

Vascular endothelial growth factor is increased by human pulmonary cells stimulated with *Dermatophagoides* sp. extract

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ABSTRACT

Airway remodeling may lead to increased secretion of TGF- β . TGF- β is known to activate fibroblasts and to stimulate the secretion of vascular endothelial growth factor (VEGF). Soluble and cell-associated VEGF have been identified in subjects with chronic severe asthma. *Dermatophagoides* sp. has been shown to contribute to the pathogenesis of asthma. The aim of this study was to determine if the *Dermatophagoides* sp. extract can stimulate confluent A549 (cA549) to express soluble or cell-associated VEGF and to secrete other factors that stimulate normal human lung fibroblasts (NHLFs) to secrete VEGF. Immunocytochemistry for cell-associated VEGF (pg/mL) was performed on cA549 stimulated with 300, 600, and 1000 AU/mL of dialyzed *Dermatophagoides* sp. extract for 24 hours with serum-free media (SFM). The subsequent conditioned media (CM) was transferred to NHLF for 24 hours (CM-NHLF). VEGF in CM, CM-NHLF, and control media (CTLM; extract without cA549) were measured by ELISA and normalized to cell density as measured by absorbance of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenol tetrazolium bromide at A550 nm (VEGF pg/mL:A550). Parametric data were analyzed by t-test or ANOVA at $\alpha = 0.05$. *Dermatophagoides* sp. extract increased normalized VEGF secretion by cA549. Immunochemical VEGF expression by cA549 was increased qualitatively by *Dermatophagoides* sp. extract. The 1000 CM-NHLF showed increased normalized VEGF relative to CTLM. *Dermatophagoides* sp. stimulates cA549 to increase soluble and immunochemical VEGF and to secrete mediators that stimulate NHLF to increase secretion of VEGF. This suggests that inhalation of dust-mite allergen may stimulate airway cells to increase VEGF secretion, potentially contributing to edema in airway remodeling.

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Key words: Alveoli, asthma, *Dermatophagoides*, fibroblasts, VEGF

A review of the literature indicates that chronic severe asthma is associated with damaged airway epithelial cells that secrete inflammatory cytokines and growth factors.¹ The epithelial cell damage includes discontinuous tight junctions caused, in part, by eosinophil-derived mediators.² The epithelial cell damage has been proposed to result in reactivation of the epithelial-mesenchymal trophic unit, accompanied by fibroblast growth and TGF- β secretion that promotes airway remodeling.³ TGF- β has been identified in samples from asthmatic subjects^{4,5} and stimulates the secretion of vascular endothelial cell growth factor (VEGF)⁶ from human bronchial fibroblasts from asthmatic subjects.^{7,8} VEGF, also known as vascular permeability factor, has been identified in the submucosa of asthmatic subjects⁷ and in induced sputum with

increased bronchial hypersensitivity and vascular permeability.⁹ Investigators also have shown that VEGF may contribute to edema with increased vascularity, hence leading to wall thickening.^{10,11} VEGF secretion may be induced by TGF- β in the human lung¹² and by stimulation of protease-activated receptors (PARs)¹³ by dust mite-derived proteases.¹⁴ This is supported by a clinical study that showed that allergen challenge to asthmatic subjects resulted in increased TGF- β signal transduction molecules such as Smad2 by epithelial and fibroblasts.^{12,15} These data suggest that dust-mite-derived proteases contribute to TGF- β - and VEGF-mediated airway remodeling in chronic severe asthma accompanied by airway epithelial cells with discontinuous tight junctions.^{16–19}

Other investigators have presented data that suggest that alveolar inflammation may occur in asthmatic subjects. For example, alveolar epithelial cells can be a source of TGF- β resulting in stimulation of mesenchymal cells to increase secretion of matrix proteins, hence contributing to alveolar wall thickening.⁴ Eosinophils and CD4⁺ cells associated with alveolar tissue obtained by distal alveolar tissue transbronchial biopsy corresponded with decreased forced expiratory volume at 1 second in nocturnal asthmatic subjects.^{20,21}

