Effect of 1,25(OH)$_2$D$_3$ on Collagen Post–Translational Modifications in a Fibroblastic Cell Culture System

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Abstract
It has been well documented that 1,25(OH)$_2$D$_3$ regulates the synthesis of collagen, however, little is known about its effect on the quality of collagen. Most recently, we have demonstrated that 1,25(OH)$_2$D$_3$ upregulates the expression of specific isoforms of lysyl hydroxylases (LHs) and lysyl oxidases (LOXs) in murine osteoblastic cell line, MC3T3–E1 cells resulting in specific collagen cross–linking pattern (1). However, at present, it is not clear whether or not this is a generalized effect or cell type specific. In order to address this, we investigated the effects of 1,25(OH)$_2$D$_3$ on gene expression of LHs and LOXs and the extent of lysine (Lys) hydroxylation of collagen using an fibroblastic cell line, NIH3T3 cells. The effect was compared to controls where cells were treated with cholecalciferol (VD$_3$) or ethanol (Et–OH). When compared to the controls, gene expressions of LH1 and LH2 were upregulated and LOXL2 was markedly upregulated by 1,25(OH)$_2$D$_3$, at 48 hours of culture. The extent of Lys hydroxylation of collagen was only slightly higher in the 1,25(OH)$_2$D$_3$ treated group. This study indicates that 1,25(OH)$_2$D$_3$ regulates the expression of collagen modifying enzymes in fibroblastic cells in a similar manner as osteoblastic cells but the extent of the effects could vary depending on the cell types.

Introduction
The biologically most active hormonal form of vitamin D$_3$, 1,25(OH)$_2$D$_3$, which is formed after two sequential hydroxylations in the liver and kidney of cholecalciferol (VD$_3$), has a broad range of physiological effects including calcium homeostasis, bone metabolism, immune system and cancer (2). These functions are mediated through a single vitamin D receptor (VDR), a member of the nuclear hormone receptor superfamily (3). The overall positive effects of vitamin D on prevention of bone fracture are well recognized (4–8). Interestingly recent studies have indicated that vitamin D also helps prevent falls suggesting its positive effects not only on bones but also connective tissues in general.

Fibrillar type I collagen, the predominant matrix component in vertebrates, is the building block to provide tissues and organs with form and connectivity. One of the characteristic features of collagen is its extensive post–translational modifications that take place intra- and extracellularly. The covalent intermolecular cross–linking is the final collagen post–translational modification crucial for the stability of the fibrils. The type and quantity of cross–links are primarily determined by the extent of hydroxylation and oxidative deamination of the specific lysine residues in collagen catalyzed by lysyl hydroxylases (LHs) and lysyl oxidase(s) (LOX), respectively. The specific cross–linking pattern appears to be vitally important for tissue’s functions as indicated by in vitro studies (9, 10). For instance, the dynamic cross–linking pattern appears to be associated with its regulatory role in the process of mineralization (11–13). Alterations in cross–linking
pattern result in defective fibrillogenesis and collagen mineralization (14, 15).

Despite the numerous studies on the effect of vitamin D on collagen, at present, little is known about its effect on collagen post-translational modifications. The objectives of this study were to investigate the effects of 1,25(OH)$_2$D$_3$ on gene expression of LHs (LH1, 2, 3) and LOX/LOX–like proteins (LOXL1–4) and the extent of lysine (Lys) hydroxylation of collagen using an fibroblastic cell culture system.

**Materials and Methods**

**Cell culture**

NIH3T3 cells were purchased from American Type Culture Collection (CRL–1658). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Atlanta, Lawrenceville, GA, USA), 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate in a 5% CO$_2$ atmosphere at 37°C. The medium was changed twice a week, and cultured up to 14 days. Cell morphology was observed under an inverted light microscope at day 14 of culture (ECLIPSE TE300, Nikon, Tokyo, Japan).

**Cell growth**

NIH3T3 cells were plated onto a 24-well plate in triplicate at a density of 2×10$^4$ cells/well, cultured overnight and treated with Et–OH, 10$^{-8}$ M VD$_3$ or 10$^{-8}$ M 1,25(OH)$_2$D$_3$ for up to 14 days. The former two served as controls. At days 2, 5, 7, 10, and 14 of culture, cell number was determined by cell counting.

