

# Vitamin A Deficiency Induces Fluid Hyposecretion from the Airway Submucosal Glands of Mice<sup>1–3</sup>

Sang Cheol Kim,<sup>4</sup> Hyun Jae Lee,<sup>7</sup> Jung-Hee Joo,<sup>5–7</sup> Joo-Heon Yoon,<sup>4–7</sup> and Jae Young Choi<sup>4,6,7\*</sup>

<sup>4</sup>Department of Otorhinolaryngology, <sup>5</sup>Research Center for Natural Human Defense System, <sup>6</sup>The Airway Mucus Institute, and <sup>7</sup>BK 21 Project for Medical Science, Yonsei University College of Medicine, Seoul, South Korea

## Abstract

Vitamin A deficiency (VAD) alters the phenotype of airway epithelium and attenuates the epithelial defense system, and many studies have reported the association of VAD with respiratory disease. In this study, we investigated changes in submucosal glands (SMG) in a mouse model of VAD. C57BL/6 mice were fed a vitamin A-devoid diet and the others were fed a control diet (1.2 mg retinol/kg). The areas of serous and mucous cells of SMG were measured in 4-, 8-, and 20-wk-old male mice. The volume and lysozyme concentration of glandular secretions were also measured. The 2 groups did not differ in body weight or general morbidity at 3–10 wk of age, although serum retinol concentrations were greater in the control mice than in the VAD mice after 4 wk. Upon histological evaluation, we found that the areal ratio of serous cells: total SMG cells was significantly lower after 8 wk in the VAD mice compared with the control mice, although the total area of SMG did not differ between groups throughout the 20-wk experiment. The number of secretory bubbles did not differ between the groups, but total secretion volume was reduced by 35% in 8-wk-old VAD mice compared with controls. Furthermore, the concentration of lysozyme in secretions from 8-wk-old VAD mice was also less than in controls, compounding the effect of diminished secretion volume. In this study, we found serous cell hypotrophy/hypoplasia and dysfunction in VAD mice, which may contribute to the susceptibility to airway infection linked to VAD. J. Nutr. 142: 739–743, 2012.

## Introduction

Retinoic acid, an active metabolite of vitamin A, regulates the development, maintenance, and differentiation of epithelial cells (1). Furthermore, vitamin A modulates immunity in the skin (2) and the gastrointestinal tract. VAD<sup>8</sup> alters innate immunity and the bacterial population in the gastrointestinal tract (3) and can be a risk factor for diarrheal disease (4). VAD may also be involved in airway diseases. Senaidy (5) reported that serum vitamin A concentrations are lower among children with asthma. Several studies have supported the hypothesis that airway infection is associated with lower serum retinol (vitamin A) and carotenoid levels (6,7). The association of VAD with respiratory infectious diseases such as measles and tuberculosis has also been reported (8,9).

Airway SMG supply ~95% of upper airway mucus (10), which is composed of ~60% serous and 40% mucous cells by volume. Both of the cell types secrete mucus. Serous cells are the major source of the fluid and antimicrobial peptides and mucous cells provide most of gel-forming mucin components (11,12). In the absence of SMG, the airway becomes susceptible to infection (13). Furthermore, the dysfunction of airway glands is thought to be one of the causes of airway infection in patients with cystic fibrosis (14).

VAD is strongly associated with respiratory infection and SMG are essential for maintaining the airway free from pathogens. However, there are no published studies on the relationship between VAD and SMG, although there are reports that VAD decreases mucin production from cultured airway epithelial cells and causes squamous metaplasia (15–17). Therefore, we conducted this study to investigate the effects of VAD on SMG in a mouse model of VAD. Our data indicate that VAD induces serous cell dysfunction and increases the severity of airway inflammation caused by *Pseudomonas aeruginosa* infection.

## Materials and Methods

**VAD mice.** C57BL/6 mice purchased from Orient Bio were bred and handled according to protocols approved by the Institutional Animal

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<sup>3</sup> Supplemental Figures 1–3 are available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at [jn.nutrition.org](http://jn.nutrition.org).

<sup>8</sup> Abbreviations used: EGFR, epidermal growth factor receptor; SMG, submucosal gland; VAD, vitamin A deficiency.