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Human A549 cells (alveolar type II epithelium-like cells) secreted eotaxin and up-regulated the cognate receptor CCR3 in the presence of IL-4.²² We previously showed that dialyzed standardized extracts of *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* stimulated confluent A549 (cA549), which approximate differentiated type II cells on attaining confluence²³ and show discontinuous tight junctions²⁴ similar to alveolar epithelial cells from asthmatic subjects,²⁵ to aggregate and secrete soluble factors that caused dysregulation of human pulmonary fibroblast and microvascular endothelial cell growth.²⁶ We also showed that TGF- β 1 secretion by cA549 was 10–15% greater than control in the presence of dust-mite extract.²⁶ Additionally, A549 secreted increased amounts of soluble inflammatory mediators that were modulated by dust-mite extracts and drugs.^{27–30} Other investigators showed that A549 cells are capable of secreting VEGF³¹ that appears to be inducible³² and can be inhibited by budesonide.³³ VEGF also may be cell-associated with airway epithelial cells as shown by electron microscopy.³⁴ Based on this information, we hypothesized that *Dermatophagoides* sp. extract stimulates the secretion and cell-associated storage of VEGF by cA549. Furthermore, dysregulated fibroblast growth is a component of the reactivation of the epithelial-mesenchymal trophic unit³ in chronic severe asthma. Therefore, we tested a second hypothesis that *Dermatophagoides* sp. extract-treated cA549 secrete soluble mediators that affect the secretion of VEGF by human pulmonary fibroblasts.

METHODS

Dialysis of Standardized Dust-Mite Extract

Standardized extracts of *D. pteronyssinus* and *D. farinae* (10,000 AU/mL stock in 50% glycerin; a gift from Alk-Abello, Inc., Port Washington, NY) were dialyzed in 8-kDa molecular weight cutoff dialysis bags (Spectra/Por Biotech RC Irradiated Membrane, Spectrum Laboratories Inc., Rancho Dominguez, CA) with several changes of sterile endotoxin-screened PBS (Invitrogen Gibco, Carlsbad, CA) at 1–6°C in a 48-hour period. The vendor claimed <1% of culture medium material in the extract was used to grow dust mites.

Cell Culture

All cells were grown in standard cell culture conditions using a humidified 37°C cell culture incubator with 5% CO₂. A549 cells (passage 85–110; ATCC, Cat No. CCL-185) were grown to confluence (cA549) in Dulbecco's Modified Eagle's media (DMEM) supplemented with 5% fetal calf serum (FCS) and 100 U/mL of penicillin G plus 100 μ g/mL of streptomycin (FCS-DMEM). Stock cultures of normal human lung fibroblasts (NHLF; Cambrex Bio-Science, Walkersville, MD)

were grown and maintained in proprietary growth factor-defined, serum-reduced media (FGM2, fibroblast growth media 2, Cambrex Bio-Science). All experiments that used dialyzed dust-mite extracts were performed with serum-free media (SFM) that included DMEM supplemented with 1X insulin-transferrin-selenium (ITS; supplied as 500X; Cambrex Bio-Science) and 100 U/mL of penicillin G plus 100 μ g/mL of streptomycin.

Generation of Conditioned Media and Control Media

CM and CTLM were generated as described in a previous work.²⁶ Briefly, FCS-DMEM was removed, and the cA549 were washed with PBS (Invitrogen Gibco). Zero, 300, 600, or 1000 AU/mL of dialyzed standardized *D. pteronyssinus* or *D. farinae* extract in SFM was added immediately. After incubation in standard cell culture conditions for 24 hours, the SFM was labeled 0 CM, 300 CM, 600 CM, and 1000 CM. CTLM was generated by incubating 0, 300, 600, or 1000 AU/mL of *D. pteronyssinus* or *D. farinae* extract in SFM without cA549. At the end of the incubation period, the SFM was labeled 0 CTLM, 300 CTLM, 600 CTLM, and 1000 CTLM. The CM and CTLM were removed at the same time and were stored at –40°C until ready for VEGF measurements by ELISA or for addition to subconfluent NHLF.

The Use of CM or CTLM to Generate NHLF CM (CM + NHLF) and CTLM (CTLM + NHLF)

NHLF were subcultured at 40–60% of confluence in proprietary culture media (FGM2). The FGM2 was removed, and the NHLFs were washed with PBS. Then, CM or CTLM were immediately added to the NHLF and incubated for 24 hours, hence, generating CM + NHLF and CTLM + NHLF. For negative controls, CM or CTLM were added to cell culture wells that were NHLF free (CM without NHLF and CTLM without NHLF). At the end of incubation, these media were removed and assayed for VEGF by ELISA (see Methods section). The NHLFs were assayed for viability and cell number with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenol tetrazolium bromide (MTT) incorporation assay (see Methods section).