In a separate set of experiments, cells were plated onto a 96-well plate in triplicate at a density of 1×10$^4$ cells/well, treated and subjected to MTS cell proliferation assay (CellTiter 96; Promega, Madison, WI, USA) at days 2, 5, and 7 of culture as reported previously (16).

**Quantitative real–time polymerase chain reaction (PCR)**

Cells are plated onto 35 mm dishes at a density of 2×10$^5$ cells/dish. After reaching confluence, the medium was replaced with that containing Et–OH, VD$_3$ or 1, 25(OH)$_2$D$_3$. At 48 hrs of culture, total RNA was isolated using TRizol reagent (Invitrogen, Carlsbad, CA, USA) and the first–strand cDNA was synthesized using an Omniscript Reverse Transcriptase kit (Qiagen, Valencia, CA, USA). Real time PCR was performed in triplicate using the specific primers–probe for Cbfa1/Runx2 (Applied Biosystems, ABI assay number: Mm00501578_m1), Col1a2 (Mm00483888_m1), LH1 (Mm00599925_m1), LH2 (Mm00478767_m1), LH3 (Mm00478798_m1), LOX (Mm00495386_m1), LOXL1 (174595), LOXL2 (Mm00804740_m1), LOXL3 (Mm00442953_m1) and LOXL4 (Mm00446385_m1) or glyceraldehyde–3–phosphate dehydrogenase (GAPDH, ABI assay number: 4308313), and analyzed by the ABI Prism 7,000 Sequence detection system (Applied Biosystems, Foster City, CA, USA). The mean fold changes in gene expression relative to GAPDH were calculated using the values obtained from Et–OH treated NIH3T3 cells as a calibrator by means of 2$^{-ΔΔC}_{t}$ method (17). All analyses were done in triplicate and the results were confirmed by three independent experiments.

**Amino acid analysis**

To determine amino acid composition, NIH3T3 cells were maintained in the same manner as described, cultured in the medium containing 50 μg/ml ascorbic acid and treated with Et–OH, VD$_3$ or 1,25 (OH)$_2$D$_3$. After 14 days of culture, cells/matrices were scraped, washed with phosphate buffered saline (PBS) and distilled water, and lyophilized. Aliquots of dried samples (~2 mg) were hydrolyzed with double-distilled 6N HCl and an aliquot of each hydrolysate was subjected to compete amino acid analysis on a Varian high–performance liquid chromatography (HPLC) system (Prostar 240/310, Varian, Walnut Creek, CA, USA) with a strong cation exchange column (AA–911, Transgenomic, San Jose, CA, USA) (18) and calculated as residues per 1,000. The extent of Lys hydroxylation of collagen was calculated as moles of hydroxylysine (Hyl)/mole of collagen based on a value of 300 residues of hydroxyproline (Hyp) per collagen molecule. The analyses were done in four independent experi-
ments.

Statistical analysis

All statistical analyses were performed using Student's t-test. Data were expressed as means \( \pm \) SD and a p value less than 0.05 was considered significant.

Results

Cell growth

Cell numbers were counted, plotted and shown in Fig. 1A. At all time-points examined, there were no significant differences in the cell growth between the two control groups. However, the cell growth rate of the 1,25(OH)\(_2\)D\(_3\) treated group was constantly lower than controls. The results were also confirmed by MTS assay performed at days 2, 5, and 7 (Fig. 1B). In the 1,25(OH)\(_2\)D\(_3\) treated group, cell morphology was significantly changed into an osteoblast–like round shape at day 14 as previously reported (19). It was noted that the 1,25(OH)\(_2\)D\(_3\) treated cells tended to be larger than those in the two control groups and acquired a stellate morphology rather than the cuboid morphology (Fig. 1C).

Expression of osteogenic markers, and LH and LOX isoforms

The mRNA expression patterns at 48 hr are shown in Fig. 2. In the two control groups, the expression patterns of all genes examined were similar to each other. In the 1,25(OH)\(_2\)D\(_3\) treated group, when compared to controls, Cbfa1/Runx2 expression was significantly upregulated. Col1a2 expression was not significantly different among the three groups. Of LH isoforms, both LH1 and LH2 expressions were 2.5 times higher in the 1,25(OH)\(_2\)D\(_3\) treated group compared to controls. No significant effect was observed on LH3 expression. Of LOX isoforms, LOXL2 expression was most responsive to 1,25(OH)\(_2\)D\(_3\) showing a 25 fold increase at 48 hr. Other LOX isoforms, LOX, LOXL1, 3, 4 did not show significant differences.

