Vitamin D modulates the composition of extracellular matrix produced by smooth muscle cells and endothelial cells from human aorta.

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Abstract
Vitamin D is involved in a wide variety of metabolic processes, including calcium homeostasis, immune modulation, hormone secretion, cell proliferation and differentiation. Hypovitaminosis D has been increasingly recognized as an independent risk factor for cardiovascular and related diseases. However, the cellular mechanisms by which these vitamin D effects are mediated remain poorly understood. Impaired mechanical stability and function of the arterial wall, including pathological changes in its extracellular matrix composition, are essential factors in the development of atherosclerotic lesions, leading to clinical disease manifestations. In this study we addressed a possible regulatory role of vitamin D in the composition of extracellular matrix (ECM) produced by resident arterial wall cells. ECM produced and deposited by cultured endothelial cells (AoEC) and smooth muscle cells (AoSMC) isolated from human aorta was analyzed for collagens type I and IV, Heparan sulfate (HS), Chondroitin sulfate (ChS) and hyaluronic acid (HA) content by immunochemical assay. Supplementation with 1 µM 1,25-dihydroxyvitamin D(3) stimulated collagen type I ECM deposition by AoSMC in a dose-dependent manner, whereas it had inhibitory effect on type IV collagen deposition. Vitamin D significantly reduced ascorbic acid (AsA)-dependent stimulation of type IV collagen ECM deposition in AoSMC, whereas it did not affect its stimulatory effects on collagen type I. The deposition of collagens type I and IV in AoEC was not significantly affected by either vitamin D or AsA. HS content in ECM deposited by both cell types decreased under vitamin D supplementation counteracting stimulatory effects of AsA. ChS and HA ECM content remained unaffected by Vitamin D in both cell types. Possible health implications of these findings are discussed. We conclude that beneficial Vitamin D effects on the cardiovascular system could in part be mediated through its involvement in regulating ECM composition of the arterial wall.

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Introduction

Hypovitaminosis D, among other medical and metabolic conditions, is increasingly recognized as an independent predicting factor for the development of cardiovascular and related diseases (CVD). The biological pathways through which these effects are mediated remain poorly understood, but may be linked to vitamin D regulation of relevant fibrotic pathways. Vitamin D, especially its most active metabolite 1,25-dihydroxyvitamin D(3) or calcitriol, is essential in regulating a wide variety of metabolic processes, including calcium homeostasis, immune modulation, hormone secretion, cell proliferation and differentiation.

It was suggested that active vitamin D may have a systemic impact on the progression of CVD through its anti-inflammatory potential. As such, it was reported that active vitamin D essentially diminishes many detrimental effects of glomerular and/or interstitial inflammation in response to various injuries, possibly through the inhibition of inflammatory infiltration. Another possible action of vitamin D is an inhibition of renin gene expression. Li and colleagues demonstrated that the renin gene is negatively controlled by vitamin D receptor. Thus, active vitamin D is mechanistically linked to the renin-angiotensin system, which plays a critical role in the regulation of hemodynamic adaptation and fibrogenic responses in CVD.

Atherosclerosis is a major process in the development of clinical manifestations of CVD. It significantly affects the arterial wall structure and function, causing dramatic changes in the composition and properties of the arterial wall connective tissue. Extracellular components of the vascular connective tissue are produced and secreted by arterial wall resident cells, such as endothelial cells and smooth muscle cells. Extracellular matrix (ECM) plays a critical role in maintaining optimum structural integrity and function of the arterial wall and as such it has drawn much research attention in recent decades. The critical role of vitamin C in preventing vascular wall dysfunction and development of atherosclerosis has been widely recognized.

Though some effects of vitamin D on the expression and deposition of ECM by different cell types have been reported, the collated results are so far inconsistent (addressed in detail in the Discussion). The discrepancies could result from the diverse actions of vitamin D in different tissues. Studies of vitamin D effects on the composition of ECM produced by arterial wall cells are scarce.

In this study we investigated effects of 1,25-dihydroxyvitamin D(3) (vitamin D) administered individually and together with vitamin C (ascorbic acid) on the extracellular deposition of major ECM proteins—collagens type I and IV, and major glycosaminoglycans (GAGs)- heparan sulfate (HS), chondroitin sulfate (ChS) and hyaluronic acid (HA), by cultured endothelial cells (AoEC) and smooth muscle cells (AoSMC) isolated from human aorta.

Materials and methods

Reagents

All reagents were from Sigma-Aldrich (St. Louis, MO) except when indicated differently.

