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Review

1,25(OH)$_2$ vitamin D suppresses macrophage migration and reverses atherogenic cholesterol metabolism in type 2 diabetic patients

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ABSTRACT

Reduced monocyte infiltration into the vessel wall and increased macrophage cholesterol efflux are critical components in atherosclerotic plaque regression. During inflammation, monocyte chemotactic protein 1 (MCP-1) signaling activation and cholesterol deposition in macrophages induce endoplasmic reticulum (ER) stress, which promotes an increased inflammatory response. Increased macrophage ER stress shifts macrophages into an M2 macrophage phenotype with increased cholesterol uptake and deposition. In type 2 diabetes, a population with elevated baseline risk of cardiovascular disease (CVD), vitamin D deficiency doubles that risk. We have found that 1,25-dihydroxy vitamin D [1,25(OH)$_2$D] prevents foam cell formation during macrophage differentiation by suppressing ER stress. However, it is unknown whether suppression of ER stress by 1,25(OH)$_2$D decreases monocyte infiltration and reverses atherogenic cholesterol metabolism in previously differentiated, vitamin D-deplete macrophages. We collected peripheral monocytes from type 2 diabetic patients and differentiated them into macrophages under vitamin D-deplete or 1,25(OH)$_2$D-supplemented conditions. 1,25(OH)$_2$D supplementation suppressed macrophage migration in response to MCP-1 and mRNA expression of chemokine (C–C motif) receptor 2 (CCR2), the MCP-1 receptor, compared to vitamin D-deplete cells. Furthermore, inhibition of ER stress with phenyl butyric acid resulted in similar effects even in vitamin D-deplete cells, while induction of ER stress with Thapsigargin under 1,25(OH)$_2$D-supplemented conditions increased macrophage migration and CCR2 expression, suggesting that the effects of vitamin D on migration are mediated through ER stress suppression. To determine whether the detrimental pattern of macrophage cholesterol metabolism in vitamin D depletion is reversible, we assessed cholesterol uptake in macrophages differentiated under vitamin D-deplete conditions as described above, then supplemented with 1,25(OH)$_2$D or maintained in vitamin D-deplete conditions. Cholesterol uptake was decreased in 1,25(OH)$_2$D-supplemented compared to vitamin D-deplete cells, suggesting slowed cholesterol deposition with active vitamin D. 1,25(OH)$_2$D supplementation also suppressed cholesteryl ester formation and enhanced cholesterol efflux in M2 macrophages compared to vitamin D-deplete cells, suggesting facilitation of cholesterol egress in the presence of 1,25(OH)$_2$D. We thus provide further evidence that active vitamin D is an ER stress reliever that may have a role in atherosclerotic plaque regression.

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Contents

Abbreviations: MCP-1, monocyte chemotactic protein 1; ER, endoplasmic reticulum; CVD, cardiovascular disease; 1,25(OH)$_2$D, 1,25-dihydroxy vitamin D; CCR2, chemokine (C–C motif) receptor 2; IFN, interferon; IL, interleukin; IC, immunocomplex plus lipopolysaccharide; 25(OH)D, 25-hydroxy vitamin D; M-CSF, macrophage colony-stimulating factor; PBA, phenyl butyric acid; qPCR, quantitative RT-PCR; oxLDL, oxidized low density lipoprotein; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl indocarbocyanine percholate; HDL, high density lipoprotein; ApoA-I, apolipoprotein A-I.

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1. Introduction

In recent years, vitamin D has been shown not only to be important for bone and calcium metabolism but also for homeostasis of critical tissues involved in vascular disease in patients with type 2 diabetes. In diabetics, the prevalence of deficiency of 25-hydroxy vitamin D [25(OH)D], the principal storage form of vitamin D, is almost twice that for non-diabetics, and low vitamin D levels nearly double the relative risk of developing CVD compared to diabetic patients with normal vitamin D levels [1–3]. A growing body of evidence from animal and human studies shows that vitamin D improves peripheral insulin action, suppresses the renin–angiotensin system, decreases systemic inflammatory mediators of vascular disease, and prevents foam cell formation [4–7], revealing the influence of vitamin D on multiple known mechanisms responsible for the increased vascular inflammation seen in diabetic patients.

