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Original Research Article

Vitamin D mitigates atherosclerosis risk in TNF-α-induced endothelial cells

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Abstract

Purpose: *To determine the effect of vitamin D on endothelial function in a cell line model of inflammation.*
Methods: *Human saphenous vein cells were treated with 1,25-dihydroxyvitamin D3 (1,25-OHD) and/or TNF-α, and the expression levels of 20 different atherosclerotic cytokines were examined in the treated cells using a human atherosclerosis antibody array. The STRING and PANTHER analyses were also performed to determine the possible pathway interactions of these four pro-atherosclerotic and two anti-atherosclerotic cytokines.*
Results: *The expression levels of pro-atherosclerotic cytokines GM-CSF, CCL20, CCL5, and TNF-α decreased upon 1,25-OHD administration, whereas those of anti-atherosclerotic cytokines PDGFB and TGF-β3 increased.*
Conclusion: *Vitamin D could mitigate cardiovascular risks by mediating the expression of inflammatory cytokines; however, this needs to be verified via in vivo studies.*

Keywords: *Atherosclerosis, Cytokines, Vitamin D, Inflammation, TNF-alpha*

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INTRODUCTION

According to World Health Organization data for the year 2015, cardiovascular diseases (CVD) are the leading cause of global mortality, accounting for 17.8 million deaths which corresponds to 31% of deaths worldwide [1]. Atherosclerosis, the underlying condition of CVD, is recognised as a low-grade inflammation of the endothelial wall instead of an ordinary lipid storage disease [2].

In the atherosclerotic process, immune responses are orchestrated by a wide range of cytokines, including IL-1, IL-6, and tumor necrosis factor (TNF)-α [3]. TNF induces the expression of adhesion molecules and other pro-inflammatory cytokines as well as chemokine receptors via the activation of endothelial cells, which in turn promotes the migration of activated leukocytes to the inflammatory region. During the development of atherosclerosis, TNF may organise the inflammatory pathway in the arterial

wall, in part by promoting endothelial dysfunction (ED) [4].

ED is a potential CVD-inducing factor responsible for the decreased availability of vasodilators, such as nitric oxide, leading to a lack of muscle relaxation which predisposes the blood vessels to enter the atherosclerotic cascade. This cascade consists of several critical steps, such as endothelial cell activation, migration of leukocytes to the inflammatory region, differentiation of monocytic cells, and formation of foam cells, followed by fibrotic plaque formation [5, 6].

Vitamin D is a fat-soluble steroid hormone endogenously produced by the human skin [7]. In addition to its role in bone and mineral metabolism, vitamin D has been reported to be involved in both cardiovascular and immunological processes, including cytokine expression, vascular cell differentiation, proliferation, migration, immune response organisation, and all steps of endothelial activation and dysfunction [8]. Large-scale epidemiological studies have associated vitamin D deficiency with increased cardiovascular risk due to accelerated atherosclerosis [9-11]. Vitamin D deficiency was observed in a high proportion of patients with myocardial infarction [12]. Besides, vitamin D deficiency induces upregulation of major proinflammatory cytokines through the nuclear factor kappa B pathway, mimicking endothelial behaviour in atherosclerotic conditions [13].

The current study was designed to determine the effect of vitamin D administration on human saphenous vein cells (HSaVECs) under TNF-α-induced inflammatory conditions.

EXPERIMENTAL

Cell culture

HSaVECs (C-12231), purchased from Promocell (Heidelberg, Germany), were cultivated in Promocell Endothelial Cell Growth Medium (C-22010). In brief, 25 cm^2 culture flasks were coated with type I collagen. HSaVECs were incubated at 37 ºC in a 5% CO_2 humidified atmosphere until 70 – 80% confluence. The cells were used for the experiments at the 3rd passage. The cells were then plated in 6-, 12-, and 24-well collagen-coated plates for further analysis. The average cell number was 6,000 cells per 1 mL of medium.

Vitamin D (1,25-Dihydroxyvitamin D3) administration

Vitamin D [1,25-Dihydroxyvitamin D3-1,25(OH)2D3, Sigma C-9756, Sigma-Aldrich Chemie GmbH, Steinheim, Germany] was prepared by dissolving in absolute ethanol. The administered TNF-α concentration was 10 ng/mL. The study groups were as follows: Control group (non-treated cells), vehicle (ethanol only) group, TNF-α only group, vitamin D only group (10^{-8} M), and vitamin D + TNF-α group. The administration durations of vitamin D and TNF-α were 24 h and 6 h, respectively. In the vitamin D + TNF-α group, vitamin D was administered first, followed by TNF-α administration (Table 1).