Amino acid composition

Amino acid compositions of two control groups and the group treated with 1,25(OH)\(_2\)D\(_3\) are shown in Table 1. Although overall compositions were somewhat similar among the three groups, there was a

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

Fig. 1. Effect of 1,25(OH)\(_2\)D\(_3\) on cell growth and cell morphology. The cell number of each group is shown by histogram at each time point indicated (n=3) (A). The cell viability was plotted by measuring the absorbance at 490 nm at each time point examined (B). Effect of 1,25(OH)\(_2\)D\(_3\) on cell morphology in each group at days 14 (C). Et OH : ethanol, VD\(_3\) : cholecalciferol.
Fig. 2. Effect of 1,25(OH)₂D₃ on the gene expression of osteogenic markers and collagen modifying enzymes. After NIH3T3 cells were treated with Et-OH, VD₃ or 1,25(OH)₂D₃, the gene expression at 48 hr was analyzed by real-time PCR. Note that the gene expressions of LH1 and 2, and LOXL2 were significantly upregulated by 1,25(OH)₂D₃ treatment. Et-OH: ethanol, VD₃: cholecalciferol. *p<0.005 (n=4).

Table 1. Amino acid composition in hydrolysate of NIH3T3 study

<table>
<thead>
<tr>
<th></th>
<th>Et-OH</th>
<th>VD₃</th>
<th>1,25(OH)₂D₃</th>
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<tr>
<td>Hydroxyproline</td>
<td>19.1</td>
<td>17.4</td>
<td>15.4</td>
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<tr>
<td>Aspartic acid</td>
<td>73.2</td>
<td>75.0</td>
<td>81.7</td>
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<td>Threonine</td>
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<td>Serine</td>
<td>68.3</td>
<td>68.3</td>
<td>71.3</td>
</tr>
<tr>
<td>Glutamic acid</td>
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<td>110.3</td>
<td>115.5</td>
</tr>
<tr>
<td>Proline</td>
<td>58.9</td>
<td>57.9</td>
<td>59.3</td>
</tr>
<tr>
<td>Glycine</td>
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<td>139.5</td>
<td>129.9</td>
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<tr>
<td>Alanine*</td>
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<tr>
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<td>50.6</td>
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<tr>
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<td>7.1</td>
<td>7.2</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>41.2</td>
<td>41.4</td>
<td>43.3</td>
</tr>
<tr>
<td>Lercine</td>
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<td>75.9</td>
<td>76.8</td>
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<tr>
<td>Tyrosine</td>
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<td>31.4</td>
<td>33.3</td>
</tr>
<tr>
<td>Phenylalanine</td>
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<td>30.9</td>
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<td>15.8</td>
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<tr>
<td>Hydroxylysine</td>
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<td>3.5</td>
</tr>
<tr>
<td>Lysine</td>
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</tr>
<tr>
<td>Arginine</td>
<td>55.6</td>
<td>54.0</td>
<td>53.7</td>
</tr>
<tr>
<td>Total</td>
<td>1,000</td>
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</tr>
</tbody>
</table>

Et-OH: ethanol, VD₃: cholecalciferol. n=4, *p<0.05 Residues per 1,000 residues (mean±S.D.).

Table 2. Extent of lysine hydroxylation

<table>
<thead>
<tr>
<th></th>
<th>Et-OH</th>
<th>VD₃</th>
<th>1,25(OH)₂D₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>60.7</td>
<td>62.1</td>
<td>68.2</td>
<td></td>
</tr>
<tr>
<td>(16.4)</td>
<td>(13.1)</td>
<td>(13.1)</td>
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trend to show relatively more aspartic acid, threonine, but relatively fewer glycine, lysine and alanine in the 1,25(OH)₂D₃ treated group when compared to controls. Of those amino acids, only alanine showed a statistically significant difference.

