

Short communication

Beneficial metabolic effects of Vitamin D on arterial wall cells *in vitro*

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Introduction

In recent years, many studies have shown positive effects of vitamin D on health, including various benefits to inflammation and immune response, cognitive function, protection against metabolic syndrome and cardiovascular disease.¹ Vitamin D deficiency has been closely related to the development of coronary heart disease and its mortality.² Vitamin D involvement in mitigating the pathogenesis and progression of CHD may occur at different levels, such as by regulation of renin-angiotensin system to lower blood pressure, promotion of insulin secretion, decrease in insulin resistance, regulation of cell differentiation and proliferation, and anti-inflammatory and anti-atherosclerosis effects, but the specific mechanisms have not yet been fully defined.^{3,4}

Atherosclerosis, a major underlying pathological process in the development of coronary heart disease, is characterized by dramatic metabolic changes in vascular wall resident cells, which constitute, build and maintain the structure and function of the arterial wall.⁵ The most inner part of the arterial wall is lined by a dense single layer of endothelial cells which form a selective and protective barrier between the bloodstream and arterial wall. We have demonstrated previously that structural impairment and lost integrity of the endothelial cell barrier due to vitamin C and other micronutrient deficiency is a triggering factor in the early stages of atherosclerosis.^{6,7,8} Vitamin C and other micronutrients can also mitigate migration and proliferation of smooth muscle cells, pathological processes that lead to intima layer thickening, increased arterial wall rigidity, impaired contraction function, and other pathological events involved in the cascade of atherosclerotic lesion development.⁹ Although various nutritional

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factors and micronutrients have been shown to modulate important metabolic cellular events in the vascular wall, there is no consistent data on the role of vitamin D in relation to cardiovascular cell metabolism.

In this *in vitro* study we investigated the effects of vitamin D on important characteristics of vascular cells, such as their capacity to grow and migrate. As an experimental model we used cultured human endothelial cells isolated from umbilical vein (HUVEC) and human smooth muscle cells isolated from aorta (HASMC).

Experimental procedures

Reagents

Vitamin D3 (Calcitriol 1,25(OH)2D3), ascorbic acid, 3H-thymidine and other reagents were from Sigma-Aldrich. Nutrient mixture of L-ascorbic acid, L-lysine, L-proline, L-arginine, N-acetyl cysteine, selenium, copper and green tea extract was supplied by Dr Rath International. (see Table 1 for composition). Human aortic smooth muscle cells (HASMC) and human umbilical vein endothelial cells (HUVEC) were supplied by Clonetics. HASMC were cultured in DMEM cell culture

medium supplemented with 10% fetal bovine serum (FBS, supplied by ATCC) at 37°C in 5% CO₂ and used in experiments at 5-8 passages. HUVEC were cultured in EGM-2 medium (Clonethics) and used in experiments at 5-8 passages.

HUVEC growth

Cells were seeded in 48-well plastic plates at density 2,000 cells per well in EGM-2 cell growth medium. After attachment, cells were incubated for 72 hours at 37°C 5% CO₂ with indicated amounts of tested compounds in cell growth medium and supplemented with DNA precursor, tritium labeled thymidine (3H-thymidine). Cell growth rate was determined as amount of DNA-associated radioactivity as described in details previously.¹⁰

HASMC growth

Cells were seeded in 48-well plastic plates at density 2,000 cells per well in cell growth medium (DMEM/2% FBS). After attachment, cells were supplemented with indicated concentrations of test compounds in cell growth medium. After incubation for 72 hours at 37°C 5% CO₂, cell number was determined using MTT proliferation kit (Boehringer Ingelheim) as described by manufacturer.

