on TIMP, the endogenous inhibitor of MMP activity. Ergocalciferol had no effect on TIMP at any time point, even though the activity of both enzymes decreased and remained suppressed at all time points. TIMP levels were increased by $1\alpha,25(OH)_2D_3$, but only at later time points, whereas active collagenase was reduced within 4 h. In animals treated with $24R,25(OH)_2D_3$, TIMP activity was blocked completely at early time points. Although neutral metalloproteinase activity was not reduced, it also was not stimulated. Another possible explanation for the rapid decrease in collagenase levels after treatment with $1\alpha,25(OH)_2D_3$ that does not include TIMP might involve the collagenase-3 receptor *(52,53)*. Although only reported to be present in UMR-106-01 cells, one could imagine that this may be an important mechanism for controlling collagenolysis in many tissues, since it removes collagenase from the matrix quickly *(52)*, and production of the receptor has been shown to be regulated by parathyroid hormone (PTH) *(53)*.

The differential effect of $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ was also noted with respect to plasminogen activator activity. Whereas $1\alpha,25(OH)_2D_3$ stimulates plasminogen activator, $24R,25(OH)_2D_3$ inhibits this enzyme activity. The fact that $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ elicit differential responses that in effect cancel each other out when examining the entire tissue may explain in part the confusion surrounding the roles of $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ in healing rickets and in normal growth plate development. Our results suggest that both metabolites are needed. $24R,25(OH)_2D_3$ appears to be required for regulating proteoglycan degradation, facilitating maturation of a calcification-competent matrix. $1\alpha,25(OH)_2D_3$ appears to be responsible for appropriate modulation of collagen, facilitating terminal differentiation of hypertrophic chondrocytes.

The consequence of vitamin D metabolite treatment was evident morphologically. $1\alpha,25(OH)_2D_3$ appeared to have a more predominant effect on the organization of the chondrocytes in the hypertrophic cartilage, whereas $24R,25(OH)_2D_3$ exerted its effects on the less differentiated cells in the growth plate. All of the 4 to 24-h treatment regimens caused a decrease in cell number in the proliferative zone, suggesting that they were promoting chondrocyte differentiation and maturation. However, $1\alpha,25(OH)_2D_3$ caused an increase in the number of cells in the zone of maturation (prehypertrophic zone) after 24–48 h, whereas $24R,25(OH)_2D_3$ caused a reduction in the number of cells between 4 and 24 h, indicating that the metabolites were affecting different pathways.

The effect of vitamin D treatment could be observed within 4 h. This was not too surprising for the two vitamin D metabolites, since they were administered intramuscularly. However, the effect of ergocalciferol was less expected, because it was given orally. We believe that some of the observed effects at the earlier time points were owing to the direct action of the secosteroids on the chondrocytes. At the later time points (e.g., after 24 h), the effect of chondrocyte-mediated 1α- and 24R-hydroxylation of $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$, respectively, to form $1,24,25(OH)_3D_3$ cannot be ruled out in the interpretation of the data. It is well known that this trihydroxylated derivative has biologic effects in rats *(54–56)*. Hunter et al. *(57)* examined the morphology of the metaphyseal microvasculature in rickets and during reversal of rickets. By 8 h after administration of vitamin D, changes in the microvasculature could be readily observed, and by 96 h, the metaphyseal region had returned to an apparently normal state. In another study, Seo et al. *(27)* injected radiolabeled $25(OH)D_3$, $1,25(OH)_2D_3$, and $24,25(OH)_2D_3$ into vitamin D–replete rats and then assessed the tissue distribution and metabolism of the metabolites. The results showed that growth plate cartilage was by far the major site for accumulation of radiolabeled $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ in the rat. The results of these two studies, taken together, suggest that vitamin D or its metabolites can quickly accumulate in growth plate cartilage and account for our observed results.

