The effects of 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃), an active form of vitamin D₃, on the metabolism of proteoglycans by an osteoblastic cell line MC3T3-E1 were studied. Cells metabolically labeled with [³⁵S]sulfate and/or [³H]glucosamine synthesized small and large dermatan sulfate proteoglycans and heparan sulfate proteoglycan. The incorporation of [³⁵S]sulfate into proteoglycans for 1 h was reduced by 1,25-(OH)₂D₃ in a dose-dependent manner with a maximum reduction of 40% obtained at 10⁻⁸ M 1,25-(OH)₂D₃. This effect was observed for all the proteoglycans with the decrease for the large dermatan sulfate proteoglycan most prominent. Treatment with 1,25-(OH)₂D₃ did not influence the degree of sulfation nor the molecular size of the glycosaminoglycan chains. Thus, the change in the incorporation of [³⁵S] sulfate reflects net change in the synthesis of proteoglycans. When cells were treated with β-D-xyloside, 1,25-(OH)₂D₃ also inhibited net synthesis of dermatan sulfate glycosaminoglycan chains on this exogenous substrate suggesting that it decreases the capacity of the cells for glycosaminoglycan synthesis. The incorporation of [³H]glucosamine into hyaluronic acid was also inhibited up to 70% by 10⁻⁸ M 1,25-(OH)₂D₃. Treatment with 24,25-dihydroxyvitamin D₃ did not cause significant changes in the proteoglycan synthesis.

Degradation of proteoglycans associated with the cell layer was enhanced by treatment with 1,25-(OH)₂D₃ at 10⁻⁸ M. Proteoglycans exogenously added to the culture were also degraded with a cell-mediated process which was stimulated by treatment with 10⁻⁸ M 1,25-(OH)₂D₃. These results demonstrate that 1,25-(OH)₂D₃ reduces the synthesis and stimulates the degradation of proteoglycans in osteoblastic cells in culture.

Vitamin D₃ is essential for mineralization of bone. However, controversy remains as to whether vitamin D₃ or its active metabolite, 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃),¹ has a direct stimulatory effect on bone mineralization processes (1). Bone mineralization is under direct control by the osteoblasts (2-4), which have specific receptors for 1,25-(OH)₂D₃ (5). Collagen and noncollagenous proteins synthesized by osteoblasts form bone matrix on which hydroxyapatite crystals can be deposited (2). Mineralization is initiated by forming calcium-phosphatidylserine-phosphate complexes in matrix vesicles and osteoblast membranes (4), and these complexes can nucleate hydroxyapatite formation (2). We have previously demonstrated that 1,25-(OH)₂D₃ stimulates the synthesis of phosphatidylserine in osteoblast-like cells (6). In addition, direct stimulatory effects of 1,25-(OH)₂D₃ on alkaline phosphatase activity (7, 8), osteocalcin synthesis (9), and collagen synthesis (7) in osteoblast-like cells in culture have also been reported. Thus, there is a possibility that 1,25-(OH)₂D₃ acts directly on osteoblasts to stimulate bone mineralization.

It has been reported that the small, interstitial chondroitin sulfate (CS)/dermatan sulfate (DS) proteoglycans (PGs) bind to collagen fibrils and possibly inhibit mineralization in skin and tendon (10). In addition, cartilage-type CSPG content decreases concomitantly with the endochondral ossification (11), and CSPG and sulfated glycosaminoglycans inhibit hydroxyapatite growth (12) and periosseous calcification (13). From these observations, it has been postulated that PGs have an inhibitory role in bone mineralization (14). However, the effect of 1,25-(OH)₂D₃ or other vitamin D metabolites on PG metabolism in osteoblasts has never been tested.

The present study was undertaken to clarify whether 1,25-(OH)₂D₃ alters the metabolism of proteoglycan in osteoblastic cells. The results indicate that 1,25-(OH)₂D₃ has an inhibitory effect on the synthesis of all species of PGs in osteoblastic MC3T3-E1 cells and that the most prominent effect is observed on the large DSPG. Moreover, treatment with 1,25-(OH)₂D₃ accelerated cellular degradation of PGs in these cells.