Table 1 – Composition of Nutrient Mixture

Component	Concentration of components in 1 mg/ml solution
L-ascorbic acid	900 µM
L-lysine	1,100 µM
L-proline	1,100 µM
L-arginine	500 µM
N-acetyl cysteine	250 µM
Selenium	85 µM
Green tea extract	150 µM (as EGCG)
Copper	7 µM
Manganese	4 µM

Cell invasion

HASMC were seeded in 75 cm² plastic flask. After attachment, cells were radioactively labeled by incubation for 72 hours in cell growth medium supplemented with 3H- thymidine. Cell layer was washed 3 times with phosphate-buffered saline (PBS). Cells were detached by standard treatment with trypsin (ATCC), suspended in serum-free DMEM and seeded on porous (8 nm pores) membrane covered with natural extracellular matrix (Matrigel) in the upper part of invasion chambers (Becton-Dickinson). Indicated amounts of vitamin D and nutrient mixture (NM) were added to cell culture after cell attachment. Cell migration through Matrigel-covered membrane toward lower part of the invasion chamber filled with DMEM/10%FBS was assessed after 24 hours incubation at 37°C 5%CO₂ by wiping out remaining cells from the upper part of the chamber and measuring radioactivity associated with lower part of the chamber.

Statistical evaluation

Results are expressed as percentage of unsupplemented control values (averages from triplicate samples +/- SD). Statistical analysis was done with T-test (Microsoft Excel). Statistical significance was accepted at p<0.01.

Results and Discussion

Endothelial cells isolated from human umbilical vein (HUVEC) used in our study share the same origin and major metabolic, behavioral and morphological characteristics of the arterial endothelial cells. As presented in Figure 1, vitamin D stimulated growth of HUVEC cells in dose-dependent manner. As such, we observed 7% cell growth increase at 20 nM vitamin D and 20% at 100 nM.

Previous reports on the effects of vitamin D on regulation of cell growth cycle of endothelial cells were inconsistent and often contradictory. Pittarella et al.

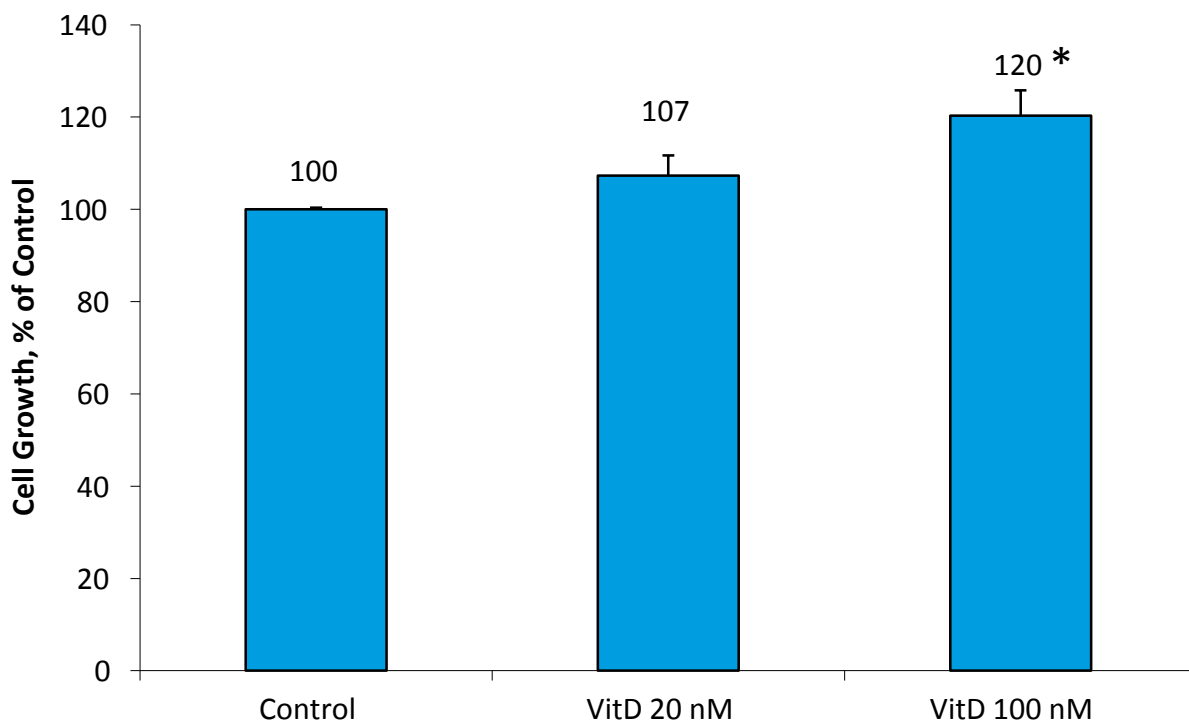


Figure 1 Effects of vitamin D3 on growth rate of cultured human umbilical vein endothelial cells (HUVEC).