\* To whom correspondence should be addressed. E-mail: [jychoi@yuhs.ac](mailto:jychoi@yuhs.ac).

Ethics Committee of Yonsei University. Pregnant female mice at ~10 d of gestation were arbitrarily distributed into 2 groups. One group was fed a vitamin A-devoid diet containing 0 mg retinol/kg (ST2P, AIN-93M without vitamin A, Purina Mills) and the other group was fed a control diet containing 1.2 mg of retinol in the form of retinyl palmitate/kg (AIN-93M, Feedlab). Both groups consumed food ad libitum. Pups were weaned at 3 wk of age and maintained on the same diet as their dams. Male mice were used for all experiments.

We checked the body weight and general morbidity of the mice every week by the age of 10 wk and measured serum retinol concentrations at the ages of 4, 8, and 20 wk. Mice were anesthetized at the ages of 4, 8, and 20 wk by i.p. injection of 30 mg/kg Zoletil (Virbac S) and 10 mg/kg Rompun (Bayer Healthcare Korea). Whole blood was obtained from the right ventricle of the heart, which was exposed after thoracotomy. Serum retinol concentrations were analyzed by HPLC (HP1100 series, Hewlett Packard).

**Histological evaluation of SMG.** Tracheae of 4-, 8-, and 20-wk-old mice were dissected and fixed in 10% phosphate-buffered formalin for 24 h. After paraffin embedding, 4- $\mu$ m horizontal sections were cut at the level of the cricoid cartilage and stained with hematoxylin and eosin. The images of the sections were obtained at 400 $\times$  magnification. We calculated the area of the serous and mucous cells by manually tracing out the area on the images using NIH Image J software (Scion).

**Optical measurements of secretion volume.** Tracheae were extracted from 8-wk-old mice, opened along the ventral midline, and fixed with the mucosal side up using cactus spines on a Sylgard-lined plastic petri dish (Dow Corning). The serosal side was bathed in ~60  $\mu$ L Krebs-Ringer solution and the other side was exposed to the air. Water-saturated mineral oil was placed on the mucosal surface and the petri dish was placed in a chamber maintained at 37°C with high humidity using a thermistor-controlled warming chamber and humidifier (Medical Systems). Droplets of mucus within the oil layer were visualized by illumination and images were taken with the macro mode of a digital camera (Moticam 2300, Motic) at 30-s intervals (Supplemental Fig. 1). After 10 min, the bath solution was replaced with Krebs-Ringer solution containing 10  $\mu$ M/L carbachol (Sigma-Aldrich), which is a cholinergic agonist, and recording of digital images continued. The stored images were analyzed by NIH Image J software and the volumes of secreted droplets between the vocal cords and the lower margin of the first tracheal ring were calculated. We defined the total volume of all bubbles secreted for 10 min under the bath of only Krebs-Ringer solution as basal secretion. We then compared the volume obtained after subtracting the basal secretion from the total volume of all the bubbles measured 1 min after treatment with carbachol.

**Measurement of lysozyme concentration.** Tracheae were carefully dissected and extracted from 8-wk-old mice. The tracheae were opened along the dorsal midline and submerged in Krebs-Ringer solution containing 100  $\mu$ M/L carbachol for 30 min. The concentration of lysozyme in the supernatant was measured by ELISA (Uscn Life Science) according to the manufacturer's instructions.

**Western blotting for total EGFR (epidermal growth factor receptor) and phosphorylated EGFR** We extracted trachea from VAD and control mice at the age of 8 wk and chopped and homogenized them in lysis buffer containing 1 mmol/L PMSE, Halt protease/phosphatase inhibitor (Thermo Fisher Scientific), and 0.5% SDS. After insoluble materials were removed via 10 min of centrifugation at 15,700  $\times$  g, the homogenates containing equal amounts of proteins were electrophoretically separated in 8% polyacrylamide gels and transferred to a polyvinylidene fluoride membrane for 1 h. The blots were incubated overnight at 4°C with primary antibody, rabbit monoclonal anti-EGFR antibody (EP38Y, Abcam) for detecting total EGFR, or rabbit monoclonal anti-phosphorylated EGFR antibody (Tyr1068, Cell Signaling Technology) for activated EGFR. After washing, the blots were further incubated for 1 h at room temperature with secondary anti-rabbit antibody (Jackson ImmunoResearch Laboratories) and visualized by enhanced chemiluminescence (Thermo Fisher Scientific). Each blot was also probed for  $\alpha$ -tubulin to confirm equal loading.