Lysine hydroxylation of collagen

The Hyl contents in the two control groups were comparable but it was slightly higher in the 1,25(OH)₂D₃ treated group (68.2±13.1 vs. 60.7±16.4 in Et-OH and 62.1±13.1 in VD₃) though the difference did not reach a significant level (Table 2).

Discussion

Vitamin D was discovered as an anti-rachitic agent. Vitamin D deficiency leads to impairment of bone mineralization, resulting rickets in infants and osteomalacia in adults (14), and mice lacking VDR exhibited impaired bone formation (20). Administration of vitamin D to rachitic or ovariectomized animals cures the impaired bone mineralization (21) and vitamin D treatment appears to reduce a bone fracture risk of patients with age-related bone loss (4) and osteoporosis (5, 22, 23). In addition, recently it has been reported that 1,25(OH)₂D₃ may prevent falls (24–26). From these studies, it was postulated that the effect of vitamin D is not only on bone/osteoblasts but also on connective tissues supporting skeletal system including tendon and ligament. In this study, we utilized fibroblast cells which may represent the predominant cell type in non–mineralized connective tissues, and explored the potential direct effects of 1,25(OH)₂D₃ on collagen post-translational modification (i.e. lysine hydroxylation) and related gene expressions. Our study demonstrated that the rate of cell growth was slower and cell morphology significantly changed by 1,25(OH)₂D₃, which are consistent with previous studies (19). In regard to the gene...
expression of LHs and LOXs, it was found that the expression of LH1, LH2 and LOXL2 were upregulated by 1,25(OH)$_2$D$_3$. Since LH1 is likely the major LH for the helical domain of collagen (27) and LH2 the telopeptides (28), the results indicate that the Lys residues of collagen synthesized by the 1,25(OH)$_2$D$_3$ treated cells are more hydroxylated, and likely cross-linking pattern reflects this. The biochemical analysis indeed to some extent confirmed this notion. The extent of Lys hydroxylation of collagen in the 1,25 (OH)$_2$D$_3$ treated group tended to be higher than controls. More analysis using purified collagen needs to be performed to determine the quantitative differences.

We have recently reported that in an osteoblastic cell culture system, the extent of lysine hydroxylation of collagen was significantly increased by the 1,25(OH)$_2$D$_3$ treatment, i.e. 56.2±2.6 vs. 38.8±5.3 in Et-OH and 39.0±4.4 in VD$_3$ groups (p<0.01) (I). Though there was a trend showing higher lysine hydroxylation of collagen deposited by the 1,25(OH)$_2$D$_3$ treated NIH3T3 cells, the difference between the treated and control groups did not reach a significant level. It could be due to the fact that collagen synthesized by NIH3T3 cells already possesses a high extent of lysine hydroxylation even without 1,25(OH)$_2$D$_3$ treatment (~60 mol/mol collagen in NIH3T3 cells vs ~40 in MC3T3-E1 cells) (I), thus, obscuring the effect. It could also be that some specific lysine residues (for instance, those involved in cross-linking) can be effectively hydroxylated by LH1 and 2 upregulated by 1,25(OH)$_2$D$_3$ but does not confirmed by amino acid analysis as they are incorporated into cross-links. The quantitative cross-link analysis of the deposited collagen should provide insights into these possibilities. In either case, the results suggest that the effect of 1,25(OH)$_2$D$_3$ could be cell-type specific. One of the most striking effects of 1,25(OH)$_2$D$_3$ common to both NIH3T3 and MC3T3-E1 cells was induction of LOXL2 expression. Without the treatment, the expression was very low in both cell types but with the treatment the expression was clearly induced. At present, the biological function of LOXL2 is not clearly defined. It could be associated with cell migration, cancer invasion, but if LOXL2 is involved in oxidative deamination of lysine/hydroxylysine residues of collagen, thus, initiating a cascade of cross-linking reactions is not established. Further studies are warranted to elucidate the role of LOXL2 induced by of 1,25(OH)$_2$D$_3$.

**Conclusion**

This study demonstrates that 1,25(OH)$_2$D$_3$ regulates the gene expression of specific isoforms of lysyl hydroxylase and lysyl oxidase in fibroblastic cells that may result in deposition of collagen that is post-translational highly modified. The extent of the outcome, however, may vary depending on the cell type.

**Acknowledgments**

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**References**