HUVEC were seeded in 48-well plates and grown in cell growth medium supplemented with vitamin D3 and H3-thymidine for 48 h at 37°C. Cell growth rate was determined as amount of cellular DNA- associated radioactivity. See experimental procedure for details.

* p<0.01 as compared to Control.

reported stimulatory effects of vitamin D on endothelial cell proliferation and migration, possibly through NO-mediated mechanisms.¹¹ In contrast, there were other reports showing inhibition of endothelial cell growth in the presence of vitamin D.^{12,13} Efficient multiplication of endothelial cells is important during replacement of detached endothelial cells and for quick repair of damaged endothelial barrier. Our data support beneficial effects of vitamin D on endothelial cell growth.

In normal, healthy, adult blood vessels, smooth muscle cells proliferate at an extremely low rate. However, in response to pathological stimuli they undergo phenotypic changes and start to grow and migrate in the tissue. Therefore, we tested the effects of vitamin D on the growth and migration of human aortic smooth muscle cells —applied both individually and in combination with vitamin C. Growth of HASMC was stimulated by addition of fetal bovine serum to cell culture medium. The results presented in Figure 2 show that HASMC cell

growth rate decreased in dose-dependent manner by vitamin D. As such, in the presence of 0.1 mcM vitamin D, smooth muscle cell growth decreased by 23%, and by 34% at 1 mcM. This growth inhibitory effect of vitamin D was potentiated by vitamin C (ascorbic acid). In the presence of 500 mcM ascorbic acid, growth of HASMC decreased by 8%. However, its combination with vitamin D had more pronounced cell growth inhibitory effects. Thus, a combination of 500 mcM ascorbate together with 0.1 mcM vitamin D decreased HASMC growth rate from 77% (with vitamin D used alone) to 56% in the presence of these 2 vitamins together. In the presence of the same concentration of ascorbic acid (500mcM) but higher amounts of vitamin D (1 mcM), cell growth rate further decreased from 66% observed when vitamin D was used alone to 50% in the presence of these vitamins combined. The effective growth inhibition achieved with the combination of these 2 nutrients was higher based on their additive effects.