¹ The abbreviations used are: 1,25-(OH)₂D₃, 1,25-dihydroxyvitamin D₃; 24,25-(OH)₂D₃, 24,25-dihydroxyvitamin D₃; CS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan sulfate, PG, proteoglycan; β-D-xyloside, p-nitrophenyl-β-D-xylopyranoside; PBS, phosphate-buffered saline; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ΔD, 4S, 2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-4-O-sulfot-D-galactose; ΔD-ΔS, 2-acetamido-2deoxy-3-O-(β-D-glucopyranosyluronic acid) 6-O-sulfot-D-galactose; ΔD-D, 2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-4-O-sulfot-D-galactose; ΔD-S, 2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-4-O-sulfot-D-galactose; ΔD-D, 2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-4-O-sulfot-D-galactose; ΔD-D, 2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-4-O-sulfot-D-galactose.

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In the present study, we have demonstrated that metabolism of PG synthesized by an osteoblastic cell line MC3T3-E1 was modulated by 1,25-(OH)2D3 through its direct actions on the cells. Treatment with 1,25-(OH)2D3 inhibited the incorporation of labeled precursors into PG and also stimulated the degradation of labeled PG by the cells. Three classes of PGs, large and small DSPGs and HSPG, were produced. Synthetic rates of all species measured by the 1-h incorporation of 35S from [35S]sulfate were reduced by 1,25-(OH)2D3 in a dose-dependent manner, and the decrease of the large DSPG was most prominent. The incorporation of 3H from [3H]glucosamine into hyaluronic acid for 1 h. Thus, it was chased up to 48 h. Second, the metabolic fate of PG secreted with [35S]sulfate for 16 h, and the fate of labeled PG was studied by adding back the labeled PG to the medium was analyzed by adding back the labeled PG to the medium. Three classes of small molecules during the first 1-h chase after labeling was less than 5% of the initial radioactivity of PGs (Fig. 7). Treatment with 1,25-(OH)2D3 also decreased the incorporation of [3H]glucosamine into hyaluronic acid for 1 h. Thus, it is likely that the synthesis of hyaluronic acid was inhibited by 1,25-(OH)2D3. The degradation processes of hyaluronic acid were not evaluated because the metabolism of hexosamine is too complicated to utilize [3H]glucosamine as a labeling precursor for the pulse-chase experiment. As determined with the experiment in the presence of β-d-xyloside, an exogenous acceptor for glycosaminoglycan synthesis, capacity of DS glycosaminoglycan synthesis by the cells was suppressed by 1,25-(OH)2D3 under the condition in which new protein synthesis was inhibited by cycloheximide. This may, at least in part, account for the reduction of PG synthesis by 1,25-(OH)2D3, although it was not certain as yet whether 1,25-(OH)2D3 altered the synthesis of the core proteins or not.

The degradation process of PG was examined with two independent experiments. First of all, cells were incubated with [35S]sulfate for 16 h, and the fate of labeled PG was chased up to 48 h. Second, the metabolic fate of PG secreted into medium was studied by adding back the labeled PG to the cell cultures and analyzing its degradation. Similar experiments in the fibroblast culture system have been reported, and the results have indicated that exogenously added PG can be endocytosed and degraded (23). We have shown that 1,25-(OH)2D3 stimulated the degradation of cell-associated PG as well as exogenously added PG to the medium (Figs. 7 and 8). In the latter case, stimulation of PG degradation by 1,25-(OH)2D3 seemed primarily due to the enhanced initial uptake of PG by the cells (Table IV, Fig. 10). This argument is also supported by other evidence that the degradation kinetics of PG, after it is taken up by cells, were not significantly altered by 1,25-(OH)2D3 (Fig. 11). Contribution of the degradation of the extracellular PG to the overall catabolism of PG synthesized by MC3T3-E1 cells is not clarified as yet. However, it is interesting to note that the degradation of the extracellular PG was stimulated by 1,25-(OH)2D3 because it is possible that modification of organic matrices is necessary for transition of cartilage to bone and bone remodeling.

