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Vitamin D_3 supplementation decreases a unique circulating monocyte cholesterol pool in patients with type 2 diabetes

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ABSTRACT

Cross-sectional studies indicate consistent associations between low 25(OH)D concentration and increased risk of cardiovascular disease (CVD), but results of randomized control trials (RCTs) are mixed. However, the majority of the RCTs do not focus on type 2 diabetics, potentially obscuring the effects of vitamin D in this population. In vitro $1,25(OH)_2D_3$ downregulates macrophage cholesterol deposition, but the in vivo effects are unknown. To explore potential mechanisms of the effects of vitamin D on CVD risk in patients with type 2 diabetes, we isolated monocytes in a subset of 26 patients from our RCT of diabetics with baseline serum 25(OH)D < 25 ng/mL randomized to vitamin D_3 4000 IU/day or placebo for 4 months. Upon enrollment, the mean 25(OH)D level was 17 ng/mL, which increased to 36 ng/mL after vitamin D and remained unchanged in the placebo group. Before randomization, groups demonstrated similar mean hemoglobin A1c and plasma lipids levels, none of which was significantly altered by vitamin D supplementation. Moreover, assessment of oxidized LDL uptake in monocytes cultured in the patient's own serum before vs. after treatment resulted in > 50% reduction in the vitamin D group with no change in the placebo group. This was mediated through suppression of endoplasmic reticulum stress and scavenger receptor CD36 protein expression. The reduction in monocyte cholesterol uptake was reflected in a 19% decrease in total monocyte cholesterol content. Interestingly, cross-sectional analysis of circulating monocytes from vitamin D-deficient vs. sufficient diabetic patients revealed 8-fold higher cholesteryl ester content, confirming the capacity of these monocytes to uptake and carry cholesterol in the circulation. This study identifies a unique circulating cholesterol pool within monocytes that is modulated by vitamin D and has the potential to contribute to CVD in type 2 diabetes.

1. Introduction

Type 2 diabetes (T2DM) remains a widely prevalent condition; in the U.S. alone, it affects more than 29 million adults. T2DM often co-exists with dyslipidemia and hypertension, and despite potent medications to treat all three risk factors, the risk of myocardial infarction or cardiovascular disease (CVD) death remains nearly twice that of non-diabetics [1], prompting continued interest in the discovery of other modifiable CVD risk factors for this population. A potential target lies in modulating mononuclear cells prior to the development of atherosclerosis. Monocytes and macrophages are critical to cholesterol deposition in the atherosclerotic plaque. Macrophages recruited to the subendothelial space increase expression of scavenger receptors, most notably scavenger receptor A1 (SR-A1) and cluster of differentiation 36 (CD36), enabling them to uptake modified low density lipoprotein (LDL) cholesterol in an unregulated manner. The resulting accumulation of cholesteryl esters transforms the macrophages into foam cells, which are the major contributor to the lipid core of atherosclerotic

Abbreviations: ABC, ATP binding cassette; CD36, cluster of differentiation 36; CHOP, CEBP homologous protein; CVD, cardiovascular disease; DBP, diastolic blood pressure; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl indocarbocyanine percholate; ER, endoplasmic reticulum; FH, familial hypercholesterolemia; GC/MS, gas chromatography mass spectrometry; JNK, c-Jun N-terminal kinase; IU, international units; oxLDL, oxidized low density lipoprotein; PPARα, peroxisome proliferator-activated receptor gamma; p-PERK, phospho-pancreatic ER kinase; RCT, randomized controlled trial; SBP, systolic blood pressure; SEM, standard error of the mean; SR-A1, scavenger receptor class A, type 1; T2DM, type 2 diabetes mellitus; 25(OH)D, 25-hydroxy vitamin D; $1,25(OH)_2D$, 1,25-dihydroxy vitamin D

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plaques [2]. The importance of cholesterol metabolism of circulating monocytes may be underrecognized in this process; monocytes from patients with familial hypercholesterolemia (FH) become cholesterol-laden in circulation, suggesting a possible capacity to carry cholesterol into the vessel wall [3]. Our mouse model of atherosclerosis and diabetes has confirmed this capacity for monocyte cholesterol transport into atherosclerotic plaques [4]. Interestingly, monocytes from type 2 diabetics are known to have higher baseline expression of CD36, which is unchanged by acute hyperglycemia [5,6], suggesting that other environmental conditions may regulate monocyte cholesterol metabolism and represent potential treatments for CVD.

Observational studies demonstrate a consistent association between low 25-hydroxy vitamin D [25(OH)D] levels and both T2DM and CVD. 25(OH)D levels < 30 ng/mL have been found in 81% of diabetics from National Health and Nutrition Examination Survey (NHANES) data [7]. Within patients with T2DM, low 25(OH)D levels nearly double the relative risk of developing CVD compared to individuals with normal vitamin D levels and T2DM [8]. Several systematic reviews and meta-analyses of randomized clinical trials of the effects of vitamin D on cardiovascular outcomes have been negative [9–12], but these trials have not targeted patients with uncomplicated diabetes. Our group has previously demonstrated that vitamin D and its metabolites have significant effects on the atherogenic functional properties of both macrophages and peripheral blood monocytes from patients with diabetes without known CVD. Ex vivo analysis of human monocyte-derived macrophages from subjects with type 2 diabetes show that vitamin D deficiency in culture increases cholesterol uptake, leading to foam cell formation via an endoplasmic reticulum (ER)-stress-dependent mechanism [13]. Furthermore, the increase in cholesterol uptake is reversible in culture with 1,25-dyhydroxy vitamin D_3 [$1,25(OH)_2D_3$] supplementation [14]. Next, to mimic in vivo conditions more closely, we looked at freshly isolated human peripheral blood monocytes from type 2 diabetics, finding that systemic 25(OH)D levels correlate inversely with cellular adhesion and migration capabilities and expression of the surface molecules that facilitate these processes, also in an ER-stress-dependent manner [15]. The increased adhesion and migration is suppressed with 25(OH)D supplementation in culture [16]. To investigate these atherogenic properties in vivo, we generated a mouse model with selective knockout of the vitamin D receptor in myeloid cells and found that these mice have cholesterol-laden monocytes with increased adhesion and migration capabilities enabling them to carry cholesterol into atherosclerotic plaques [4]. The effects of vitamin D on monocyte cholesterol metabolism in mice prompted us to investigate whether vitamin D supplementation could alter this mechanism of atherosclerosis in patients with type 2 diabetes.

