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Effects of Retinoids on Augmentation of Club Cell Secretory Protein

To the Editor:

Club cell secretory protein (CC16; encoding gene, *SCGB1A1*) is a homodimeric pneumoprotein that is produced mainly by club cells and other nonciliated epithelial cells in both proximal and distal airways (1). Higher airway expression and circulating levels of CC16 have been associated cross-sectionally with better lung function and lower prevalence and severity of chronic obstructive pulmonary disease (COPD) (2). Prospective studies have also shown that increased serum levels of CC16 at baseline are protective against subsequent development of COPD and accelerated FEV_1 decline (3). In explaining these protective effects, growing evidence supports antiinflammatory and antioxidative properties of CC16 in the lungs, although results from animal models have been to some extent inconsistent. In line with direct protective effects, recombinant human CC16 has been shown to inhibit the cigarette smoke extract–induced release of IL-8 from bronchial epithelial cells isolated from patients with COPD (4). Thus, CC16 augmentation may be beneficial in the prevention and treatment of COPD.

Retinoic acid (RA)—an active metabolite of vitamin A—is known to play a key role in early lung development (5). These effects are largely dose specific (6). Studies on RA-induced alveolization in mice also indicate the importance of retinoic acid receptor (RAR) subtypes in mediating these effects of vitamin A, with decreased alveolar number selectively shown by RARγ null animals (7). In humans, despite conflicting results from reports that used estimates of vitamin A intake, epidemiological and clinical studies that actually measured circulating levels of vitamin A found consistently lower circulating levels of retinol and carotenoids in patients with COPD as compared with control subjects. In line with this scenario, baseline serum concentrations of β-carotene and retinol were inversely associated with respiratory symptoms in smokers from the ATBC (Alpha-Tocopherol, Beta-Carotene Cancer Prevention) Study (8), and vitamin A serum levels correlated significantly with FEV_1 values among NHANES (National Health and Nutrition Examination Survey) III participants, particularly smokers (9). Of note, mice that were fed a purified diet containing reduced levels of vitamin A and were exposed to cigarette smoke for 3 months had increased susceptibility to lung emphysema, suggesting a potential causal link between vitamin A deficiency and smoking-related COPD (10).

Because of the effects of vitamin A on lung epithelial development, differentiation, and homeostasis, it is plausible that CC16—a major airway epithelial marker—may mediate some of the above associations. Yet, whether vitamin A affects CC16 production remains unknown. Here, we report that *in vivo* circulating levels of CC16 are up-regulated by vitamin A treatment and that *in vitro* CC16 expression in airway epithelial cells is increased by all-*trans*-retinoic acid (*t*-RA) acting mainly via RARα and RARγ.

In Vivo Studies

CC16 levels were measured with a commercially available ELISA kit (BioVendor, Asheville, NC) in serum samples collected from 71 subjects who participated in a placebo-controlled vitamin A trial for severely sun-damaged skin at baseline and at 3, 6, and 12 months of treatment (11). All participants had sun-damaged skin on their forearms, but they were otherwise required to be in general good health. Subjects were randomized to receive placebo or vitamin A (retinyl palmitate) at 25,000, 50,000, or 75,000 IU/d for 12 months. Overall, 65% of participants were male and 44% were never-smokers. Their mean (SD) age was 62 (7) years. Figure 1 shows the geometric means of circulating CC16 across the four treatment groups at baseline and at 3, 6, and 12 months of treatment. We found significant vitamin A treatment effects on serum CC16 as early as 3 months after initiation of treatment. By the completion of the trial all treatment groups had higher CC16 levels than the placebo group, with the 50,000-IU/d treatment having the highest levels. In addition, circulating CC16 levels increased significantly between baseline and the completion of the trial in both the 50,000- and 75,000-IU/d groups ($P = 0.002$ and 0.005, respectively). Similar trends for effects of vitamin A on CC16 levels were found in analyses stratified by smoking (data not shown), with CC16 levels at the end of the trial being significantly higher in the 50,000-IU/d group compared with the placebo group, both among the 31 never-smokers ($P = 0.02$) and among the 40 ever-smokers ($P = 0.02$). Thus, in these studies vitamin A treatment increased significantly the circulating levels of CC16 *in vivo*.