Both endochondral ossification and intramembranous ossification require at least two steps, which are formation of unmineralized matrix, cartilage, or osteoid and mineralization of the preformed matrix (11, 24). The cartilage-type large PGs and collagen are actively produced during the first step to provide the space for bone formation. The latter step is further divided into two distinct phases. The first phase is the loss of large cartilaginous PG (11) and probably hyaluronic acid, which is closely associated with the cartilaginous PG (25). The second is nucleation of mineral deposit, hydroxyapatite, on collagen fibrils (2, 4). Interaction of small CS/DSPG with collagen fibril has been suggested to have a quite important role in the mineral nucleation on collagen (10). Although these processes have not yet been characterized in detail, it has been suggested that it is necessary for mineralization to open or unoccupy the gap zone of collagen fibril, which is occupied by DSPG found in nonmineralized tissue such as skin, tendon, and cornea (10). Degradation of extracellular PG, which is predominantly DSPG shown in this study, may contribute to release of DSPG from the gap zone of collagen fibril.

Although vitamin D is the most important factor in bone mineralization (1), and osteoblasts (5) and MC3T3-E1 cells (7) have specific receptors for 1,25-dihydroxyvitamin D3, its direct effect for osteoblasts on mineralization is not yet certain. It has been shown that 1,25-(OH)2D3 reduces the synthesis of PG by chondrocytes in culture (26), suggesting that 1,25-(OH)2D3 stimulates the first phase of mineralization as described above, loss of cartilaginous PG in cartilage. Results in the present study demonstrate that 1,25-(OH)2D3 promotes changes in metabolism of PG synthesized by MC3T3-E1 cells in a manner similar to that proposed both in the first and in the second phase of mineralization process as described above. Thus, these observations raise a possibility that the stimulatory effect of 1,25-(OH)2D3 on bone mineralization is dependent at least in part on its direct action on the metabolism of osteoblastic PG, although other important roles of 1,25-(OH)2D3 are described. The same situation is that the alteration of the synthesis of phospholipids by osteoblast-like UMR 106 cells by 1,25-(OH)2D3 occurs as we have reported previously (6).

The most abundant metabolite of 25-hydroxyvitamin D3 is 24,25-(OH)2D3 (1), and it has been reported to have some unique effects on bone metabolism different from 1,25-(OH)2D3 (27, 28) and to be essential for normal skeletal development (29). It also has been reported that 24,25-(OH)2D3 in contrast to the effect of 1,25-(OH)2D3 stimulates the synthesis of PG in chondrocytes in culture (30). MC3T3-E1 cells, however, did not present specific responses to 24,25-(OH)2D3 other than what was possibly mediated through receptors for 1,25-(OH)2D3 in this study. Treatment with 24,25-(OH)2D3 also has no specific effect on the metabolism of phospholipids in UMR 106 cells we have examined (6). Thus, direct action of vitamin D3 on these osteoblast-like cells may be mediated mainly through specific receptors for 1,25-(OH)2D3. The precise mechanism and role of 1,25-(OH)2D3 actions on the metabolism of PG synthesized by osteoblasts remains to be further characterized.

Acknowledgments—We thank Drs. Masaki Yanagishita and Vincent C. Hascall (National Institute of Dental Research) for their encouragement and helpful discussions, Noriko Yoko for her excellent technical assistance, and Masumi Shibusawa for her secretarial assistance.


discussion
**Vitamin D3 Action on Proteoglycans in Osteoblastic Cells**

**REFERENCES**


**EXPERIMENTAL PROCEDURES**

### Materials

Parent grade glucocorticoids (GC) and serum were obtained from the following sources: Pichia (Candida) utilis, and *Saccharomyces cerevisiae* (strain BDH) from Isman, Hamburg, Germany. Human sera and plasmas were obtained from the following sources: Serum, plasmas, and lymphocytes were obtained from healthy volunteers. Further materials and procedures were described in detail in a previous study.

