Cigarette smoke exposure is associated with vitamin D3 deficiencies in patients with chronic rhinosinusitis

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Background: Cigarette smoke (CS) plays a role in the exacerbation of chronic rhinosinusitis (CRS); however, the mechanism for this is unknown. We hypothesize that CS impairs human sinonasal epithelial cell (HSNEC) conversion of 25(OH) D3 (25VD3) to 1,25-dihydroxyvitamin D3 (1,25VD3) and, furthermore, that supplementation with 1,25VD3 will reverse smoke-induced inflammatory responses by HSNECs.

Objective: We sought to determine the effect of CS on vitamin D3 (VD3) levels, conversion, and regulation of CS-induced inflammation in control subjects and patients with CRS.

Methods: Blood and sinus tissue explants were collected at the time of surgery from control subjects, patients with chronic rhinosinusitis without nasal polyps, and patients with chronic sinusitis with nasal polyps (CRSwNP). Expression of VD3 metabolizing enzymes were measured by using RT-PCR. Primary HSNECs were cultured from tissue explants. 25VD3 with and without cigarette smoke extract (CSE) was used to examine conversion of 25VD3 to 1,25VD3, as well as HSNEC production of proinflammatory cytokines.

Results: CS exposure was associated with reduced circulating and sinonasal 25VD3 levels in all groups compared with those seen in CS-naive, disease-matched counterparts. CS exposure decreased expression of *CYP27B1* and was especially pronounced in patients with CRSwNP. CSE impairs control HSNEC conversion of 25VD3. HSNECs from patients with CRSwNP also demonstrate an intrinsic reduction in conversion of 25VD3 to 1,25VD3. Exogenous 1,25VD3 reduces CSE-induced cytokine production by HSNECs.

Conclusions: Exposure to CS is associated with reduced 25VD3 levels and an impaired ability of HSNECs to convert 25VD3 to 1,25VD3. Addition of 1,25VD3 reduces the proinflammatory effects of CS on HSNECs. Impaired VD3 conversion by CS exposure represents a novel mechanism through which CS induces its proinflammatory effects. (J Allergy Clin Immunol 2014;134:342-9.)

Key words: *Vitamin D, secondhand smoke, sinusitis, epithelial cell*

Chronic rhinosinusitis (CRS) is a health condition characterized by diffuse inflammation of the sinonasal mucosa and is composed of a variety of clinical phenotypes. Patients with chronic rhinosinusitis without nasal polyps (CRSsNP) have increased levels of T_H1 and T_H2 mediators, resulting in a mixed neutrophilic and eosinophilic infiltrate.[1-3] Chronic rhinosinusitis with nasal polyps (CRSwNP) is the most difficult form of the disease to treat and displays a T_H2-skewed immune phenotype similar to that of asthmatic patients, including increased mast cell and eosinophil numbers.[4,5] Although the exact cause of the inflammation associated with CRS is unknown, it is thought to be the result of numerous interactions between environmental factors and intrinsic alterations in the host immune system.

Active smoking and environmental tobacco smoke (ETS) exposure have been shown to cause or exacerbate numerous diseases of the upper and lower airways, including CRS.[6-9] Exposure to ETS during childhood and adulthood has been identified as a risk factor for the development of a number of respiratory diseases, including CRS.[10-13] In the upper airway, cigarette smoke (CS) has been shown to induce nasal irritation and congestion, as well as increased nasal obstruction. Lastly, *in vitro* studies have shown that cigarette smoke extract (CSE)–treated human sinonasal epithelial cells (HSNECs) produce a robust inflammatory response demonstrated by a significant increase in proinflammatory cytokine levels.[14,15] Although CS-induced respiratory inflammation is becoming more widely accepted, the mechanism through which CS exacerbates CRS is unclear.