MTT Incorporation Assay for NHLF Viability and Cell Number

At the end of the incubation of NHLF with CM and CTLM, 250 μ g/mL of MTT (Sigma, St. Louis, MO) in SFM was added immediately to the NHLF followed by incubation for 3 hours at 37°C. During this incubation period viable NHLF incorporated and enzymically converted the MTT to a blue formazan end product. The MTT/growth media was then removed and the

cells with (viable) and without (not viable) blue formazan color were counted microscopically. The percent viability was then calculated ([number of formazan-positive cells per field/{formazan-positive cells plus formazan negative cells per field}100]). To quantify the relative NHLF cell numbers CM versus CTLM, the blue formazan color was dissolved with 70% isopropanol in 0.04 N HCl for 1 hour at 21°C, and the optical density was measured at A₅₅₀ using a 96-well plate reader with the background subtracted at A₆₅₀. Sufficient volume of isopropanol/HCl with plate sealing was used to minimize differences in well-to-well evaporation. The intensity and intracellular distribution of the blue formazan end product was uniform by all cells tested in all conditions.

Normalization of VEGF Secretion in CM and CTLM from cA549 and NHLF

All CM and CTLM (CM, CTLM, CM + NHLF, and CTLM + NHLF) as well as negative CTLM were harvested at 24 hours, stored at -40°C, and assayed for VEGF by ELISA (Quantikine ELISA; R & D Systems, Minneapolis, MN). R & D Systems claimed that the anti-VEGF used in the ELISA recognizes the soluble VEGF₁₆₅ and VEGF₁₂₁ isoforms. In some experiments, VEGF concentration was normalized to cell density by dividing the absolute concentration in picograms per milliliter by the absorption units (A₅₅₀) of the blue formazan end product derived from the absorbed MTT substrate by cA549 or NHLF. Therefore, normalized VEGF secretion was expressed as VEGF picograms per milliliter/A₅₅₀ in arbitrary units.

Immunocytochemistry

Preliminary studies based on the absence of positive staining with the secondary antibody alone and optimal signal-to-noise ratio of positive staining with the primary plus secondary antibodies determined the final antibody dilutions stated in this section. cA549 incubated with 0, 300, 600, or 1000 AU/mL of *D. pteronyssinus* or *D. farinae* for 24–48 hours or 48–72 hours, respectively, were fixed with 2% paraformaldehyde for 1–2 hours at 21°C. Cells were washed with PBS and endogenous peroxides quenched with 0.03% hydrogen peroxide for 5 minutes or until bubbling ceased. After PBS wash, 1% bovine serum albumin (BSA) in PBS was used to block nonspecific binding sites for 5 minutes at 21°C. The BSA was removed and 1:25 rabbit anti-human VEGF (1–20 N terminus; Santa Cruz Biotechnologies, Santa Cruz, CA) was added in 1% BSA for 1 hour at 21°C or 18–24 hours at 4°C; the negative control received PBS only. The cells were washed three times with PBS followed by the addition of 1.5% goat serum in PBS for 5 minutes at 21°C. After the re-

moval of the goat serum, the cells were washed with PBS, and binding of the primary antibody was detected using the Vectastain Detection Method (Vector Laboratories, Burlingame, CA). The 1:100 biotinylated goat anti-rabbit IgG was added for 30 minutes at 21°C. The secondary antibody was removed, the cells were washed with PBS, and then horseradish peroxidase-streptavidin was added for 5 minutes at 21°C. After PBS wash, diaminobenzidine (Vector Laboratories, Burlingame, CA) was added and the color development was monitored. Color development was stopped by removing the diaminobenzidine and adding distilled water when the signal-to-noise ratio was high with the primary antibody while the absence of staining persisted in the negative control. Cells were examined for intracellular VEGF staining relative to the negative controls that included secondary antibody only or primary antibody preadsorbed with recombinant human VEGF.