Cell cultures

Human aortic smooth muscle cells (AoSMC) and human aortic endothelial cells (AoEC) were purchased from Cambrex (East Rutherford, NJ). AoSMC were maintained in DMEM medium (ATCC) containing antibiotics and 5% fetal bovine serum (FBS, ATCC). AoEC were cultured in EGM-2 medium (Cambrex). All cell cultures were maintained at 37°C and 5% CO2 atmosphere. Cell viability was monitored with MTT assay. None of the experimental conditions resulted in statistically significant cell death (data not shown).

Collagens and Glycosaminoglycans deposition by human vascular cells

For experiments, AoEC or AoSMC, at 5th to 8th passages, were seeded on collagen type I-covered plastic plates (Becton-Dickinson, collagen I isolated from rat tail tendon) at density of 25,000/cm2 and grown to confluence
for 5-7 days. 1,25-dihydroxyvitamin D(3) (vitamin D) and other tested compounds were added to cells at indicated concentrations for 72 h in DMEM supplemented with 2% FBS. Cell-produced extracellular matrix was exposed by sequential treatment with 0.5% Triton X-100 and 20 mM ammonium sulfate in phosphate buffered saline (PBS, Life Technologies) for 3 min each at room temperature (RT) as described previously.7 After 4 washes with PBS, ECM layers were treated with 1% bovine serum albumin (BSA) in PBS for 1 hour at RT and immediately used in experiments.

Immunoassays for types I and IV collagen, heparan sulfate, chondroitin sulfate and hyaluronic acid were done as described previously by sequential incubation with corresponding primary monoclonal antibodies (Sigma-Aldrich, except for anti-heparan sulfate supplied by US Biologics) in 1% BSA/PBS for 2 hours followed by 1 hour incubation with secondary goat anti-mouse IgG antibodies labeled with horse radish peroxidase (HRP).8 Retained peroxidase activity was measured after the last washing cycle (3 times with 0.1% BSA/PBS) using TMB peroxidase substrate reagent (Rockland). Optical density was read with plate reader (Molecular Devices) at 450 nm and expressed as percentage of control cell samples incubated in unsupplemented growth medium.

Statistical analysis
Results in figures are means ± SD from 3 or more repetitions from the most representative of at least 2 independent experiments. Differences between samples were estimated with a two-tailed Student’s T-test using Microsoft Excel software and accepted as significant at p-values less than 0.05.

Results
ECM deposition of collagens
Vitamin D stimulated ECM deposition of type I collagen by cultured AoSMC in a dose-dependent manner (Figure 1A). Thus, in the presence of 1 µM vitamin D the amount of extracellular collagen type I increased by 68% compared to control cells maintained in a standard cell culture medium. The effect of vitamin D on the deposition of collagen type IV by these cells was just the opposite: under vitamin D supplementation the deposition of collagen type IV decreased in a dose-dependent manner, with 22% inhibition at 1 µM vitamin D concentration compared to control (Figure 1A).

We have shown previously that ascorbic acid stimulates the ECM deposition of both types of collagen in human AoSMC.7 In this study we observed a dramatic increase in both collagen I and IV deposition by AoSMC in the presence of 100µM ascorbic acid, which reached 2023% and 1014% of control values for collagen type I and type IV, respectively (Figure 1B). Addition of vitamin D together with ascorbic acid did not affect ECM deposition of collagen type I. However, the inhibitory effects of vitamin D on the extracellular deposition of collagen type IV by AoSMC persisted in the presence of ascorbic acid, thereby reducing its stimulatory effects by 53% (from 1014% to 542% of controls).

The changes in collagen ECM deposition by AoSMC under the influence of vitamin D resulted in altered relative prevalence of different collagen types. Thus, collagen type I to type IV ratio gradually increased in proportion to the vitamin D dose. As such, in the presence of 1 µM vitamin D alone, this ratio changed from 1.0 (control) to 2.1, and in the presence of 0.1 µM vitamin D together with ascorbic acid, from 2.2 (control) to 3.8 (Figure 1C).

A similar trend was observed for ECM deposition of collagens in AoEC in the presence of vitamin D, but the effects were less pronounced (Figure 2). Thus, the amount of extracellular collagen type I increased by 11% in the presence of 1 µM vitamin D, whereas collagen type IV reduced by 11%. Ascorbic acid added individually at 100 µM decreased type I collagen deposition by AoEC by 15% compared to control values. ECM deposition of collagen type IV did not significantly differ under ascorbic acid supplementation. When ascorbic acid and vitamin D were added together, the deposition of collagens type I and type IV was reduced by 21% and 12%, respectively. Supplementation with vitamin D resulted in a change in the proportion of collagen I to IV. Thus, collagen type I to type IV ratio increased from
Figure 1: Effects of vitamin D on Collagens type I and type IV ECM deposition by AoSMC.