The height of the growth plate, as measured in the current study, also suggests that the vitamin D treatments had rapid effects on reversing the rachitic syndrome. Four hours after administration, all three forms of the vitamin caused a decrease in growth plate height. With increasing time, however, growth plate height began to increase, and by 48 h, was significantly higher than before treatment. These results were not entirely expected, because prior studies had shown that growth plate height decreased with reversal of rickets *(33,58)*. In those studies, however, the vitamins were given at much higher levels (0.1–10 μg/kg), in repeated doses, and for longer periods (daily for 3 d or every other day for 1 wk). In addition to these differences in vitamin administration, there was also variability in whether the rachitic rats were restored to normal diet or maintained on the rachitogenic regimen. This would have a significant impact on the robustness of the healing, because dietary phosphate is very important in mineral formation and vascular invasion at the metaphyseal border. A possible explanation for the initial decrease in growth plate height, in the absence of restoring dietary phosphate, is the fact that nucleotide levels are increased in the cartilage fluid surrounding the hypertrophic chondrocytes *(59)*. These may have served as an initial source of phosphate on hydrolysis, resulting in a transient reduction in growth plate height. In any event, the rapidity and magnitude of the effect observed in our study could not have been predicted, since longitudinal bone growth has been shown to be much slower *(18,60,61)*.

It is tempting to speculate how the vitamin D metabolites might indirectly exert their effects on the growth plate. It has been reported that vitamin D deficiency is associated with decreased deposition of TGF-β in rat bone *(62)*. If this were true in the growth plate as well, chondrocytes in the upper and middle hypertrophic cell zones would enlarge

and accumulate as seen in rickets. On administration of vitamin D or the metabolites, TGF-β production would be turned back on, resulting in the inhibition of MMPs associated with chondrocyte hypertrophy and the arrest of maturation prior to the terminally differentiated state *(63)*. Our own in vitro studies have shown that TGF-β1 and 24R,25$(OH)_2D_3$ can stimulate resting zone chondrocyte differentiation *(32,64)* as well as increased production of vitamin D metabolites *(25)*. This latter effect of TGF-β1 and 24R,25$(OH)_2D_3$ in chondrocyte cultures would complete an autocrine loop that may be involved in regulating other portions of the differentiation program and also account for the speed with which an effect is observed.

It is also possible that PTH and PTH-related peptide (PTHrP) are involved in the reversal of rickets by vitamin D. It is known that PTH levels are high in rickets, and this may account for the increased production of metalloproteinases in the growth plate *(65)*. High levels of PTH in rickets have also been associated with a downregulation of the PTH/PTHrP receptor *(66)*. These two apparently conflicting observations may be owing to species or age differences in the animal models or selection of tissues examined. Healing of rickets by vitamin D metabolites could act to directly stimulate renewed synthesis of PTHrP, or may stimulate the production of TGF-β1, which will result in increased production of PTHrP *(67)* and its receptor. Because it is well-known that PTHrP is an important regulator of hypertrophic chondrocyte differentiation *(68,69)*, a resumption in the expression of receptors for this growth factor would appear important for healing of the rachitic lesions.

Materials and Methods

Animal and Tissue Preparation

All procedures involving the use of animals were approved by the Institutional Animal Care and Use Committee at the University of Miami School of Medicine and the Miami VA Medical Center.