Statistics

All data shown in the graphs conformed to a Gaussian distribution as indicated by the D'Agostino-Pearson omnibus test. Parametric data were analyzed at $\alpha = 0.05$ by comparing two groups (treatment to control) using the two-tailed unpaired *t*-test or by comparing three or more groups to each other by ANOVA. An ANOVA of $p < 0.05$ was followed by the Dunnett's *post hoc* test for comparison of multiple groups to control, or by the Student-Newman-Keuls *post hoc* test for between-group comparisons. Pearson correlation was used for linear regression analysis of parametric data. All parametric data are expressed as the mean $\pm$ 2 SD. All data showed power in the range of 0.6–0.9 at $\alpha = 0.05$ and $\beta = 0.2$. Statistical software used included GraphPad (GraphPad Software Inc., San Diego, CA) and SigmaStat (Systat Software Inc., San Jose, CA).

RESULTS

cA549 Treated with *Dermatophagoides* Species Extract Secrete VEGF

Three hundred, 600, and 1000 AU/mL of *D. pteronyssinus* stimulated some cA549 cells to aggregate with weak adhesion and other cA549 to strongly adhere as previously reported.²⁶ In this study, we report that *D. pteronyssinus* stimulated cA549 to increase VEGF secretion normalized to cell density in 24 hours relative to control (Fig. 1 A). No dose-response was observed. Absolute VEGF secretion was increased also over control in some experiments (data not shown). Aggregated cA549 showed a dose-dependent increase in VEGF secretion normalized to cell density when cultured with 300, 600, and 1000 AU/mL of *D. farinae* relative to control in 48 hours (Fig. 1 B).

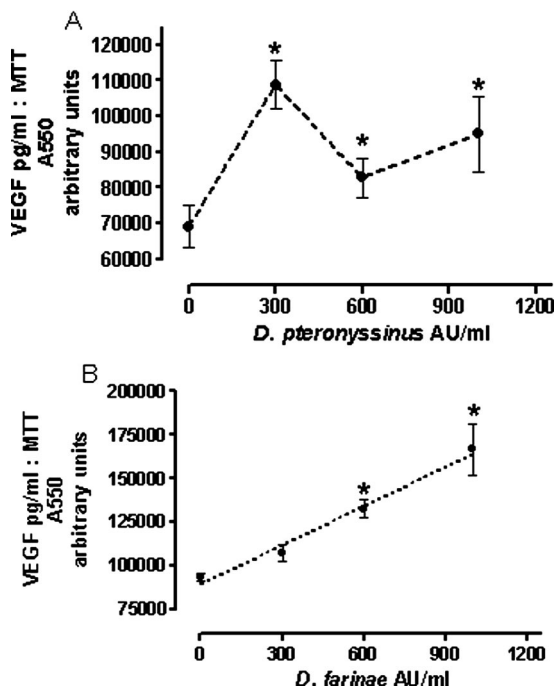


Figure 1. (A) Normalized VEGF secretion by *D. pteronyssinus*-stimulated cA549 after 24 hours in culture in SFM. Asterisk shows $p < 0.05$ ANOVA, followed by Dunnet's post hoc test relative to control ($n = 3$; bar = 2 SD). Representative of ten experiments. (B) Normalized VEGF secretion by *D. farinae*-stimulated cA549 after 24 hours in culture in SFM. Asterisk in shows $p < 0.05$ ANOVA, followed by Dunnet's post hoc test relative to control ($n = 5$; bar = 2 SD). Pearson correlation analysis showed $r^2 = 0.98$ and $p = 0.007$. Representative of three experiments.

Cell-Associated VEGF Is Increased by *D. Pteronyssinus*- and *D. Farinae*-Treated cA549

To determine if *D. pteronyssinus* extract stimulated cA549 to increase cell-associated VEGF above basal levels, immunocytochemistry was performed. cA549 cultured in SFM without *D. pteronyssinus* extract for 24 hours showed a baseline heterogeneous expression of cell-associated VEGF that supports the results of a reported perinuclear distribution of VEGF immunostain³¹ (Fig. 2 A). Approximately 25% of the cA549 showed no VEGF expression (Fig. 2 A). At no time was a signal observed with secondary antibody alone or anti-VEGF adsorbed with recombinant human VEGF. When cA549s were stimulated for 24 hours with 300, 600, or 1000 AU/mL of *D. pteronyssinus* extract, a qualitative increase in cell-associated VEGF expression was observed in the adhered, nonaggregated cA549 (Fig. 2, B–D). Detachment of cA549 during quenching of endogenous peroxidase with H₂O₂ increased with 600 and 1000 AU/mL of *D. pteronyssinus* extract, probably because of enzymic degradation of cA549 adhesion molecules by mite-derived proteases. This did not occur with 0 and 300 AU/mL of *D. pteronyssinus* extract

or with CTLM. The VEGF expression of the detached cA549 is not known at this time.