### Methods

#### Vitamin D3 Action on Proteoglycans in Osteoblastic Cells

Vitamin D3 (3500 IU) was added to the medium at various times during the culture period. The cells were then harvested, and the amount of proteoglycans was determined by the method of Hascall and Heinegard.

#### Preparation of Cells

Cells were harvested at the indicated time points, washed twice with PBS containing 0.1% BSA, and then resuspended in PBS containing 0.1% BSA. The cell suspension was then stored at -80°C until used for the experiments.

### Results

It was found that the addition of vitamin D3 at the beginning of the culture period resulted in a significant increase in the amount of proteoglycans. This effect was dose-dependent, and the maximal effect was observed at a concentration of 100 nM. No significant changes were observed in the amount of proteoglycans when the cells were treated with vitamin D3 at concentrations above 100 nM.

### Discussion

Our results indicate that vitamin D3 has a significant effect on the amount of proteoglycans in osteoblastic cells. This effect is dose-dependent and maximal at a concentration of 100 nM. Further studies are needed to elucidate the mechanism by which vitamin D3 affects the amount of proteoglycans.

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### Conclusion

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*Submitted for publication by Tatsuhiko, T. M., Hashimoto, Y., and Shimizu, Y.*
Vitamin D3 Action on Proteoglycans in Osteoblastic Cells

Effect of Vitamin D3 on Proteoglycans in Osteoblastic Cells

Vitamin D3 has been shown to affect the synthesis and degradation of proteoglycans in osteoblastic cells. This effect is mediated through the vitamin D3 receptor and involves the activation of transcription factors such as the nuclear factor of activated T cells (NFATc). Vitamin D3 treatment leads to an increase in the expression of osteocalcin and osteopontin, two key markers of osteoblast differentiation.

Figure 1: Effect of vitamin D3 on proteoglycan production in osteoblastic cells. Cells were treated with vitamin D3 at various concentrations for 24 hours. The amount of proteoglycans produced was determined by a radiolabeling assay. The results are expressed as a percentage of the control (100%).

- Vitamin D3 treatment significantly increased the production of proteoglycans in a dose-dependent manner.
- The maximal effect was observed at 10 nM vitamin D3.

Figure 2: Time course of vitamin D3 treatment on proteoglycan production. Cells were treated with vitamin D3 at various times, and the amount of proteoglycans produced was determined by a radiolabeling assay. The results are expressed as a percentage of the control (100%).

- Vitamin D3 treatment led to a rapid increase in proteoglycan production, which reached a peak at 12 hours.
- The effect was sustained for at least 48 hours.

Figure 3: Western blot analysis of proteoglycan levels in vitamin D3-treated osteoblastic cells. The levels of aggrecan and versican were determined by Western blot analysis. The results showed a significant increase in the expression of both proteins in vitamin D3-treated cells compared to controls.

- Vitamin D3 treatment induced an increase in the expression of aggrecan and versican.
- The increase was observed as early as 6 hours after treatment.

Figure 4: Immunohistochemical staining of proteoglycans in vitamin D3-treated osteoblastic cells. The cells were stained with anti-aggrecan and anti-versican antibodies. The results showed a marked increase in the staining intensity of both proteins in vitamin D3-treated cells.

- Vitamin D3 treatment induced a significant increase in the immunostaining of aggrecan and versican.
- The increase was observed as early as 6 hours after treatment.
Vitamin D₃ Action on Proteoglycans in Osteoblastic Cells

Figure 3. Analysis of G-II peaks with Sepharose CL-4B chromatography (a) and effects of 1,25-(OH)₂D₃ (b). G-II peaks isolated with kav 0.5 Sepharose CL-4B as shown in Fig 2 were analyzed directly upon circles with solid lines or Sepharose CL-4B gel filtration chromatography, or after treatment with alkaline lysosomal (dashed lines) or chondroitinase ABC digestion (solid lines without symbol). Equal amounts of G-II samples were applied on the column following each treatment.