Male Sprague-Dawley rats (35–45 g) (Caesarian-derived, viral antibody-free, strain K-92; Charles River Breeding Laboratories, Wilmington, MA) at 21 d of age were assigned to five different treatment regimens. Group 1, normal controls, were kept for 21–23 d on standard rat chow diet (0.93% Ca and 0.65% P; Ralston Purina, St. Louis, MO) and water ad libitum. Group 2 rats were kept in the dark and fed U.S. Pharmacopaeia Rachitogenic Diet #12 (Teklad Test Diets, Madison, WI), which is low in phosphorus (1.64% Ca and 0.25% P) and lacks vitamin D, to induce rickets (–VDP) as described before *(5,70)*. Group 3, vitamin D–healing rachitic rats, were raised on the –VDP regimen, but they received a single oral dose of vitamin D_2 (ergocalciferol [Ergo], 2000 IU; Eli Lilly, Indianapolis, IN) 4, 24, 48, or 72 h prior to euthanasia (–VDP + Ergo). Group 4, 1α,25$(OH)_2D_3$-healing rachitic rats (–VDP + 1,25), were raised on the –VDP regimen but received a single im injection of 1α,25$(OH)_2D_3$ (a gift from Dr. Milan Uskokovic, Hoffmann-LaRoche, Nutley, NJ; 1.0 μg/kg of body weight in propylene glycol) 4, 16, 24, 48, or 72 h prior to euthanasia. Group 5, 24R,25$(OH)_2D_3$-healing rachitic rats (–VDP + 24,25), were raised on the –VDP regimen but received a single im injection of 24R,25$(OH)_2D_3$ (3.0 μg/kg of body weight in propylene glycol; TEVA Pharmaceuticals, Jerusalem, Israel) 4, 16, 24, 48, or 72 h prior to euthanasia.

During healing, group 3 animals were given normal rat chow and water ad libitum, while animals in groups 4 and 5 were maintained on the rachitogenic diet. Thus, the group 3 animals were not only given vitamin D_2, but returned to a phosphate-replete diet to induce a robust reversal of the rachitic syndrome; part of the robustness of the healing was probably owing to the action of 24-hydroxylase, which is present in rat intestine *(71)*. These animals were our positive control and allowed an assessment of the effect of each vitamin D_3 metabolite alone. Those animals receiving vitamin D metabolite treatment intramuscularly were maintained on the rachitogenic diet so that the effect of the secosteroid alone could be observed without the complicating effects of phosphate restoration and metabolism or absorption of the vitamin D metabolite in the intestine *(71)*. Ergocalciferol (vitamin D_2) was chosen as a positive control because of its increased ability to induce healing of rickets in rats. It is well known that rats convert ergocalciferol to 25$(OH)D_2$ more efficiently than cholecalciferol to 25$(OH)D_3$ *(72)*, that 1,25$(OH)_2D_2$ has the same antirachitic activity as 1,25$(OH)_2D_3$ *(73)*, and that 1α-$(OH)D_2$ and 1α-$(OH)D_3$ are equipotent in stimulating calcium transport *(74)*. The doses and times of treatment for all three secosteroids were based on studies in our own laboratory and those of other investigators *(33,70,75,76)*. Low doses of the vitamins and short treatment times were intentionally selected in order to detect early changes in metalloproteinase activity and morphology.

Animals were euthanized with an overdose of sodium pentobarbital (10% in sterile water), and the growth cartilage from the proximal tibiae of both legs was excised. The tissues surrounding the growth plate were removed under a dissecting microscope to exclude unwanted metaphyseal tissue, capillaries, and perichondrium. The tissue was washed with sterile 0.9% NaCl, blotted on tissue paper, and then prepared for biochemical analysis.

Histology and Morphometry

For histology, tissues were fixed in 10% neutral formalin, decalcified in 0.5% formic acid, embedded in paraffin, cut into 3 to 5-μm serial sections, and stained with safranin O and counterstained with light green SF yellow. To identify sites of calcification, sections were prepared as described earlier, but not decalcified, and stained with von Kossa and counterstained with H&E.

Growth plate height, number of cells in each zone (proliferative cell zone, zone of maturation, zone of elongation, and hypertrophic cell zone), and extent of calcification into the hypertrophic cell zone were determined using standard morphometric methods. Growth plates were prepared for histology as just described and sectioned longitudinally in series to find the center of the growth plate. Six adjacent sections from the center of the growth plate were then analyzed and 20 measurements performed on each section; an average for the six sections was then calculated. This was done for tissues from three to six rats.