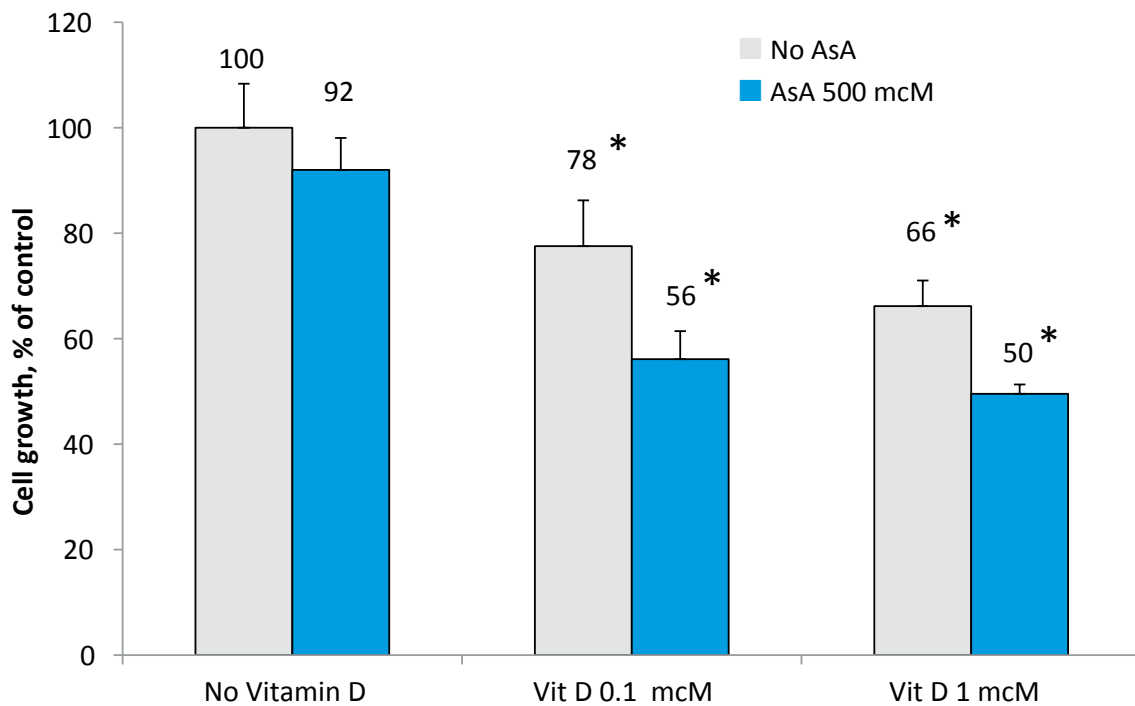


Figure 2 Effects of vitamin D and vitamin C on growth rate of cultured human aortic smooth muscle cells (HASMC).

HASMC were seeded in 48-well plates and grown in cell growth medium supplemented with vitamin D only (light bars), vitamin D3 (1,25(OH)2D3), and ascorbic acid (dark bars) for 72 h at 37°C. Cell growth rate was determined with MTT proliferation assay. See experimental procedures for details.

* $p < 0.01$ as compared to unsupplemented Control.

Inhibitory effects of vitamin D on smooth muscle cell growth rate have been reported previously and similar effects have been demonstrated in several other cell types, including human cell breast cancer cells.^{14,15} Conversely, negative effects of vitamin D on growth of vascular endothelial and smooth muscle cells could relate to their distinctive structural and functional roles in the body. Endothelium is formed by a single layer of endothelial cells that line the interior surface of blood vessels and lymphatic vessels and serve as a barrier, regulate blood vessel tone and perform other functions.¹⁶ Vascular smooth muscle cells constitute the major cellular type within the arterial wall, supporting contractile function of the vessels and providing structural support by synthesis and deposition of extracellular matrix—an essential part of arterial wall interstitium.¹⁷ Accelerated growth and migration of smooth muscle cells in the vascular wall represents vascular pathology leading to vascular wall thickening and atherosclerotic plaques. Therefore, our findings

that vitamin D used alone and especially in combination with ascorbic acid (vitamin C) can significantly inhibit pathological HASMC growth have important health implications.

Moreover, our results indicate that vitamin D and other micronutrients can also affect motility of HASMC. As presented in Figure 3, the migration of HASMC through naturally produced extracellular matrix, Matrigel, stimulated by chemo attractive action of FBS components, significantly decreased (by 22%) in the presence of 20 nM vitamin D. In addition, we tested the combined effect of vitamin D and a specific nutrient mixture (NM), previously shown in our studies to inhibit extracellular matrix invasion of different types of human cancer cells.¹⁸ Our earlier study showed that NM—which contains natural components, such as vitamin C, polyphenols from green tea, amino acids lysine, proline and arginine, N-acetyl cysteine, and others—inhibits invasion of aortic smooth muscle