Cytotoxicity analysis

The levels of lactate dehydrogenase enzyme in the culture media of the different groups were quantified using an ELISA-based kit in triplicate, comparing drug-administered groups to negative control groups. A cytotoxicity detection kit (lot no: 11 644 793 001, Roche, Mannheim, Germany) was used to determine the cytotoxicity levels in these groups.

Human atherosclerosis antibody array

RayBio C-Series Human Atherosclerosis Antibody Array C1 (AAH-ATH-1, Raybiotech, Norcross, GA, USA) was used according to the manufacturer's instructions to measure the expression levels of 20 different atherosclerosis-associated cytokines and chemokines: Eotaxin-1, Granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating Factor (GM-CSF), glial cell-derived neurotrophic factor (GDNF), intercellular adhesion molecule 1 (ICAM-1), interleukin 1 β (IL-1β),

Table 1: Dose and administration duration of Vitamin D and TNF-α in the groups

Group	Duration and dose of Vitamin D (1,25(OH)2D3) administration	Duration and dose of TNF-α administration
Control	-	-
Vehicle (ethanol)	-	-
TNF-α-only	-	6 h (10 ng/mL)
Vitamin D-only	24 h (10^{-8} M)	-
Vitamin D + TNF-α	24 h (10^{-8} M)	6 h (10 ng/mL)

Table 2: Distribution of proinflammatory protein antibodies in the Human Atherosclerosis Antibody Array

A	B	C	D	E	F	G	H
Positive Control	Positive Control	Negative Control	Negative Control	Eotaxin-1 (CCL11)	G-CSF	GDNF	GM-CSF (CSF2)
ICAM-1 (CD54)	IL-1 R1	IL-1 R2	IFN-γ (IFNG)	IL-1 A (IL-1 F1)	IL-1 Beta (IL-1 F2)	MCP-1 (CCL2)	M-CSF
MIP-3α (CCL20)	PDGF-BB (PDGFB)	RANTES (CCL5)	TGF-β1 (TGFB1)	TGF-β2 (TGFB2)	TGF-β3	TNF-α	TNF-β (LTA)
Blank	Blank	Blank	Blank	Blank	Blank	Blank	Positive control

interferon γ (IFN-γ), interleukin 1 α (IL-1α), interleukin-1 soluble receptor type I (IL-1sRI), interleukin-1 soluble receptor type II (IL-1sRII), macrophage colony-stimulating factor (M-CSF), monocyte chemoattractant protein-1 (MCP-1), TNF-β, TNF-α, transforming growth factor β 1 (TGF-β1), TGF-β2, TGF-β3, macrophage inflammatory protein-3 α (MIP-3α), regulated upon activation normal T cell expressed and presumably secreted (RANTES), and platelet-derived growth factor-BB (PDGFBB). The experiments were performed three times in duplicates. The structure of the dot blot membrane is presented in Table 2.

Statistical analysis

SPSS software [IBM, Chicago, USA (version 22.0)] was used to analyse the data. Means, standard deviations, medians, and highest-lowest values were used in the descriptive statistics. The Kolmogorov-Smirnov test was used to analyse data distribution. None of the data was within the normal distribution range. Thus, the Kruskal-Wallis and Mann-Whitney U tests were used to evaluate the study data. Statistical significance was set at $p \leq 0.05$. Dunn's multiple comparisons test was used to compare the groups for each pro-inflammatory cytokine and chemokine.

RESULTS

Proinflammatory cytokines and chemokines

Using a dot-blot array, the levels of 20 different proinflammatory cytokines and chemokines in five different groups were determined (Figure 1). According to the statistical analysis of the array results, a significant difference was found between the groups concerning the expression of six proinflammatory cytokines including GM-CSF (CSF2), MIP-3α (CCL20), TGF-β3 (TGFB3), PDGF-BB (PDGFB), TNF-α (TNF), and RANTES (CCL5). The differences in the expression levels of GM-CSF, MIP-3α, TNF-α, and RANTES were statistically significant in the TNF-α only and vitamin D + TNF-α groups when compared to the control and vitamin D-only groups ($p < 0.001$, Figure 2).

GM-CSF expression levels were lower in the vitamin D-only group than in the TNF-α-only and vitamin D + TNF-α groups. Similar expression profiles were observed for MIP-3α, TNF-α, and RANTES.