Atherosclerotic plaque progression depends on the accumulation of monocyte-derived cells within the plaque. This process results from the imbalance of monocyte recruitment, macrophage survival within the plaque, and macrophage egression capabilities. In ApoE$^{-/-}$ mouse models of atherosclerosis, ApoE rescue lowers plasma cholesterol and increases HDL, leading to plaque regression mediated by suppression of monocyte recruitment with stable rates of macrophage apoptosis [8]. In a surgical murine model, transplantation of plaque-bearing ApoE$^{-/-}$ aortae into wild-type mice results in rapid plaque regression mediated by emigration of macrophages expressing CCR7, an M1 macrophage marker [9]. Monocytes recruited to the subendothelial space respond to environmental signals such as cytokines and modified cholesterol to stimulate differentiation into macrophages with diverse functional programs. Interferon (IFN)γ induces the M1 macrophage subtype, characterized by proinflammatory cytokine production to accelerate additional immune cell recruitment [10]. Interleukin (IL)-4, IL-10, and immunocomplex plus lipopolysaccaride (IC) induce the multiple M2 macrophage subtypes, more heterogeneous cells with both pro- and anti-inflammatory functions [11–13], but all with increased cholesterol uptake and cholesteryl ester formation [14]. In mouse models of atherosclerosis, alteration of the cytokine microenvironment triggers the conversion of macrophage subtypes already present in the lesion and changes their location within the plaque [15]. We have demonstrated that ER stress is a functional switch controlling macrophage differentiation in diabetics; suppression of ER stress shifts M2-predominant macrophages to M1-predominant cells and decreases foam cell formation [14]. We have also showed that 1,25(OH)$_2$D suppresses macrophage ER stress. This leads to M1-predominant macrophage differentiation and prevention of foam cell formation through downregulation of scavenger receptors CD36 and SRA-1 [7,16], suggesting that vitamin D promotes an anti-atherogenic macrophage phenotype. Recently, we found that 25(OH)D deficiency in diabetics is also associated with increased monocyte ER stress, leading to similarly M1-predominant markers and increased monocyte adhesion to the endothelium [16]. However, it is unclear whether suppression of ER stress by vitamin D affects mechanisms implicated in plaque regression, including suppression of monocyte infiltration and shift of differentiated macrophages toward a phenotype with lower cholesterol content.

2. Materials and methods

Subjects with type 2 diabetes were voluntarily recruited for a single venous blood draw and provided written informed consent, approved by the Human Research Protection Office of Washington University School of Medicine. Peripheral monocytes were isolated by standard Ficoll technique and selected for CD14 marker positivity (Miltenyi Biotec). To induce differentiation into vitamin D-deplete or 1,25(OH)$_2$D-supplemented macrophages, monocytes were cultured for 5 days in vitamin D-deplete media [deplete of both 25(OH)D and 1,25(OH)$_2$D: DMEM plus 10% charcoal/dextran-treated FBS] plus macrophage colony-stimulating factor (M-CSF; 100 ng/ml; Sigma) and with or without 1,25(OH)$_2$D$_3$ supplementation (10^{-8} M). ER stress inhibition was obtained by adding phenyl butyric acid (PBA; 10 mM; Calbiochem) for 16 h in macrophages following culture in vitamin D-deplete media. Induction of ER stress was obtained by adding Thapsigargin (0.25 μM, Sigma) for 24 h in macrophages following culture in vitamin D-supplemented media. Transwell migration assays were performed (Costar polycarbonate filters, 5 μm pore size) as previously described [17]. Membranes and 12-well plates were coated with fibronectin (5 μl/mL; Life Technologies) overnight at 4°. Macrophages (0.3×10^5 cells/well) were added to the upper chamber, and MCP-1 (100 ng/well; Sigma) in 0.8% agarose solution was added to the lower chamber to stimulate migration. Cells migrating into the lower chamber after 8 h of incubation were manually counted. Quantitative RT-PCR (qPCR) analyses for CCR2 expression were performed by Sybr-green methodologies and normalized to the housekeeping gene L32. Cholesterol uptake and efflux, as well as cholesteryl ester formation were performed as we previously described [7]. Briefly, cholesterol uptake was measured after macrophage incubation with 10 μg/mL oxidized low density lipoprotein (oxLDL) labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl indocarbocyanine percholate (DiI; Invitrogen) for 6 h. Cholesteryl ester formation was measured after macrophage incubation with oxLDL (200 μg/mL) with ^{3}H oleic acid (0.1 mM) (American Radiolabeled Chemicals Inc.) for 6 h. Cholesterol efflux was measured after macrophage incubation for 24 h with labeled oxLDL (300 μg/mL) with 5 mCi of ^{3}H cholesterol and initiation of efflux by media containing high density lipoprotein (HDL; 50 μg/mL) or apolipoprotein A-I (ApoA-I; 25 μg/mL). Cholesterol uptake was assessed in differentiated vitamin D-deplete macrophages subsequently supplemented with 1,25(OH)$_2$D$_3$ (10^{-8} M) or maintained in vitamin D-deplete conditions for 5 additional days, then incubated with oxLDL. Cholesteryl ester formation and cholesterol efflux were also assessed in differentiated vitamin D-deplete macrophages subsequently supplemented with 1,25(OH)$_2$D$_3$ or maintained in vitamin D-deplete conditions, but prior to oxidized LDL incubation, cells were stimulated with IFNγ to promote M1 macrophage formation or IL-4, IL-10, or IC to promote M2 macrophage subtypes. Experiments were carried out with duplicate or triplicate samples, with results expressed as mean ± SEM for continuous variables. Statistical significance of differences was defined by $p \leq 0.05$ using the paired t-test.

3. Results and discussion

To evaluate mechanisms involved in plaque regression, we performed migration assays in vitamin D-deplete

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