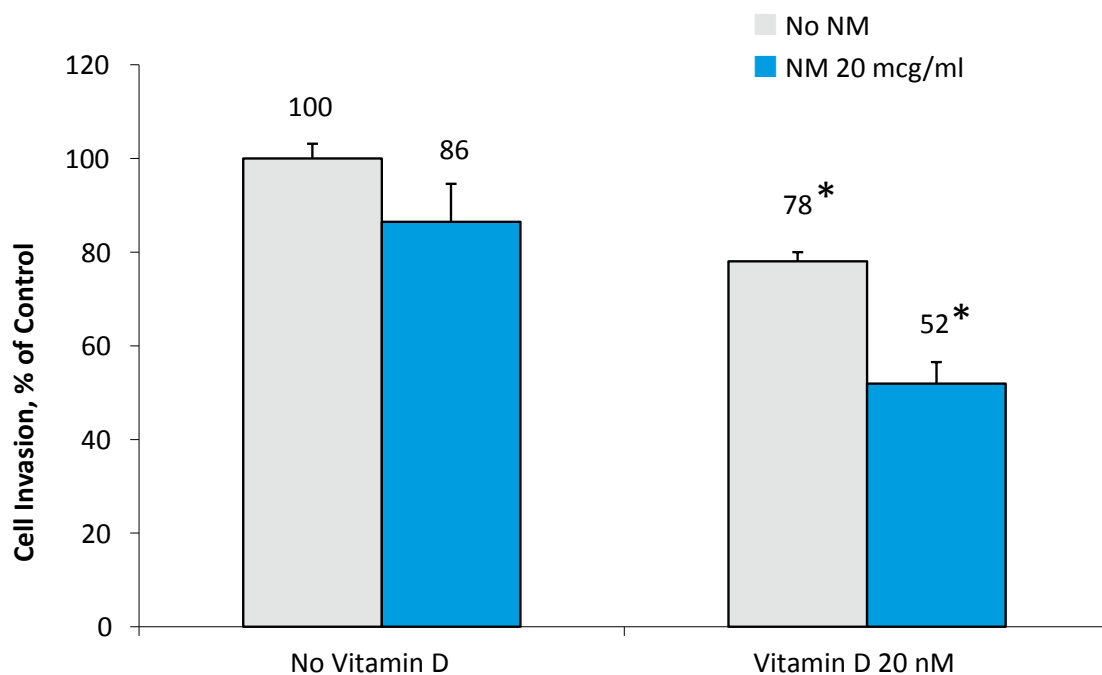


Figure 3 Vitamin D inhibits smooth muscle cell migration through extracellular matrix.

Radioactively prelabeled with 3H-thymidine, HASMC were seeded in upper part of invasion chambers on porous membrane covered with Matrigel. Cell migration was estimated after 24 h incubation at 37°C by measuring radioactivity recovered from lower part of the chambers. Light bars represent samples incubated with or without 20 nM vitamin D. Dark bars represent samples with added 20 mcg/ml nutrient mixture (NM). See experimental procedures for details.

* $p < 0.01$ as compared to unsupplemented Control.

cells in a dose-dependent manner, achieving 35% inhibition at 50 mcg/ml.⁹ In our present study we used lower NM concentration (20 mcg/ml), which by itself decreased HASMC migration through Matrigel by 14%. This amount of NM was combined with 20nM Vitamin D, which by itself could inhibit HASMC migration by 22%. We observed that when combined, both test nutritional components further decreased smooth muscle cell invasion by 48%. This level of inhibition implies synergistic interactions of this micronutrient combination, since calculated additive effects indicate 36% inhibition. The achieved effect of 48% is 12% higher than expected cumulative inhibition.

The results show that vitamin D can mitigate pathological metabolic events important in the development of atherosclerosis, and its efficacy can be enhanced by interaction with vitamin C and a specific mixture of micronutrients. Our data imply a synergistic interaction between vitamin D and NM and vitamin D and vitamin C, allowing for enhanced metabolic efficacy with lower doses of individual components. Taking advantage of nutrient synergy in developing effective approaches based on this principle has been a hallmark of our scientific approaches in various aspects of human health, from atherosclerosis to cancer.

These results further implicate the need for the development of new strategies based on natural compounds in managing arterial wall health and preventing coronary heart disease.