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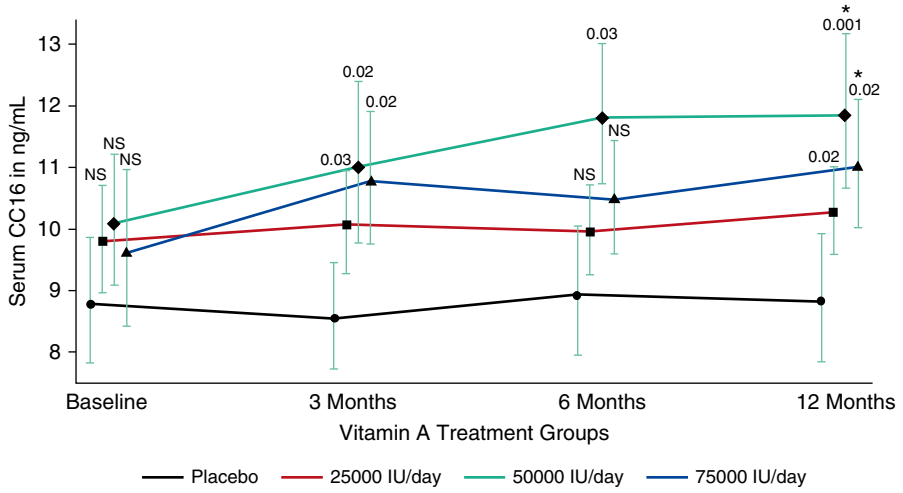

Figure 1. Serum club cell secretory protein (CC16) levels at baseline, during, and after treatment in 71 subjects who participated in a placebo-controlled vitamin A trial for severely sun-damaged skin (11). CC16 levels (geometric mean ± SE) are shown for the placebo and the three treatment groups (vitamin A [retinyl palmitate] at 25,000, 50,000, or 75,000 IU/d) at baseline and at 3, 6, and 12 months of treatment. Number of subjects: Placebo (n = 20), 25,000 IU/d (n = 18), 50,000 IU/d (n = 15), and 75,000 IU/d (n = 18). *P* values (shown above *error bars*) refer to comparison with placebo group at each time point. Data were analyzed using random coefficients models to take into account intrasubject serial correlation. All analyses were adjusted for sex, age, and smoking status. *CC16 levels at 12 months significantly higher than baseline CC16 levels for treatment groups receiving 50,000 and 75,000 IU/d. NS = not significant.

In Vitro Studies

Because lung epithelium is the major contributor of serum CC16, we tested whether the effects of vitamin A on CC16 augmentation can be replicated *in vitro* in primary epithelial cell cultures. Because CC16 is expressed by epithelial cells from the bronchi to the bronchioles (1) and because of the technical barrier to isolate bronchiolar cells, we tested only bronchial epithelial cells in this study. Briefly, we cultured primary human bronchial epithelial cells from nine individuals with no lung disease (normal) and four patients with COPD (Global Initiative for Chronic Obstructive Lung Disease stage IV, receiving lung transplant). These cells were treated with various doses of *t*-RA, a stable metabolite that mediates physiological functions of vitamin A. As shown in Figure 2, we found that *t*-RA increased CC16 secretion in both normal and COPD cells by 72 hours after treatment with various doses, according to a bell-shaped curve. In addition, because *t*-RA binds with equal affinity to each retinoic