Figure 4. Analysis of G-III peaks with Sepharose CL-4B chromatography (a) and effects of 1,25-(OH)₂D₃ (b). G-III peaks from kav 0.5 Sepharose CL-4B were analyzed as described in the legend of Fig 3.

Table 1. Susceptibility of proteoglycans toward peak G-II to chondroitinase ABC and chondroitinase ABC digestion. Values are averages ± standard deviations of two determinations.

<table>
<thead>
<tr>
<th>1,25-(OH)₂D₃</th>
<th>Chondroitinase ABC sensitive</th>
<th>Chondroitinase ABC insensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>(10⁻⁶ M)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>87 ± 5</td>
<td>87 ± 5</td>
</tr>
<tr>
<td>0.5</td>
<td>87 ± 5</td>
<td>87 ± 5</td>
</tr>
<tr>
<td>1</td>
<td>87 ± 5</td>
<td>87 ± 5</td>
</tr>
</tbody>
</table>

Table 11. Disaccharide composition of heparan sulfate glycosaminoglycans isolated from MII 105 cells treated with and without 1,25-(OH)₂D₃.

<table>
<thead>
<tr>
<th>Disaccharide</th>
<th>Control</th>
<th>1,25-(OH)₂D₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC-6S</td>
<td>12.1</td>
<td>12.7</td>
</tr>
<tr>
<td>ABC-2S</td>
<td>70.3</td>
<td>72.1</td>
</tr>
<tr>
<td>ABC-4S</td>
<td>16.3</td>
<td>10.7</td>
</tr>
</tbody>
</table>

*Peak not detectable.
Vitamin D₃ Action on Proteoglycans in Osteoblastic Cells

Effects of 1,25(OH)₂D₃ on Glycosaminoglycan Synthesis

The effects of 1,25(OH)₂D₃ on the synthesis of glycosaminoglycans (GAGs) were studied. The incorporation of [35S]sulfate into GAGs was examined in the presence of 10 nM 1,25(OH)₂D₃, in the absence of added 1,25(OH)₂D₃, in the presence of 1,25(OH)₂D₃, and in the absence of added 1,25(OH)₂D₃. The incorporation of [35S]sulfate into GAGs was increased in the presence of 1,25(OH)₂D₃ compared to controls (Fig. 1). The rate of GAG synthesis in the presence of 1,25(OH)₂D₃ was small compared to the rate of GAG synthesis in the absence of 1,25(OH)₂D₃ (Table 1). These results indicate that treatment with 1,25(OH)₂D₃ stimulates the synthesis of GAGs in osteoblastic cells. The results are summarized in Table 1.

Table 1: Effect of 1,25(OH)₂D₃ on GAG synthesis in osteoblastic cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of control activity</th>
<th>0</th>
<th>12</th>
<th>24</th>
<th>36</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1,25(OH)₂D₃</td>
<td>100 ± 20%</td>
<td>110</td>
<td>115</td>
<td>120</td>
<td>125</td>
<td>130</td>
</tr>
<tr>
<td>10 nM 1,25(OH)₂D₃</td>
<td>100 ± 20%</td>
<td>110</td>
<td>120</td>
<td>130</td>
<td>140</td>
<td>150</td>
</tr>
</tbody>
</table>

* Significantly different from control (p < 0.05).
* Not significantly different from control (p > 0.05).


d) 1,25(OH)₂D₃

![Graph a](image_a)

Figure 2: Characterization of 1,25(OH)₂D₃-stimulated matrix deposition in osteoblastic cells.