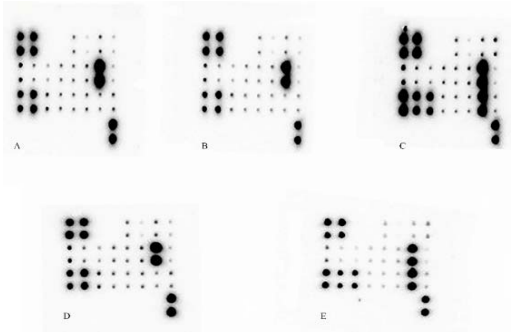

Figure 1: The expression patterns of proinflammatory cytokines and chemokines in the study groups. (A) Control group, (B) vehicle (ethanol) group; (C) TNF-α-only group; (D) vitamin D-only group (10^{-8} M); (E) vitamin D (10^{-8} M) + TNF-α group

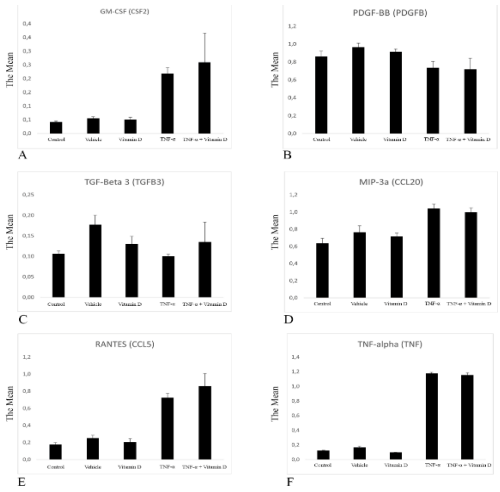

Figure 2: Graphical presentation of the expression levels of six different proinflammatory cytokines (GM-CSF, MIP-3α, PDGF-BB, RANTES, TGF-β3, and TNF-α) in the five experimental groups

Moreover, the difference in PDGF-BB expression levels was statistically significant in TNF-α-only and vitamin D + TNF-α groups when compared to the vitamin D-only group ($p < 0.001$ for both). PDGFB expression levels were higher in the vitamin D-only group than in the TNF-α-only and vitamin D + TNF-α groups. Similarly, the decrease in TGF-β3 expression levels was statistically significant in the TNF-α-only group compared to that in the vitamin D-only group ($p < 0.01$) (Figure 2). There were no statistically significant differences in the expression levels of CCL11, GCSF, GDNF, CD54, IL-1R1, IL-1R2, IFNG, IL-1F1, IL-1F2, CCL2, MCSF, TGFB1, TGFB2, and LTA among the groups.

Protein interaction

Proteins that were shown to have increased expression levels were analysed for their possible interactions, co-expression, and intervening pathway status. According to the biological network analysis of protein list integration for gene prioritisation and predicting gene function with some additional nodes from Wang-Yang-2011 [14] and GeneMANIA; CCL5, CCL20, and TGFB3 were observed to have physical interactions with SDC4, CHMP2A, SLC2A5, TRIP6, EDC4, and RANGAP1 through positive regulation of DNA replication, DNA metabolic process, and phosphatidylinositol 3-kinase (Figure 3).

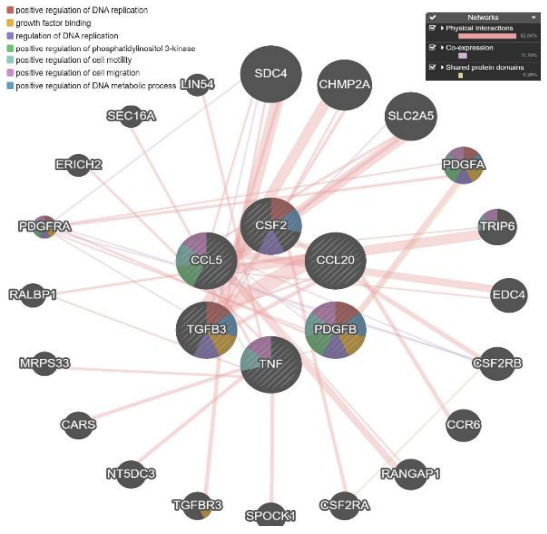

Figure 3: The statistical query results of the six proteins. Physical interactions were 82.04% and the co-expression rates were 11.70%. The pink colour on the edges between the nodes shows the possible relationship of physical contact. Nodes of query genes are in rounded order in the interior of the network. The edge sizes between nodes were in different thicknesses according to statistical value

Pathway analysis data

The six proteins identified above (CSF2, CCL20, TGFB3, PDGFB, TNF, and CCL5) were then analysed for their possible pathway associations using the online tool PANTHER. As shown in Figure 4, two out of six genes were associated with inflammation mediated by chemokine and cytokine signalling pathways. In addition, each of these six genes were also associated with one of the following: Wnt signalling, angiogenesis, TGF-β signalling, apoptosis signalling, and PDGF signalling pathways (Figure 4).