CM, but Not CTLM, Stimulate NHLF to Secrete VEGF

Investigators have suggested that growth factors and cytokines secreted by epithelial cells may modulate the secretion of mediators by other cell types in the asthmatic lung.^{1,3} This may be affected by dust-mite proteases in a PAR-dependent manner. Hence, 1000 CM from dust-mite extract-treated cA549 was used to stimulate NHLF for 24 hours and the resultant media were assayed for VEGF. 1000 CM stimulated NHLF (CM + NHLF) to increase VEGF secretion relative to CM without NHLF (CM without NHLF; Fig. 3; 1004 ± 59 pg/mL versus 611 ± 44 pg/mL, respectively). CTLM generated by incubating SFM with 1000 AU/mL of *D. pteronyssinus* or *D. farinae* extract for 24 hours showed negligible levels of VEGF (Fig. 3; CTLM without NHLF). When NHLFs were incubated for 24 hours with dust-mite extract, 198 ± 39 pg/mL of VEGF was secreted into the CTLM (Fig. 3; CTLM + NHLF). These data, taken together, indicated that extract-stimulated cA549 secreted a putative factor or factors into CM that stimulated NHLF to secrete VEGF almost fivefold over CTLM + NHLF (1004 ± 59 pg/mL versus 198 ± 39 pg/mL, respectively) and by 64% over CM without NHLF (1004 ± 59 pg/mL versus 611 ± 44 pg/mL, respectively). Similar results were observed with CM generated with 600 AU/mL of dust-mite extract. When the VEGF pg/mL were normalized to MTT at A₅₅₀, an increase in VEGF secretion was observed that was dependent on the concentration of dust-mite extract used to generate the CM from the cA549 relative to the CTLM (Fig. 4).

DISCUSSION

The pathogenesis of asthma emphasizing the role of airway epithelium, fibroblast activation, and growth factors has been reviewed.³⁵ Allergens such as dust-mite-derived proteases may contribute to asthma pathogenesis.^{15–17} Previous work showed that 300, 600, and 1000 AU/mL of *D. pteronyssinus* extract induced aggregation of cA549 in a dose-dependent manner at 24 hours of incubation and with *D. farinae* at 48 hours of incubation.²⁶ Because other investigators showed that VEGF secretion may be PAR dependent,^{36,37} this study focused on the effects of *Dermatophagoides* sp. extract on VEGF secretion by human pulmonary cells *in vitro*. In the first part of the study, cA549 secreted VEGF normalized to cell density 25–50% above baseline levels when stimulated with either *D. pteronyssinus* or *D. farinae* extract (Fig. 1 A). Furthermore, *D. farinae* stimulated normalized VEGF secretion in a dose-dependent man-