2. Material and methods

2.1. Subjects

Subjects were obtained from two settings for longitudinal and cross-sectional assessments.

Studies were approved by the Washington University Human Research Protection Office, and all subjects gave informed consent. All procedures were carried out at Washington University School of Medicine in St. Louis, MO.

For longitudinal studies, monocyte assessment was performed in a subset of patients from our randomized clinical trial of the effects of vitamin D_3 supplementation on blood pressure in subjects with type 2 diabetes, hypertension, and 25(OH)D level < 25 ng/mL. Patients were recruited and enrolled between September 2006 and November 2015. Subjects were screened and included if they were between age 25 and 80 with a diagnosis of type 2 diabetes and hypertension, as well as serum 25(OH)D level < 25 ng/mL. Diabetes diagnosis was confirmed by hemoglobin A1c (A1c) > 6.5% or ongoing hypoglycemic medications for at least 3 months, and subjects had to have A1c 5.5–9.5% and not be on insulin therapy. Due to the American Diabetes Association recommendations for lower blood pressure (BP) targets in diabetic patients [17], hypertension meeting inclusion criteria was defined as a mean systolic blood pressure (SBP) of ≥120 mm Hg and/or mean diastolic blood pressure (DBP) of ≥80 mm Hg as assessed by 24-h ambulatory blood pressure monitoring after stopping antihypertensive medications for at least 2 weeks. Antihypertensives were held for the duration of the study. Subjects were excluded from participation for mean SBP > 160 mm Hg or mean DBP > 100 mm Hg, cardiovascular disease, arrhythmia, congestive heart failure, stage 4 or worse chronic kidney disease, > 2+ proteinuria on urine dipstick, untreated thyroid disorders, calcium disorders, recurrent nephrolithiasis, osteoporosis, oral or intravenous immunomodulatory medications, heavy alcohol consumption (males > 2 drinks per day and females > 1 drink per day), drug use, weight change > 5% in the 3 months prior to screening, extreme diets, unwillingness to stop supplements containing calcium or vitamin D for the study duration, and pregnancy. Subjects were then randomized in a 1:1 ratio to one of two groups: vitamin D_3 4000 international units (IU) or matching placebo daily (supplied by Tishcon Corp.) for 4 months, with treatment allocation blinded to both investigators and participants. Both groups received calcium carbonate 500 mg twice daily. For safety, patients were seen at 2 weeks, 1 month, 2 months, 3 months, and 4 months for assessment of blood pressure, hypercalciuria, and hypercalcemia, as well as any other adverse events. Monocytes were isolated from venous blood draw at baseline and 4 months.

For cross-sectional studies, patients were screened and included if they were between age 25 and 80 with a self-identified diagnosis of type 2 diabetes and varying levels of 25(OH)D. They were excluded if known to have stage 4 or worse chronic kidney disease, cardiovascular disease, arrhythmia, congestive heart failure, or calcium disorders, if on oral or intravenous immunomodulatory medications, or if pregnant. Subjects underwent a single venous blood draw for serum 25(OH)D level and monocyte isolation.

2.2. Clinical laboratory assessment

Serum 25(OH) vitamin D was quantified by liquid chromatography-tandem mass spectrometry (LC–MS/MS) at Mayo Medical Laboratories (Rochester, MN). Lipid levels were quantified by enzymatic colorimetry (with Friedewald calculation for LDL if triglycerides < 400 mg/dL), A1c levels were quantified by turbidimetric inhibition immunoassay, and monocyte counts were quantified by electrical impedance at the Core Lab for Clinical Studies at Washington University School of Medicine (St. Louis, MO).

2.3. Monocyte isolation

Peripheral blood monocytes were isolated as we have previously described from lithium heparin blood collection tubes by standard Ficoll isolation techniques, followed by positive selection with CD14 microbeads (Miltenyi Biotec) [15]. Monocytes were stabilized for 3 h in the subject's plasma to mimic in vivo conditions prior to experimentation.

2.4. Monocyte cholesterol metabolism

Cholesterol uptake was assessed as we have previously described [13]. Briefly, isolated monocytes were incubated 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, then lysed using radioimmunoprecipitation assay (RIPA) buffer. DiI-oxLDL cholesterol uptake was detected using a microplate reader and normalized to cellular protein. Monocyte protein extracts were analyzed by Western blot for CD36 (Santa Cruz) and ER stress protein expression [phospho-pancreatic ER kinase (pPERK, Cell Signaling) and