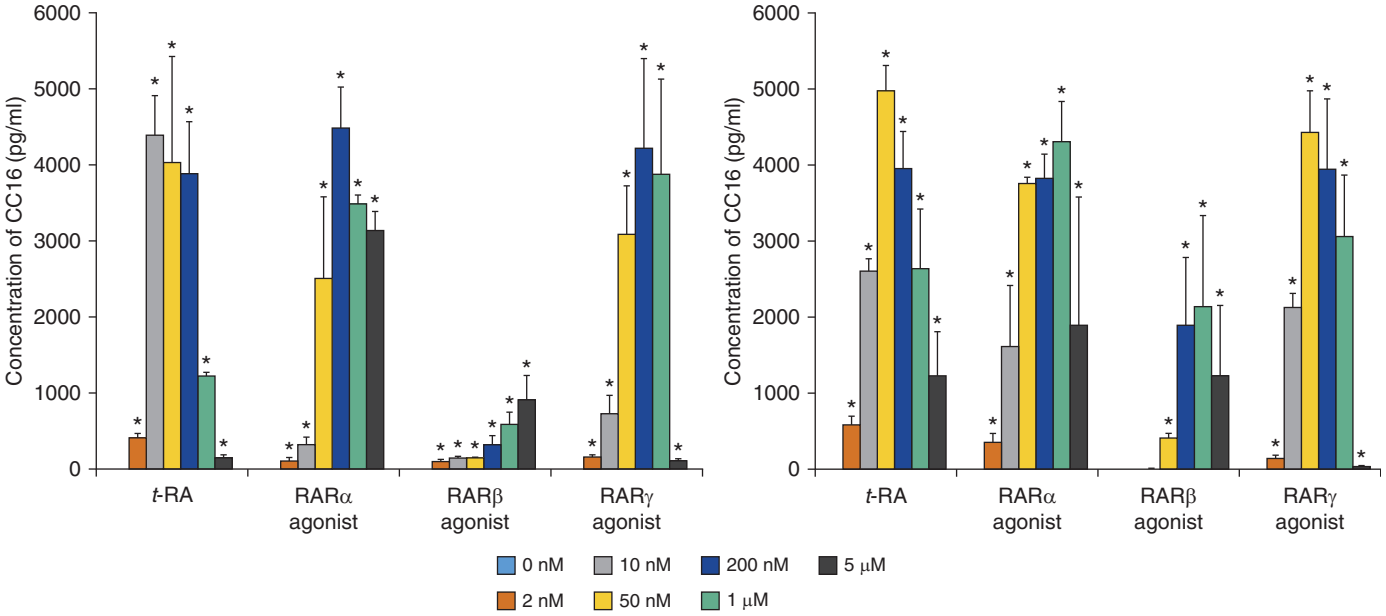

Figure 2. Dose-dependent club cell secretory protein (CC16) production in primary human bronchial epithelial cell cultures from normal individuals with no respiratory diseases (*left*) and from patients with COPD (GOLD stage IV; *right*) under immersed conditions in response to various doses of *t*-RA or RAR-specific agonist treatment. RAR-specific agonists included AM580 for RARα, CD2314 for RARβ, and CD1530 for RARγ. Secreted CC16 was measured in the culture medium. Values represent means ± SEM 72 hours after treatment. Number of subjects: Normal (n = 9), patients with COPD (n = 4). *$P < 0.01$ compared with untreated. COPD = chronic obstructive pulmonary disease; GOLD = Global Initiative for Chronic Obstructive Lung Disease; RAR = retinoic acid receptor; *t*-RA = all-*trans*-retinoic acid.

acid receptor subtype (RARα, RARβ, and RARγ) (12), we tested whether any of the three subtypes was preferentially involved in the regulation of CC16. As also shown in Figure 2, by using agonists that are specific to RAR subtypes we found that both RARα-specific (AM580) and RARγ-specific (CD1530) agonists induced CC16 expression in a dose-dependent manner with comparable potency as *t*-RA, and that these responses were largely similar between normal and COPD cells. In contrast, the RARβ-specific agonist CD2314 induced CC16 expression with much less potency, although COPD cells tended to have stronger RARβ-specific responses than normal cells.

In summary, our studies indicate that retinoids increase CC16 secretion in human bronchial epithelial cells from both normal individuals and patients with COPD; that these effects are mediated mainly through RARα and RARγ; and that, *in vivo*, vitamin A treatment results in a significant increase in circulating CC16 levels in individuals with no COPD. Whether these results simply support the importance of vitamin A dietary intake or may also have pharmacological implications in the early and preclinical stages of COPD remains to be determined. In this context, two considerations are noteworthy. First, previous large randomized cancer prevention trials (13, 14) have reported an increased risk for lung cancer among participants receiving β-carotene supplementation. Thus, any intervention aimed at using carotenoids to increase CC16 production would first need to address and minimize the potential effects of these agents on lung cancer risk, particularly among smokers. Second, two previous clinical trials (15, 16) that tested oral γ-selective retinoid agonists in patients with moderate to severe COPD did not find significant effects in the advanced stages of disease. The rationale for these trials was largely based on the postulated effects of retinoids on alveologenesis. However, if the main mechanism of action of retinoids is related to up-regulation of CC16 production, their effects would be expected to be strongest at the early and preclinical stages of COPD, when the irreversible airway remodeling and parenchymal destruction have not been established and a sufficient number of CC16-producing cells are still present in the airways of patients. Further studies are warranted to evaluate these scenarios. ■