Extraction of Enzymes and TIMP

Extraction of the enzymes and TIMP was performed as previously described *(16)*, but with slight modification for this study. Growth plates (50–75 mg of wet wt) were homogenized in 20 vol of ice-cold 50 m*M* Tris-HCl, 10 m*M* $CaCl_2$ (pH 7.5), buffer containing 1.0 *M* guanidine-HCl (for TIMP) or 2.0 *M* guanidine-HCl (for enzymes) with a ground glass homogenizer (Duall #20; Kontes, Vineland, NJ). The homogenate was then gently stirred at 4°C for 2 h, followed by centrifugation at 20,000*g* for 30 min at 4°C. The supernatants (extracts) used for measuring enzyme activity were split into two aliquots (80:20). The larger aliquot was dialyzed overnight against metalloproteinase assay buffer (50 m*M* Tris-HCl; 0.2 *M* NaCl; 10 m*M* $CaCl_2$; 0.05% Brij 35; 0.02% sodium azide, pH 7.5) in 3500 mol wt cutoff dialysis tubing (Spectra/Por 3; Spectrum Medical, Los Angeles, CA) at 4°C. The smaller aliquot was dialyzed in an identical fashion, but into plasminogen activator assay buffer (0.05 *M* Tris-HCl, pH 8.5), and assayed immediately for activity. This extraction method, originally described for human articular cartilage *(77)* and subsequently modified for use with growth cartilage in these studies, is capable of extracting >90% of the collagenase, neutral metalloproteinase, plasminogen activator, and TIMP present in the cartilage.

Preparation of Radiolabeled Collagen and Assay of Collagenase Activity

The method for measuring collagenase activity was essentially the same as previously described *(16,78)*; all metalloproteinases capable of digesting helical collagen (e.g., MMP-1, -2, -8, -13, and -14) are measured by this method, and no distinction between the various types of MMPs can be made. For this reason, the term *collagenase* has been used to describe this activity.

Acid-soluble type I collagen was obtained from rat skins by extraction in 0.5 *M* acetic acid. The solubilized collagen was purified, pepsinized, and then labeled with [^{3}H]-acetic anhydride as previously described *(78)*. The specific activity of the final product was approx 5.2×10^3 dpm/μg and was homogeneous on sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Telopeptide-free, radiolabeled rat skin collagen (16.7 μg), prepared as described in the last paragraph, was incubated with enzyme for 18 h at 30°C in a total volume of 50 μL. Active enzyme was measured without any other addition to the assay, and total enzyme (latent + active forms) was measured by adding 1.0 m*M* aminophenylmercuric acetate (APMA). At the end of the incubation, the reaction was stopped by the addition of EDTA and unlabeled type I collagen (50 μg), followed by trypsin and chymotrypsin to digest the products of collagenase action. Ice-cold trichloroacetic acid was added to a final concentration of 10% to precipitate undigested collagen; this was pelleted by centrifugation for 10 min at 4°C in a microfuge. An aliquot of the supernatant was added to 6 mL of Aquasol scintillation fluid (Packard, Meriden, CT) and counted in a liquid scintillation counter. Results were calculated as percentage of digestion after correction for buffer and 1,10-phenanthroline (=100% inhibition of all collagenase present) blanks to determine the micrograms of collagen digested during the 18-h incubation. One enzyme unit was defined as the amount of enzyme digesting 1 μg of collagen/min at 30°C, and the data are presented as enzyme units/gram of wet wt cartilage.