**Pseudomonas infection.** *P. aeruginosa* PA01, generously provided by S.S. Yoon at Yonsei University, was grown overnight in Luria-Bertani broth at 37°C. The bacterial concentration was determined by counting colonies after serial dilution. The tracheae of 8-wk-old mice were exposed via vertical neck incisions under anesthesia. The bacteria were suspended in 50  $\mu$ L PBS and administered intratracheally ( $1 \times 10^6$  CFU/mouse). After 24 h, the mice were killed and the lungs were extracted through thoracotomy. The left upper lobes were harvested, fixed overnight with 10% formalin, embedded in paraffin, cut in 4- $\mu$ m-thick sections, and stained with hematoxylin and eosin to evaluate the severity of inflammation. The right upper lobes from mice were homogenized in Trizol (Invitrogen) and stored at -70°C.

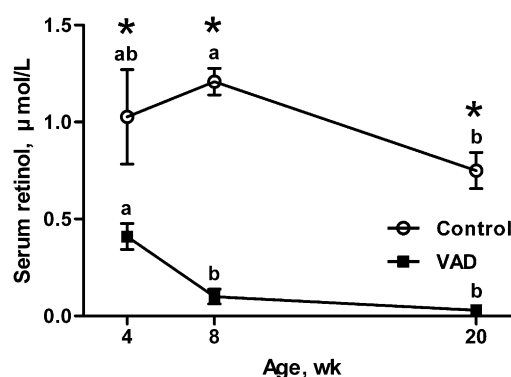
**RT-PCR.** Total RNA was isolated from right upper lobes and reverse transcribed with oligo primers. PCR was performed with sequence-specific primers for macrophage inflammatory protein-2 $\alpha$  (MIP2 $\alpha$ ), which is analogous to IL-8 in humans, and  $\beta$ -actin. Amplified PCR products were analyzed on a 1% agarose gel containing ethidium bromide and visualized by UV illumination.

**Statistical analysis.** All data were presented as mean  $\pm$  SEM. The number of bubbles and the volume of secretions were analyzed with a Student's *t* test. Body weights were compared between the age and diet groups by 2-way ANOVA and post hoc Bonferroni tests. The serum retinol concentrations and SMG areas were logarithmically transformed to improve normality and to compensate for unequal variance and were analyzed by 2-way ANOVA followed by post hoc Bonferroni tests. Statistical analyses were performed using the SAS software (version 9.2, SAS Institute). Differences were considered significant when *P* < 0.05.

## Results

**Body weight and general morbidity of VAD mice.** There were no significant differences in the growth pattern of the two groups through the age of 10 wk (Supplemental Fig. 2). In contrast, the serum retinol concentration of VAD mice was  $0.41 \pm 0.07$   $\mu$ M/L at 4 wk,  $0.10 \pm 0.04$   $\mu$ M/L at 8 wk, and almost below the detection limit (0.03  $\mu$ M/L) at 20 wk, whereas that of control mice was maintained in a relatively stable manner, ranging from 0.75 to 1.21  $\mu$ M/L throughout the 20 wk (Fig. 1). VAD mice showed no general morbidity at 8 wk; however, on gross examination, the livers were yellowish and slightly enlarged in two of the 20-wk-old VAD mice.

**Histological evaluation of SMG.** The total areas of SMG at the cricoid level of VAD mice did not significantly differ from



**FIGURE 1** Serum retinol concentrations of control and VAD mice at 4, 8, and 20 wk of age. Values are means  $\pm$  SEM, *n* = 4 except for wk 8, *n* = 10. Within a diet group, means without a common letter differ, *P* < 0.05. \*Different from controls at that age, *P* < 0.01. VAD, vitamin A deficiency.

those of control mice through the age of 20 wk (Supplemental Fig. 3). However, the areal ratio of serous cells:total SMG in VAD mice was significantly lower than in control mice at 8 and 20 wk (Fig 2C). The decrease in serous cells of VAD mice was apparent upon histological evaluation (Fig. 2A, B).