Vitamin D3 (VD3) is a potent steroid hormone involved in the regulation of calcium homeostasis; antibacterial products, such as cathelicidin; and inflammatory cell processes. VD3 regulates a variety of cell types, including epithelial cells, dendritic cells (DCs), monocytes, macrophages, and T cells, and has been shown to promote immune tolerance in a number of contexts.[16-20] For example, it has been demonstrated that 1α,25-dihydroxyvitamin D3 (1,25VD3) diminishes DC stimulation of T_H1/T_H2 differentiation by blocking monocyte to DC differentiation and maturation[21,22] and also recruits regulatory T cells, which produce the anti-inflammatory cytokine IL-10.[23,24]

VD3 synthesis begins in the skin, where pro-VD3 is converted to pre-VD3. After binding to vitamin D binding protein, it is transported to the liver and converted to 25-hydroxycholecalciferol (25VD3).[25] Originally it was thought that conversion of 25VD3 to the active metabolite, 1,25VD3, by 1α-hydroxylase occurred in the kidneys.[26] However, a number

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Abbreviations used

BEGM: Basal epithelial cell growth medium
CRS: Chronic rhinosinusitis
CRSsNP: Chronic rhinosinusitis without nasal polyps
CRSwNP: Chronic rhinosinusitis with nasal polyps
CS: Cigarette smoke
CSE: Cigarette smoke extract
C_t: Cycle threshold
DC: Dendritic cell
ETS: Environmental tobacco smoke
HSNEC: Human sinonasal epithelial cell
VD3: Vitamin D3
1,25VD3: 1α,25-Dihydroxyvitamin D3
25VD3: 25-Hydroxycholecalciferol
VDR: Vitamin D receptor

of reports have shown that other peripheral tissues, including respiratory epithelial cells, contain 1α-hydroxylase[27] and can serve as a source of 1,25VD3.

Increasing evidence suggests that VD3 plays an important role in respiratory health. For instance, higher 25VD3 levels were associated with reduced likelihood for being hospitalized for asthma-related complications and reduced use of anti-inflammatory medications.[28] Serum 25VD3 levels have been shown to be inversely associated with the occurrence of upper respiratory tract infections, and this association was even stronger in those with asthma or chronic obstructive pulmonary disease.[29] In the upper airway we have previously reported that patients with CRSwNP have systemic 25VD3 deficiencies that are independent of age, race, sex, and asthma. Additionally, VD3 deficiency was associated with more severe bone erosion and increased DC infiltrates in patients with CRSwNP.[30,31] However, what is still unidentified is whether intrinsic changes in local VD3 regulation are associated with CRS or whether extrinsic insults, such as CS, alter VD3 regulation. In these studies, we explored the relationship between CS exposure and regulation of VD3 metabolism in patient with CRS. As we report here, CS exposure is associated with reduced circulating and tissue levels of 25VD3 and impaired VD3 conversion of 25VD3 to 1,25VD3. Furthermore, addition of exogenous 1,25VD3 reverses smoke-induced inflammation by HSNECs.

METHODS

Patients

The Institutional Review Board at the Medical University of South Carolina approved these studies, and written informed consent was obtained for all patients. Blood, hair, and sinus tissue were taken at the time of endoscopic sinus surgery. Inclusion criteria include patients with CRSsNP and those with CRSwNP who met the diagnostic criteria outlined by the European Position Paper on Rhinosinusitis and Nasal Polyps 2012.[32] Control subjects were undergoing surgery for cerebrospinal fluid leak repair or to remove non–hormone-secreting pituitary tumors. Disease severity was assessed by using the Lund-Mackay radiologic staging system[33] for patients with available computed tomographic scans. Scores were assigned by 2 blinded graders and then averaged to yield a mean score for each patient.

Exclusion criteria include use of oral steroids or immunomodulatory agents within the preceding 30 days; use of vitamin D supplements other than standard multivitamins; other immunologic (eg, rheumatoid arthritis, immunodeficiency, cystic fibrosis, ciliary dyskinesia, and malabsorption), renal, gastrointestinal, endocrine, or skeletal disorders; and pregnancy.