Preparation of Radiolabeled Aggrecan and Assay of Neutral Metalloproteinase Activity

The tritiated aggrecan bead assay of Nagase and Woessner *(79)*, as described in Dean et al. *(77)*, was used to measure neutral metalloproteinase activity. Aggrecan monomer was extracted from bovine nasal septum, purified by cesium chloride density gradient centrifugation, and labeled with [^{3}H]-acetic anhydride. The labeled aggrecan monomer was entrapped in polyacrylamide gel beads having a pore size that retains the undigested monomers but that permits escape of digestion products smaller than 200,000 Daltons. The assay gives a linear response with trypsin (0.3–3.0 ng) *(77)*, partially purified neutral metalloproteinase from human articular cartilage *(80)*, and extracts of rat growth plate cartilage (unpublished observation) over an assay period of 3–18 h at 37°C. Reproducibility of this assay is ±8%. Note that this assay does not distinguish among the various MMPs (e.g., MMP-1, -2, -3, -7, -8, -9, -13, and -14) or aggrecanases that are capable of digesting aggrecan. The term *neutral metalloproteinase* is used to describe this activity.

Neutral metalloproteinase activity in the extracts was determined after dialysis into metalloproteinase assay buffer (see section on Extraction of Enzymes and TIMP). Each sample (50–100 μL) was added to a 7-mL scintillation vial containing 2 mg of [^{3}H]-aggrecan beads (12,000 cpm/mg of beads; 150 μg of proteoglycan/mg of beads) and made up to a total volume of 200 μL. Active enzyme was measured without any other addition to the vial, whereas total enzyme (latent + active forms) was measured in the presence of 1.0 m*M* APMA. Blanks contained 1.0 m*M* 1,10-phenanthroline to inhibit all metalloproteinase activity. Incubation was continued for 18 h at 37°C. At the end

of incubation, 6.0 mL of Aquasol scintillation fluid was added and the vials were counted. Enzyme activity was expressed as enzyme units per gram of wet wt cartilage, in which one enzyme unit was defined as the amount of enzyme capable of digesting 1 μg of aggrecan/min at 37°C from 2 mg of beads.

Assay of Plasminogen Activator Activity

Plasminogen activator activity was measured using the method of Coleman and Green *(81)* with urokinase (#672081; Calbiochem, La Jolla, CA) as a standard. The assay consists of two distinct steps. In the first step, plasminogen is converted to plasmin by any plasminogen activators present in the sample. Plasmin is then measured by its ability to hydrolyze thiobenzyloxycarbonyl-L-lysinate, which reacts with 5,5'-dithiobis(2-nitrobenzoic acid) to form a chromogen that can be read spectrophotometrically at 412 nm. The second step is maximized for plasmin activity and minimized for plasminogen activator activity.

Assay of TIMP

TIMP activity was measured using a low M_r metalloproteinase (matrilysin, MMP-7) extracted and purified from 1-d postpartum rat uteri as previously described with slight modification *(82–84)*. The enzyme was partially purified by chromatography on Ultrogel AcA 54 (Sepracor, Marlborough, MA). The enzyme peak from the gel filtration column was applied to a 1.5-mL column of Blue Sepharose CL-6B (Pharmacia, Piscataway, NJ) in metalloproteinase assay buffer and eluted with a 0.2–2.0 *M* NaCl gradient. The enzyme was pooled and activated with 0.5 m*M* APMA for 1 h at 37°C and then dialyzed free of APMA.

For assay, active matrilysin (10–20 μL; 0.25–0.31 enzyme units), inhibitor (0–100 μL), and assay buffer were preincubated in a total volume of 300 μL. The assay was then conducted by adding 950 μL of assay buffer containing 1 mg of Azocoll (Calbiochem) and incubating for 18 h at 37°C with horizontal shaking. Blanks were prepared by the addition of 1 m*M* 1,10-phenanthroline. At the end of incubation, the optical density of the supernatant was read at 520 nm. One inhibitor unit blocked one unit of enzyme (1 enzyme unit = 1 μg of Azocoll digested/min at 37°C), and the data are presented as inhibitor units/gram of wet wt cartilage.

Statistical Analyses

Differences among groups were determined by analysis of variance and the Tukey test. In cases of single comparisons, student's *t*-test with Bonferroni's modification was used. $p \leq 0.05$ was considered significant.

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