Aortic SMC were plated on collagen covered 96 well plates in 5%FBS/DMEM and were grown to confluent layer. Additions of Vitamin D were made in the presence (Fig 1B) or absence (Fig 1A) of 100 µM ascorbic acid. After 72 hours incubation cells were removed and deposited ECM was exposed by differential treatment. ECM content for collagens type I and type IV was determined by immunoenzymatic assay (see Materials and methods for details). Fig 1C, Ratio of Collagen type I to Collagen type IV.
1.0 in control samples to 1.2 under 1 µM vitamin D supplementation, whereas its value was reduced to 0.9 when AoEC were incubated in the presence of ascorbic acid together with vitamin D (Figure 2).

**ECM deposition of glycosaminoglycans**

The effects of vitamin D and ascorbic acid on ECM deposition of GAGs by AoSMC and AoEC are presented in Figure 3. As such, heparan sulfate deposition was not significantly affected by 1 µM vitamin D supplementation in either cell type (Figure 3A). Ascorbic acid, added individually at 100 µM concentration, resulted in a significant increase in ECM heparan sulfate accumulation by AoSMC (322% as compared to control samples), whereas the amount produced by AoEC was reduced by 34%. Ascorbic acid used together with vitamin D resulted in a significant reduction of the stimulatory effects of ascorbic acid on heparan sulfate deposition by AoSMC from 322% to 172% of control samples. Inhibitory effect of ascorbic acid on heparan sulfate production by AoEC was not affected by vitamin D (Figure 3A).

ECM deposition of chondroitin sulfate by AoSMC was not affected by either vitamin D or ascorbic acid (Figure 3B). Supplementation of AoEC culture with 1 µM vitamin D resulted in a modest 10% decrease in chondroitin sulfate ECM deposition, whereas ascorbic acid showed no effects.

Ascorbic acid supplementation significantly increased ECM hyaluronic acid deposition in AoSMC and AoEC cultures, by 30% and 17%, respectively (Figure 3C). Vitamin
Figure 3 Effects of Vitamin D on Glycosaminoglycans ECM deposition by AoSMC and AoEC

Aortic SMC and EC were cultured as described in Figures 1 and 2. Additions of Vitamin D were made in the presence or absence of 100 µM ascorbic acid. After 72 hours incubation cells were removed and deposited. ECM was exposed by differential treatment. ECM content for heparan sulfate (Fig 3A), chondroitin sulfate (Fig 3B) and hyaluronic Acid (Fig 3C) were determined by immunoenzymatic assay.
D supplementation at 1 µM level did not produce any significant effects on hyaluronic acid ECM deposition in either cell type.

Discussion

Vitamin D is a pro-hormone belonging to the category of fat-soluble group of vitamins with its main sources coming from sunlight, diet, and supplementation. Vitamin D in the skin is present in the form of pro-vitamin D3 (7-dehydrocholesterol) and is converted to pre-vitamin D3 photochemically by the Sun’s ultraviolet B rays. Foods rich in vitamin D include fatty fish (e.g. salmon, mackerel), cod liver oil, and in small amounts, eggs. Relative to sun exposure, diet is a poor source of vitamin D. Vitamin D3 from the skin, food, or supplements is transported to the liver by vitamin D–binding proteins, where it is converted to 25(OH)D3 (calcidiol) through the process of hydroxylation. In the kidneys, 25(OH)D3 goes through a second hydroxylation to 1,25(OH)2D3 (calcitriol) which is the active metabolite.9,10

The diverse biological actions of vitamin D are mediated by an intracellular vitamin D receptor (VDR). Activation of the VDR through direct interaction with 1,25(OH)2D3 prompts the receptor’s rapid binding to regulatory regions of target genes whose functional activities are essential for directed changes in transcription. In most target cells, these actions trigger the expression of the networks of target genes whose functional activities combine to orchestrate specific metabolic responses. These responses are tissue-specific and range from highly complex actions essential for homeostatic control of mineral metabolism to focal actions that control the growth, differentiation, and functional activity of numerous cell types including those of the immune system, skin, the pancreas and bone.11