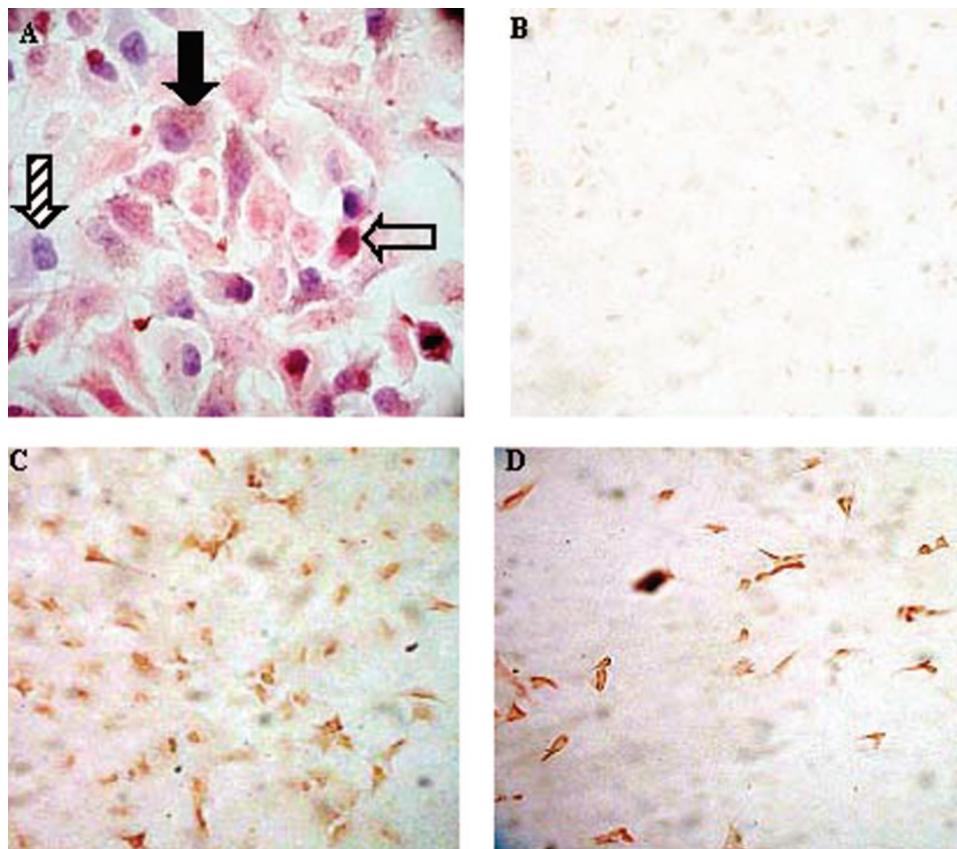


Figure 2. (A) cA549 cells in SFM without dust-mite extract immunostained for VEGF and counterstained with hematoxylin and eosin (400 $\times$). cA549 showing perinuclear VEGF expression (black arrow), nuclear distribution (open arrow), and no VEGF expression (hatched arrow) are indicated. Immunoreactive VEGF expression (without counterstain) by adhered cA549 remaining after 24 hours in culture with (B) 300 CM, (C) 600 CM, and (D) 1000 CM AU/mL of *D. pteronyssinus* extracts (100 $\times$). VEGF immunostaining of cA549 with CTLM showed results similar to panel A (data not shown). Representative of 10 experiments. Similar results were observed with *D. farinae* with three experiments.

ner as suggested by the high r^2 of 0.98 (Fig. 1 B). This was accompanied by an increased qualitative intensity of intracellular levels of immunostained VEGF. It is not known at this time if the source of the increased VEGF secretion between 300 and 1000 AU/mL of *D. pteronyssinus* and *D. farinae* was from cell-associated storage sites as shown by immunohistochemistry (Fig. 2) or from inducible VEGF transcription by the cA549 at the corresponding dust-mite concentrations. The cell-associated immunochemical VEGF expressed by cA549 corroborated the findings by previous investigators that A549 cells show a perinuclear distribution of VEGF.³¹ In addition, we also showed that although some cA549 may store VEGF in the nucleus, other cA549 showed an absence of cell-associated VEGF. These VEGF⁻ cA549 cells either did not have active VEGF gene expression at the time of fixation or the cell-associated VEGF was already released into the culture media at an earlier time point. Interestingly, the qualitative intensity of the cA549 showed an increased intensity of immunostained cell-associated

VEGF in the presence of 1000 AU/mL of *D. pteronyssinus* (Fig. 2, B–D) that paralleled the increased secreted VEGF (Fig. 1). Additional studies would need to be performed to determine if a dose-response exists between dust-mite extract concentration and cell-associated VEGF concentration. We concluded that *Dermatophagoides* sp. extract stimulated cA549 to increase cell-associated VEGF expression and secretion.