**Expression of EGFR.** We performed Western blotting of EGFR, because of activation of EGFR signaling, which is a key regulator of the airway SMG phenotype (18–20). The expression of total EGFR in VAD mice was not different from control mice, but the expression of its activated form, phosphorylated EGFR, was higher in VAD mice compared with control mice (Fig. 2D).

**Secretion of SMG.** We next examined whether there were changes in SMG secretion in 8-wk-old VAD mice that had started to show histological changes in SMG without an abnormal growth pattern or general morbidity. The total number of secretory bubbles 1 min after treatment with 100  $\mu$ mol/L carbachol was not significantly different between control mice and VAD mice (Fig. 3C). However, the total secreted volume in VAD mice ( $37.5 \pm 5.4$  nL) tended to be less than in control mice ( $57.4 \pm 8.7$  nL;  $P = 0.07$ ) (Fig. 3D).

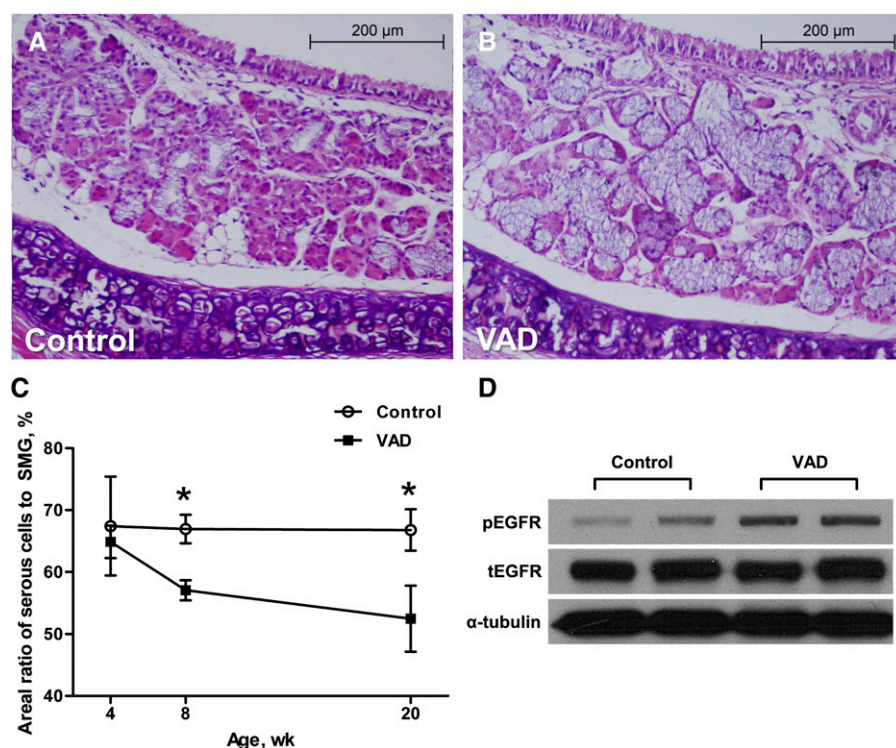
**Lysozyme concentration in airway secretions.** The concentration of lysozyme in secretions of VAD mice ( $3.0 \pm 0.6$   $\mu$ g/L) was 63% less than in control mice ( $8.2 \pm 0.9$   $\mu$ g/L;  $P < 0.01$ ), which is a much greater difference than the 22% difference in the serous cell area.

**Inflammatory response to *Pseudomonas* infection.** We intratracheally inoculated the airways of the male mice with *P. aeruginosa* to investigate whether the observed functional changes of the serous cells weakened the airway defense system. Upon histological examination, we found much more severe alveolar destruction and infiltration of inflammatory cells into

the lung parenchyma in VAD mice (Fig. 4B) than in control mice (Fig. 4A). We also measured the level of MIP2 $\alpha$ , a proinflammatory cytokine, by semiquantitative RT-PCR. We found that MIP2 $\alpha$  expression was very weak in the uninfected airways of both control and VAD mice. After infection, MIP2 $\alpha$  expression increased slightly in control mice but increased far more substantially in VAD mice (Fig. 4C).