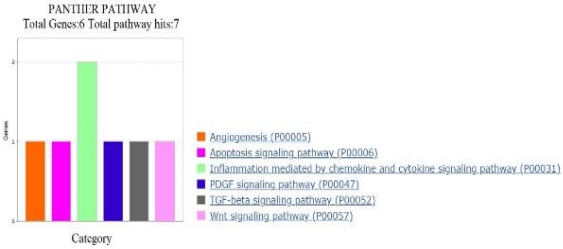

Figure 4: An overview of pathways associated with CSF2, CCL20, TGFB3, PDGFB, TNF, and CCL5 based on Panther analysis

DISCUSSION

Atherosclerosis is a major global health concern that precipitates life-threatening clinical conditions, including stroke and acute coronary syndromes. Studies devoted to the underlying cellular mechanisms have suggested the pivotal role of ED in the development and progression of atherosclerosis [15]. Cytokines, which are grouped into numerous classes, such as TNF, interferons, interleukins, colony-stimulating factors, and chemokines, orchestrate the complex inflammatory response within the atherosclerotic plaque and participate in all steps of the process, from early ED to the disruption of a vulnerable plaque [3, 16]. Specifically, TNF, IL1-β, IL-6, and MCP-1 are proatherogenic (atherosclerosis-inducing) cytokines, whereas IL-10, IL-19, and TGF-β are the antiatherogenic (atherosclerosis inhibiting) cytokines [17].

Vitamin D, which is accepted as a key player in mineral and bone metabolism, has recently been linked to immunity and cardiovascular health [8, 10]. The results of this study indicated that vitamin D administration attenuated atherosclerosis and significantly decreased the expression levels of proinflammatory cytokines, such as GM-CSF, CCL20, CCL5, and TNF-α, whereas it increased the expression levels of anti-inflammatory cytokines, including PDGFB and TGF-β3. Vitamin D appears to prevent atherosclerosis. However, updated evidence

regarding the relationship between atherosclerosis and vitamin D deficiency is still inconclusive and contradictory [18].

Several studies have found that vitamin D does not lower the incidence of major cardiovascular events. In two of these randomised, placebo-controlled, and double-blinded trials with 5110 (VİDA) [19] and 25871 patients (VİTAL) [20], vitamin D supplementation was not beneficial and did not decrease myocardial infarction or mortality. Similarly, in another trial with 305 patients, Hin *et al* concluded that vitamin D did not alter any cardiovascular risk factors, such as increased heart rate, blood lipids, and arterial stiffness [21]. This contradictory result may be because of three reasons: vitamin D levels of the patients were not tested before the studies, or low-dose vitamin D administration was preferred during treatment, or the follow-up duration of the treatment was not long enough.

More recently, there have been contradictory findings regarding the impact of vitamin D supplementation on cardiovascular diseases. In a randomised, placebo-controlled, double-blinded study with diabetic patients, Farrokhian *et al* showed improved glycaemic status and attenuated vascular inflammation after vitamin D administration [22].

Although CSF2, CCL20, TGF-β3, PDGFB, TNF, and CCL5 were found to be associated with vitamin D uptake, no associations were found with the cytokines CCL11, GCSF, GDNF, CD54, IL-1R1, IL-1R2, IFN-γ, IL-1F1, IL-1F2, CCL2, MCSF, TGF-β1, TGF-β2, and LTA. These results match those observed in earlier studies in which some of the inflammatory markers were affected, while some were insignificantly changed in the presence of vitamin D supplementation [23, 24]. The presence of elevated levels of proinflammatory cytokines, such as GM-CSF, CCL20, CCL5, and TNF-α could be accepted as clear and precise evidence of inflammatory response in atherosclerotic plaque development. Interestingly, two anti-inflammatory cytokines (PDGFB and TGF-β3) seem to have protective roles under these conditions. These data suggest that there is a balance between anti-inflammatory and inflammatory activities in atherosclerosis.

CONCLUSION

Although the study has successfully demonstrated that vitamin D supplementation could mitigate cardiovascular risk, the findings should be confirmed and validated using further molecular techniques. In addition, the experimental results should be compared with those obtained using different cell lines. Considerable effort is needed to determine the actual impact of vitamin D on the atherosclerotic process through both pro- and anti-inflammatory markers.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Burak Önal designed the study and supervised the data collection. Merve Alaylıoğlu, Erdinç Dursun and Duygu Gezen-Ak analyzed and interpreted the data. Burak Önal, Erdinç Dursun and Duygu Gezen-Ak prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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