References

1. Kunadian V, Ford GA, Bawamia B, Qiu W, Manson JE. Vitamin D deficiency and coronary artery disease: a review of the evidence. *Am Heart J.* 2014;167(3):283–91.
2. Zittermann A. Vitamin D and cardiovascular disease. *Anticancer Res.* 2014;34(9):4641–48.
3. Nitsa A, Toutouza M, Machairas N, Mariolis A, Philippou A, Koutsilieris M. Vitamin D in cardiovascular disease. *In Vivo.* 2018;32(5):977–981.
4. Legarth C., Grimm D., Wehland M., Bauer J., Krüger M. The Impact of Vitamin D in the Treatment of Essential Hypertension. *Int. J. Mol. Sci.* 2018;19(2):455. doi: 10.3390/ijms19020455
5. Libby P. Coronary artery injury and the biology of atherosclerosis: inflammation, thrombosis, and stabilization. *Am J Cardiol.* 2000;86(8B):3J–8J.
6. Cha J, Niedzwiecki A, Rath M. Hypoascorbemia induces atherosclerosis and vascular deposition of lipoprotein(a) in transgenic mice. *Am J Cardiovasc Dis.* 2015;5(1):53-62.
7. Shi L, Niedzwiecki A, Ivanov V, Rath M. Cardiovascular effects of cyclical dietary vitamin C withdrawal in mice deficient in internal synthesis vitamin C and producing human lipoprotein (a): Gulo(-/-); Lp(a)+. *Int J Cardiovasc Res.* 2018; 7:6. doi: 10.4172/2324-8602.1000397.
8. Ivanov V, Cha J, Ivanova S, Kalinovsky T, Rath M, Niedzwiecki A. Nutrient supplementation modulates angiotensin II-mediated atherosclerosis in ApoE KO mice. *Mol Med Rep.* 2010;3(3):417-425.
9. Ivanov V, Roomi M, Kalinovsky T, Niedzwiecki A, Rath M. Anti-atherogenic effects of a mixture of ascorbic acid, lysine, proline, arginine, cysteine, and green tea phenolics in human aortic smooth muscle cells, *J Cardiovasc Pharmacol.* 2007;49(3):140-145.
10. Ivanov O, Ivanova SV, Niedzwiecki A. Ascorbate affects proliferation of guinea-pig vascular smooth muscle cells by direct and extracellular matrix-mediated effects. *J Mol Cell Cardiol.* 1997;29(12):3293-303.
11. Pittarella P, Squarzanti DF, Molinari C, Invernizzi M, Uberti F, Renò F. NO-dependent proliferation and migration induced by Vitamin D in HUVEC. *J Steroid Biochem Mol Biol.* 2015;149:35-42.
12. Gonzalez-Pardo V, Martin D, Gutkind JS, Verstuyf A, et al. 1 Alpha,25-dihydroxyvitamin D3 and its

- TX527 analog inhibit the growth of endothelial cells transformed by Kaposi sarcoma-associated herpes virus G protein-coupled receptor in vitro and in vivo. *Endocrinology*. 2010;151(1):23-31.
13. Yazdani S, Poosti F, Toro L, et al. Vitamin D inhibits lymphangiogenesis through VDR-dependent mechanisms. *Sci Rep*. 2017;17;7:44403. doi: 10.1038/srep44403.
14. Kim SH, Pei QM, Jiang P, Yang M, Qian XJ, Liu JB. Effect of active vitamin D3 on VEGF-induced ADAM33 expression and proliferation in human airway smooth muscle cells: implications for asthma treatment. *Respir Res*. 2017;18(1):7.
15. Ivanov V, Ivanova S, Niedzwiecki A, Rath M. Vitamin D enhances anticancer effects of EGCG and a specific micronutrient combination in breast cancer cells. *J Cel Med Nat Health*. 2018; 6.
16. Dyer LA, Patterson C. Development of the endothelium: emphasis on heterogeneity. *Semin Thromb Hemost*. 2010;36(3):227-35.
17. Pfaltzgraff ER, Bader DM. Heterogeneity in vascular smooth muscle cell embryonic origin in relation to adult structure, physiology, and disease. *Dev Dyn*. 2015;244(3):410-6.
18. Niedzwiecki A, Rath M. Clinical nutrients in cancer therapy: A scientific review and perspective. *Dr. Mattias Rath's Cellular Health Research Series*. 2005.