## Discussion

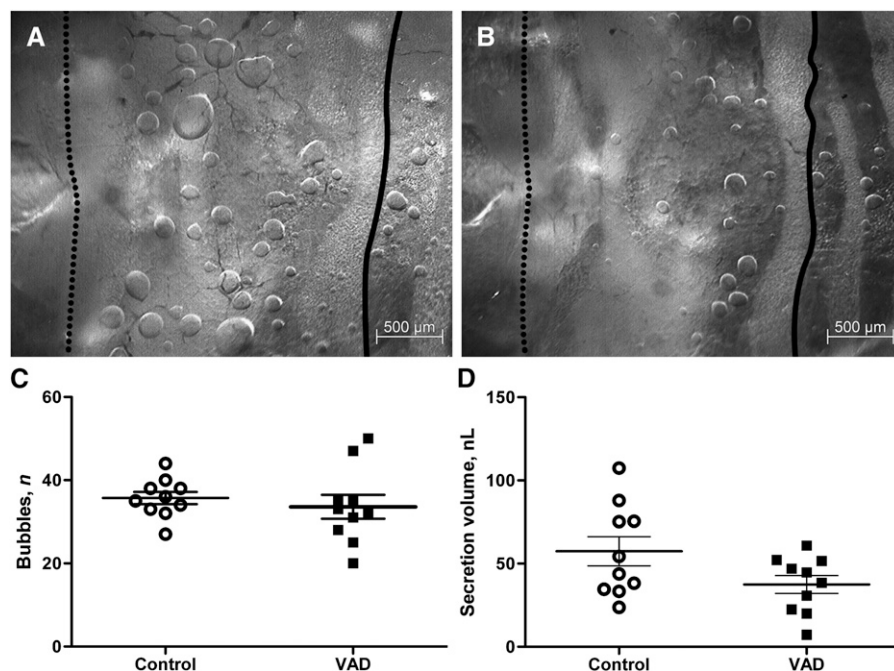
Vitamin A depletion affects exocrine glands in various ways, depending on the tissue or species. Parotid glands are modestly enlarged and their secretion increased in mildly vitamin A-deficient rats but become atrophic during profound VAD (21). Lacrimal glands are smaller and secretory granules are essentially absent under VAD conditions (22). However, there are no reports on the biological effects of VAD on airway SMG, the main source of airway mucus and antimicrobial peptides. In our study, although the number of secreted bubbles and the total area of SMG in VAD mice were not significantly different from those in control mice, the area of serous cells in SMG of VAD mice was ~22% less than that of control mice, which mirrored the decrease in the fluid secretion rate from SMG in VAD mice. Therefore, we speculate that changes of serous cells brought about the decrease in fluid secretion in VAD mice. The mucous metaplasia of airway glands in VAD mice is consistent with the results from salivary glands of VAD rats; Horn et al. (21) reported that VAD resulted in marked atrophy only in serous salivary glands of rats. The underlying mechanism of the SMG changes is not clear. One possible mechanism is the EGFR pathway. VAD induces oxidative stress (23) and production of reactive oxygen species, which can stimulate mucous metaplasia of SMG by the activation of EGFR (24–27). We showed that the expression of phosphorylated EGFR was higher in VAD mice compared with control mice. Therefore, we postulate that the



**FIGURE 2** Hematoxylin and eosin-stained tracheae of 8-wk-old male control (A) and VAD mice (B), the areal ratio of serous cells:total SMG (C), and Western blots of tEGFR and pEGFR from tracheae (D). Values are means  $\pm$  SEM,  $n = 3$  except for wk 8,  $n = 7$ . \*Different from control at that age,  $P < 0.05$ . In D,  $\alpha$ -tubulin was the internal control for protein loading. pEGFR, phosphorylated epidermal growth factor receptor; SMG, submucosal gland; tEGFR, total epidermal growth factor receptor; VAD, vitamin A deficiency.