The diverse tissue-specific effects of vitamin D may be attributed to conflicting reports on its effects on the production of ECM components. As such, it was reported that in pro-fibrotic conditions Vitamin D can lower an excessive production of ECM components. Thus, Potter and colleagues observed vitamin D-mediated decrease in α(1) (I) collagen mRNA and protein and the secretion of type I collagen by human liver stellate cells after exposure to TGFβ1.12 Artaza and Norris reported Vitamin D-dependent reduction in the expression of collagen and key profibrotic factors by inducing an antifibrotic phenotype in mesenchymal multipotent cells.13 In contrast, studies of osteogenic differentiation of multipotent mesenchymal stromal cells from human adipose tissue revealed activation of gene expression of type I collagen proteins.14

In our study we observed that vitamin D effects vary in different types of human resident arterial wall cells. Since ascorbic acid has an established role in modulating vascular wall integrity and structure, we compared the vitamin D effects to the ones observed with vitamin C. We chose the ascorbic acid concentration that was above 60 µM, considered as optimal blood plasma level from dietary ascorbic acid intake. The 100 µM ascorbic acid concentration used in our study can be reached in human blood with prolonged supplementation with ascorbic acid doses above 1 g/day or higher.

Thus, AoSMC responded to vitamin D supplementation in the range 0.1 to 1 µM by increasing ECM deposition of type I collagen and decreasing ECM deposition of collagen type IV, which affected relative proportions of these 2 collagen fractions in ECM. Interestingly while 0.1 µM can correspond to optimal vitamin D blood levels, the dose of 1 µM is rather high. It has been reported that supplementation with 20,000 IU of vitamin D daily can bring this vitamin blood level up to about 0.25 µM. However, 1 µM vitamin D in our study did not exhibit cell toxicity, confirming earlier reports that underline the safety of vitamin D used in high doses and for prolonged time periods.15 We observed that vitamin D effects were additive to those of ascorbic acid, which itself acts as a potent modulator of ECM composition in human AoSMC.7 In AoEC culture vitamin D effects on collagen production were only minor.
In a normal human artery type I collagen is found in all layers: intima, media and adventitia. It is produced by both major types of arterial wall resident cells, smooth muscle and endothelial cells. The amount of collagen type I increases along with fibrotic process during the development of atherosclerotic lesions. Formation of atherosclerotic plaques, including fibrosis in intima and media aortic layers, are being considered as a compensatory mechanism against a weakening mechanical structure of arterial wall under chronic vitamin C deficiency. Collagen type IV is predominantly found in the intima layer as a component of basic membrane underlying the endothelial cell monolayer. It is produced and deposited predominantly by arterial endothelial cells. Therefore, vitamin D-dependent increase in type I collagen deposition by AoSMC could contribute to the organism’s ability to counteract pathologically induced weakness of the arterial wall. A simultaneous vitamin D-dependent decrease in ECM deposition of collagen type IV by AoSMC would probably indicate a switch in the use of cellular synthetic resources from type IV to type I collagen, as presence of type IV collagen is not essential in the vascular media.

There is scarce literature data on vitamin D effects on GAG production. Among these, Koh and colleagues reported a dose-dependent vitamin D inhibition of GAG synthesis by rat AoSMC. Vitamin D inhibited GAG synthesis in dose-dependent fashion in rabbit craniofacial chondrocytes. Also, vitamin D inhibited synthesis and enhanced the GAG degradation in osteoblastic cells in vitro. In contrast, it was reported that long-term vitamin D supplementation can significantly increase GAG content in bone tissue of patients with postmenopausal osteoporosis.

We investigated the effects of vitamin D on extracellular deposition of all 3 major GAG species: heparan sulfate, chondroitin sulfate and hyaluronic acid in the major arterial wall resident cell types: smooth muscle and endothelial cells. Our results demonstrate that vitamin D supplementation can reduce heparan sulfate ECM deposition by both AoSMC and AoEC. However, the deposition of other GAG species, ChS and HA, remained unaffected in both cell types. The accumulation of sulfated GAG in atherosclerotic lesions can contribute to the disease progression through retention and consequent oxidative modification of blood plasma low density lipoproteins. Therefore a reduction in heparan sulfate deposition by AoEC and AoSMC under supplementation with vitamin D—as found in our study—could contribute to the prevention of this important step in initiating the atherosclerotic process in the arterial wall.

**Conclusion**

In this study we observed beneficial effects of vitamin D on the deposition of important components of extracellular matrix (ECM) produced by key resident arterial wall cells: smooth muscle cells and endothelial cells. These findings contribute to a better understanding of the cellular mechanisms and the role of vitamin D in the prevention of cardiovascular disease.

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