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Exosomal MicroRNA for Detection of Cardiac Sarcoidosis

To the Editor:

Sarcoidosis is a multisystem, granulomatous disease of unknown cause that most commonly affects adults 25–50 years of age, causing significant morbidity and mortality. Studies indicate that sarcoidosis-related mortality is on the rise, perhaps relating to improved disease detection (1). Cardiac sarcoidosis (CS) is the second leading cause of sarcoidosis-related death, and young adults are particularly at risk (2). A contributing factor is failure to detect CS during routine clinical screening, including patient history, physical examination, and electrocardiography (1). As a result, many cases are detected for the first time after a cardiovascular complication, ranging from frequent premature ventricular contractions and atrial arrhythmias to sudden cardiac death (1). Whereas high-resolution cardiac imaging techniques, such as cardiac magnetic resonance or fludeoxyglucose F 18–positron emission tomography/computed tomography ([^{18}F]FDG-PET/CT), greatly improve CS detection (1), these modalities are expensive and present certain patient risks (e.g., radiation exposure with [^{18}F]FDG-PET/CT [3]), making them impractical for routine screening for CS. To our knowledge, no circulating biomarker has been shown to reliably detect CS.

Noncoding RNAs are readily detected in human blood and are reported to serve as biomarkers of diseases including acute myocardial damage (4). A fraction of the microRNAs (miRNAs) in blood are encapsulated within lipid bilayer vesicles, referred to as *exosomes*, where they are shielded from enzymatic degradation (5). Exosomes originate from multivesicle bodies within cells containing proteins, nucleic acids, and lipids, and as such, exosomal molecular content reflects its cellular origin (6). Thus, we hypothesized that exosome-derived miRNA could serve as an informative source of biomarkers for cardiac sarcoidosis.

With institutional review board approval, we conducted a retrospective study of 21 subjects with histologically proven sarcoidosis who had clinical and radiographic evidence of CS, based on established criteria (7, 8), compared with 21 subjects with sarcoidosis with no evidence of CS (non-CS), and 11 healthy human volunteers. We randomly divided the plasma samples obtained into two groups: discovery (10 CS, 10 non-CS, and 5 control) and validation (11 CS, 11 non-CS, and 6 control) cohorts. The demographic and clinical characteristics are presented in Table 1, with no significant differences found between the groups in any of the demographic characteristics. The plasma samples were collected within the scope of the National Institutes

Table 1. Patient Demographics and Clinical Manifestations

Group	Age (*yr*) (*Mean ± SE*)	Race (*W/B/O*)	Sex (*M/F*)	Smoking Status (*N/C/F*)	MRI*	PET†	Heart Block‡	V-tach§	HF‖
First cohort (NGS analysis)									
Control (n = 5)	50.0 ± 3.4	4/1/0	2/3	3/0/2					
Cardiac sarcoidosis (n = 10)	51.8 ± 3.1	9/0/1	5/5	8/0/2	50%	30%	60%	50%	40%
Noncardiac sarcoidosis (n = 10)	52.9 ± 3.8	8/1/1	2/8	5/0/5					
Second cohort (qRT-PCR analysis)									
Control (n = 6)	44.0 ± 5.2	5/1/0	3/3	3/0/3					
Cardiac sarcoidosis (n = 11)	54.1 ± 4.1	4/7/0	4/7	10/0/1	27%	55%	64%	27%	36%
Noncardiac sarcoidosis (n = 11)	51.2 ± 3.7	8/3/0	5/6	8/0/3					

Definition of abbreviations: HF = heart failure; M/F = male/female; MRI = magnetic resonance imaging; N/C/F = never/current/former; NGS = next-generation sequencing; PET = positron emission tomography; qRT-PCR = real-time quantitative reverse transcription–polymerase chain reaction; V-tach = ventricular tachycardia; W/B/O = white/black/other.
*Late gadolinium enhancement on cardiac MRI.
†Patchy left ventricular and/or septal uptake by PET with fludeoxyglucose F 18.
‡Second- or third-degree heart block or bifascicular block.
§Nonsustained or sustained spontaneous or inducible V-tach.
‖Evidence of systolic (left ventricular ejection fraction < 50%) or diastolic left ventricular dysfunction by echocardiogram or MRI.

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