**FIGURE 3** SMG secretions of the tracheae 1 min after treatment with 100  $\mu\text{mol/L}$  carbachol in 8-wk-old male control (A) and VAD mice (B), the number of bubbles released (C), and the total volume secreted between the vocal cords (dotted lines in A and B) and the lower margins of the first tracheal rings (solid lines in A and B) (D). Values are means  $\pm$  SEM,  $n = 10$ . SMG, submucosal gland; VAD, vitamin A deficiency.



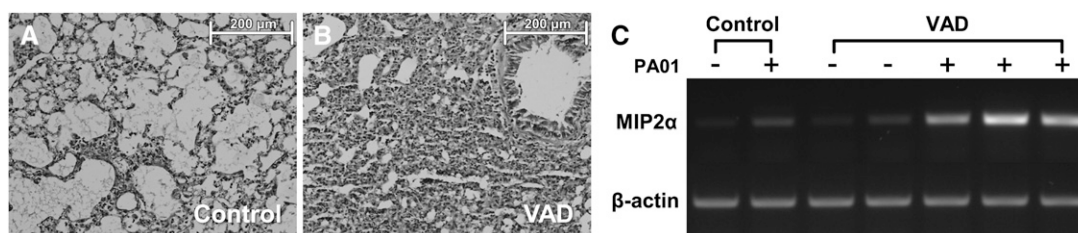
higher activation of EGFR is a key reason for the SMG changes in VAD mice. We are now studying the exact signaling pathway associated with SMG changes.

SMG are the main source of antimicrobial peptides, including lysozyme, lactoferrin, and secretory leukocyte protease inhibitor in the airway. In addition to gross morphological changes to exocrine glands, VAD is linked to alteration in secretion of several protein products. For example, VAD leads to a severe functional disturbance of intestinal epithelial enzymes in rats (28). In addition, the level of secretory IgA in VAD children was significantly lower than that in vitamin A-sufficient children (29). However, the effect of VAD on antimicrobial peptide secretion has not been investigated. Our data show that VAD induces glandular hyposecretion. More interestingly, the concentration of lysozyme in VAD mice was reduced by 63.4% compared with control mice, which was a much greater reduction than expected based on the morphological changes in serous cells. This suggests that VAD could impede the function of serous cells themselves in addition to inducing morphologic changes within the SMG. However, at this time, the precise molecular mechanism has not been elucidated and further studies regarding the role of retinoic acid in lysozyme synthesis are needed.

The incidence and severity of airway infection is higher under the conditions of VAD (30). Because long-term VAD causes

problems in adaptive immunity and intestinal malabsorption, which can heighten susceptibility to bacterial infection (3), we studied tracheal secretions, EGFR expression, and *Pseudomonas* infection in 8-wk-old mice. VAD mice at this age had lower serum retinol concentrations but were of normal body weight and without general morbidity. We found that airway inflammation was more severe in mice with VAD as early as 8 wk compared with controls. Therefore, SMG dysfunction in VAD could also be a possible mechanism of airway morbidity. SMG play a key role in protecting airways and impairment of glandular secretion can lead to an abnormal airway defense system, which may result in bacterial infection (14). In future studies, we are planning to investigate mortality due to airway infection and the bactericidal effects of the secretions from SMG in VAD mice.

VAD is still common in developing countries; it is estimated that there are >100 million preschool children with VAD throughout the world (31). The prevalence of subclinical VAD extends even further and is observed even in developed countries (32). In addition, patients with cystic fibrosis have lower serum retinol concentrations due to problems absorbing the fat-soluble vitamin. Our data demonstrate that VAD induces the mucous metaplasia of SMG and decreases the secretion of antimicrobial peptides, which may underlie the elevated incidence and severity of airway infection under conditions of VAD.



**FIGURE 4** Hematoxylin and eosin-stained sections of *Pseudomonas*-infected lung tissue in 8-wk-old male control (A) and VAD mice (B), and mRNA expression of MIP2 $\alpha$  in the presence and absence of *P. aeruginosa* PA01 infection (C). In C,  $\beta$ -actin was used as a control. VAD, vitamin A deficiency.

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S.C.K., J-H.J., and J-H.Y. designed the experiments; S.C.K. and H.J.L. conducted the experiments and analyzed the data; S.C.K. wrote the first draft; J.Y.C. revised the paper; and J.Y.C. had primary responsibility for the final content. All authors read and approved the final manuscript.

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