Smoking status determination

CS-exposed patients included active smokers and those with ETS exposure. Self-reported smoking status was obtained at the time of surgery and recorded in the preoperative medical records. Hair samples were analyzed for nicotine at the Wellington Hospital Laboratory in Newton, New Zealand, by using methods previously described.[34] One centimeter of hair provides a mean exposure over the previous month and is less vulnerable to day-to-day fluctuations in environmental exposures than other measures, such as urinary or serum cotinine. Patients were classified as (1) smoke naive (ie, those without ETS exposure and hair nicotine <2 ng/mg); (2) ETS exposed (ie, those with a hair nicotine level of 2-30 ng/mg); or (3) active smokers (ie, those with hair nicotine levels of >30 ng/mg, those who admitted to actively smoking, or both).[35] Admitted former smokers were excluded.

Tissue processing and HSNEC line establishment

Sinus tissue explants were returned immediately to the laboratory for processing on ice. Samples to be used for RT-PCR and tissue homogenate analysis were snap-frozen and stored in RNAlater at −80°C until use. Tissue lysates were prepared, as described previously.[36] The total protein concentration of each sample was determined by using a bicinchoninic acid assay (Thermo Scientific, Waltham, Mass).

Samples used to generate primary HSNEC lines were processed, as previously described.[37] Briefly, tissue explants were minced with a scalpel and then digested in Pronase (Sigma-Aldrich, St Louis, Mo) for 90 minutes at 37°C. Cell suspensions were then passed over a 70-μm cell strainer and washed in PBS. Cells were plated for 2 hours on standard tissue culture–treated plates to remove contaminating fibroblasts that adhere faster than HSNECs and then cultured with basal epithelial cell growth medium (BEGM). Cells were expanded for 5 to 7 days on collagen IV–coated 75-cm^2 dishes. Subconfluent HSNECs were trypsinized and reseeded on human collagen type IV–coated, 6-well tissue-culture dishes. Cell purity was confirmed by means of flow cytometry with positive immunostaining for the epithelial cell marker MUC1 (>95%) and negative immunostaining (<2%) for the fibroblast marker FSP. All cells were used at passage 2.

Blood processing

Peripheral blood was collected at the time of intravenous line placement and processed, as previously described.[30] Briefly, blood was collected in vacutainers containing K2EDTA. Blood was centrifuged at 500*g*, and then plasma was collected and stored at −80°C.

RNA isolation and RT-PCR analysis

An RNeasy Plus Kit (Qiagen, Hilden, Germany) was used to isolate RNA and eliminate genomic DNA. All tissue was processed according to the manufacturer's instructions. RT-PCR analysis was conducted by using the RT^2 Profiler Array System (SABiosciences, Valencia, Calif) for the following genes: *GC* (VD3 binding protein), *CYP2R1* (vitamin D 25-hydroxylase), vitamin D receptor *(VDR)*, and *CYP27B1* (1-α hydroxylase). Glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* was used for data normalization. Execution of the array was conducted with a Mastercycler ep realplex PCR system (Eppendorf AG, Hauppauge, NY). For RT-PCR analysis, Δ cycle threshold (ΔC_t) values from 5 control subjects were averaged together and then used to calculate $\Delta\Delta C_t$ values for individual noncontrol patients. Fold change was calculated from $2^{(-\Delta\Delta Ct)}$. Fold change values of less than 1 were then calculated by using the negative inverse and reported as fold downregulation.

Measurement of VD3 levels

Plasma and tissue 25VD3 and 1,25VD3 levels were measured by using an enzyme immunoassay (Immunodiagnostic Systems, Fountain Hills, Ariz), according to the manufacturer's instructions. 25VD3 insufficiency was defined as less than 32 ng/mL.[38-40] Assay detection limits were 2.4 to 200 pg/mL and 5 to 152 ng/mL for 1,25VD3 and 25VD3, respectively.

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