The second part of the study examined the NHLF as another potential source of VEGF. NHLF stimulated with CM (CM + NHLF; generated by *Dermatophagoides* sp. extract stimulation of cA549 for 24 hours) showed a 66% increase in VEGF secretion by NHLF relative to CM without NHLF (Fig. 3). This indicated that the NHLF secreted VEGF levels that exceeded the cA549-derived VEGF present in the CM. Additionally, VEGF levels in CM + NHLF were approximately fivefold greater than CTLM + NHLF. VEGF secretion by NHLF was increased by 300, 600, and 1000 CM over CTLM (Fig. 4). Based on the data in Fig. 3, the rank order of VEGF secretion therefore was CM + NHLF > CM

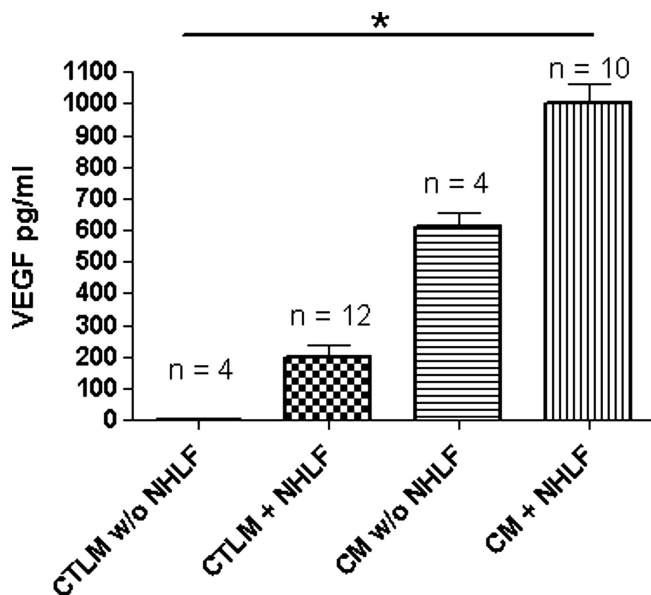


Figure 3. VEGF secretion by NHLF incubated with 1000 CM (CM $\pm$ NHLF) and 1000 CTLM (CTLM $\pm$ NHLF) for 24 hours. Data are shown as mean $\pm$ 2 SD with indicated sample sizes per condition. Asterisk represents $p < 0.05$ by ANOVA followed by the SNK post hoc test. Similar results were found with 600 CM and 600 CTLM.

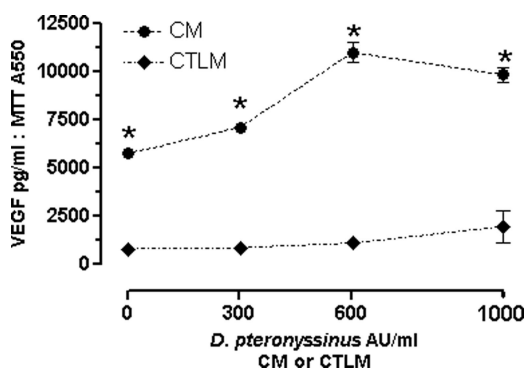


Figure 4. Comparison of normalized VEGF secretion by NHLF cultured for 24 hours in CM and CTLM. Data expressed as mean $\pm$ 2 SD, $n = 3$. Asterisk represents CM versus CTLM at each concentration of *D. pteronyssinus* extract at $p < 0.05$, two-tailed *t*-test. No difference between 300 CM and 0 CM was found ($p > 0.05$). Representative of four experiments.

without NHLF $>$ CTLM + NHLF $>$ CTLM without NHLF. This shows the presence of an unknown factor or factors in the cA549-derived CM, but not CTLM, that stimulated the NHLF to secrete VEGF.

This study presents *in vitro* support for the recent report that dust-mite proteases and PARs play a role in stimulating fibroblast-mediated events in airway remodeling.¹⁵ It is possible that the effects of dust-mite proteases may act in concert with T_H2 -derived inflammatory mediators. Epithelial and mesenchymal cell growth is important in the early years of life when

exposure to dust-mite allergen can play a role in the progression of airway remodeling from childhood to adult. The combined effects of allergen and inflammatory cytokines that may mediate this progression may be explored by expanding the culture setting described in this study to include the addition of known T_H2 -related inflammatory mediators to cocultures of dust-mite extract-treated epithelial and mesenchymal cells. This may permit the identification of cA549-derived mediators that alter the growth of mesenchymal cells thereby providing targets for novel pharmacologic interventions in chronic severe asthma.^{35,38}

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