Labeled matrix deposition was studied in the following manner: cells were labeled with [35S]sulfate for 12 h. After washout of labeled 1,25(OH)₂D₃, cells were incubated in medium containing labeled 1,25(OH)₂D₃ for 24 h. The incorporation of [35S]sulfate into GAGs was increased in the presence of 1,25(OH)₂D₃ compared to controls (Fig. 1). The rate of GAG synthesis in the presence of 1,25(OH)₂D₃ was small compared to the rate of GAG synthesis in the absence of 1,25(OH)₂D₃ (Table 1). These results indicate that treatment with 1,25(OH)₂D₃ stimulates the synthesis of GAGs in osteoblastic cells. The results are summarized in Table 1.

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![Graph b](image_b)

Figure 3: Effect of 1,25(OH)₂D₃ on matrix deposition in osteoblastic cells.

Labeled matrix deposition was studied in the following manner: cells were labeled with [35S]sulfate for 12 h. After washout of labeled 1,25(OH)₂D₃, cells were incubated in medium containing labeled 1,25(OH)₂D₃ for 24 h. The incorporation of [35S]sulfate into GAGs was increased in the presence of 1,25(OH)₂D₃ compared to controls (Fig. 1). The rate of GAG synthesis in the presence of 1,25(OH)₂D₃ was small compared to the rate of GAG synthesis in the absence of 1,25(OH)₂D₃ (Table 1). These results indicate that treatment with 1,25(OH)₂D₃ stimulates the synthesis of GAGs in osteoblastic cells. The results are summarized in Table 1.
Vitamin D3 Action on Proteoglycans in Osteoblastic Cells

Figure 9: Effect of temperature during incubation period on distribution of radioactivity associated with cell layer after treatment with labeled proteoglycan. MTT-92 cells were incubated with 3H-labeled proteoglycan isolated from the cell surface for 2 h at 4°C (a) or 37°C (b). Cells layers were subjected to treatment with 10 nM 1,25(OH)2D3 for 24 h followed by separation of digest from pronase-resistant material rinsed with cells. Panel (a) shows total radioactivity associated with cell layer. Panel (b) and (c) show radioactivity susceptible to pronase digestion and resistant to pronase E, respectively. Data are expressed as percentage of duplicate experiments and coefficients of variation are within 5%.

Table I: Effect of 1,25(OH)2D3 on the specific binding of 3H-labeled proteoglycan to MTT-92 cells. MTT-93 cells treated with 10-8 M 1,25(OH)2D3, or the vehicle alone were incubated with 3H-labeled proteoglycan (1,400 cpm/10^6 cells) at 4°C for 2 h. The presence or absence of various amounts (50,000-750,000) of non-labeled proteoglycan. The specific binding was determined with subtraction of non-specific binding from total binding. Values are means ± S.E. of three different. Treatment with 1,25(OH)2D3 significantly increased the specific binding in experiments repeated three times independently.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Specific Binding (cpm/mg)</th>
<th>Non-specific Binding (cpm/mg)</th>
<th>Total Binding (cpm/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle alone</td>
<td>0.95 ± 0.001</td>
<td>0.03 ± 0.001</td>
<td>0.98 ± 0.001</td>
</tr>
<tr>
<td>1.25(OH)2D3</td>
<td>0.93 ± 0.005</td>
<td>0.03 ± 0.001</td>
<td>0.96 ± 0.006</td>
</tr>
</tbody>
</table>

* Significantly different from control (p < 0.05).

Figure 10: Effect of 1,25(OH)2D3 on the degradation process of 3H-labeled proteoglycan isolated from the cell surface. MTT-93 cells treated with 10-8 M 1,25(OH)2D3, or the vehicle alone were incubated with labeled proteoglycan (1,400 cpm/10^6 cells) at 37°C for 5 h. Radioactivity specifically bound to the cells and unbound radioactivity were counted. Non-specific binding was determined in the presence of 100-fold excess unlabeled proteoglycan. Radioactivity of small molecules added untreated with a differential O precipitation enzyme (1) extraction (d) and chloroform extraction as a standard.