

Transforming growth factor beta: A role in the upper airway and rhinosinusitis—*Dermatophagoides pteronyssinus*–induced apoptosis with pulmonary alveolar cells

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ABSTRACT

Background: There is a link with the upper and lower airway and disruption of alveolar epithelial cells, which is a potential trigger for the reactivation of the epithelial-mesenchymal trophic unit (EMTU) and induced characteristic airway changes associated with allergic asthma. *Dermatophagoides pteronyssinus* is a common inhalant indoor allergen and is known for causing allergic rhinitis and airway inflammation. Transforming growth factor beta 1 (TGF-beta1) is a major participant in the airway remodeling of asthma, a component of cellular stress response pathways, and enhanced epithelial immunoreactivity is known to occur in allergic rhinitis.

Methods: In this study, we show the ability of *D. pteronyssinus* allergens from dialyzed standardized immunotherapy extract to induce apoptosis and increase TGF-beta1 secretion in a confluent A549 cell line model. A549 cells were treated with either 600 AU/mL dialyzed *D. pteronyssinus* immunotherapy extract (eDp) or Ctl media (Ctl) for 24 hours. Cells and supernatants were collected, washed, and treated with Annexin V-FITC Apoptosis Detection Kit II (BD Pharmingen, La Jolla, CA) and then analyzed by flow cytometry. TGF-beta1 secretion was determined by ELISA using cell culture supernatants.

Results: The eDp group showed a fourfold increase in early apoptotic cells with a twofold increase in late apoptotic cells versus the Ctl group, along with a 1.65-fold increase of TGF-beta1.

Conclusion: eDp induced viable A549 cells to undergo apoptosis determined by flow cytometry analysis with a significant increase in TGF-beta1 secretion compared with Ctl.

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Asthma is a chronic inflammatory disease of the airway that can be triggered by allergic rhinitis and sinusitis characterized by hyperresponsiveness leading to acute bronchoconstriction, airway edema, and airway wall remodeling. Exposure to allergens, environmental irritants, viral infections, cold air, or exercise leads to clinical symptoms of chronic rhinitis, wheezing, and dyspnea. The inflammation of asthma is characterized by the continued presence of increased numbers of TH2 lymphocytes, eosinophils, and neutrophils that are involved in the disruption and activation of the epithelial-mesenchymal trophic unit (EMTU).^{1–3}

The EMTU is formed by interactions between opposing layers of epithelial and mesenchymal cells separated by a basement membrane. Chronic insults by environmental factors are believed to result in the reactivation of EMTU driving airway remodeling, a hallmark of asthma.^{4,5}

Type II epithelial cells interact with the fibroblast through apertures in the endothelial basement membrane and injury or distortion of epithelial monolayer results in an increase of fibroproliferative factors.⁶ Furthermore, disruption of alveolar epithelial cells by eosinophils and macrophages can further trigger the reactivation of the EMTU leading to dysregulated mesenchymal cell growth creating thickened, hyperresponsive airways.^{7,8}

Dermatophagoides species, a common indoor inhalant allergen and known precipitating factor for allergic rhinitis and asthma, contain peptidase allergen that contribute to allergic inflammation and nasal

and airway damage.⁹ It has been shown that house-dust mite proteases can cleave cell adhesion, induce cell death, and increase the permeability of lung epithelium.¹⁰ *Dermatophagoides* sp. extract was shown to stimulate A549 cells to secrete factors that dysregulate mesenchymal cell growth *in vitro*, increase vascular endothelial growth factor (VEGF) secretion by A549 cells, and stimulates A549 cells to secrete mediators that stimulate normal human lung fibroblasts to increase secretion of VEGF.^{11,12}

Recently, it was shown that β -agonist enantiomers can modulate *Dermatophagoides* sp. extract-induced VEGF from myofibroblasts.¹³ In addition, these peptidases can induce apoptosis in a bronchial cell model independent of tight junction perturbation.¹⁴

Cytokines are known to play an important role in the activation of the EMTU and damaged airway epithelial cells are known to secrete inflammatory cytokines and growth factors.¹⁵ Transforming growth factor (TGF) β 1 is a major cytokine important in regulating the EMTU and is considered central in the hyperplasia of airway smooth muscle.¹⁶ TGF- β 1 signaling is activated during acute allergen challenges that increase fibrogenetic factor involved in airway remodeling by altering collagen synthesis and fibroblast proliferation causing airway remodeling.¹⁷ Increased gene expression for TGF- β receptor I was shown in whole nasal biopsy specimens from subjects with naturally occurring allergic rhinitis, which has advanced our understanding of the role of TGF- β in the pathogenesis of the inflammatory response in allergic rhinitis and can shed further light on the role of TGF- β role in the upper airways and paranasal sinuses.¹⁸ TGF- β 1 has been thought to play a role in allergic inflammatory disease of the upper airways and have a possible therapeutic implication including potential future applications in the treatment of nasal polyposis and reduction of postoperative scar tissue formation after endoscopic sinus surgery.¹⁹

TGF- β 1 is secreted in many inflammatory cell types including eosinophils.²⁰ Atopic asthma patients are known to have increased presence of TGF- β 1 in the bronchoalveolar lavage fluid.²¹ Furthermore, TGF- β 1 is associated with increased expression of stress response genes such as JNK, ERK, and NF- κ B and there is evidence that it attenuates the hyperresponsiveness of asthmatic airway, indicating a role of TGF- β 1 being prosurvival.^{22–24}

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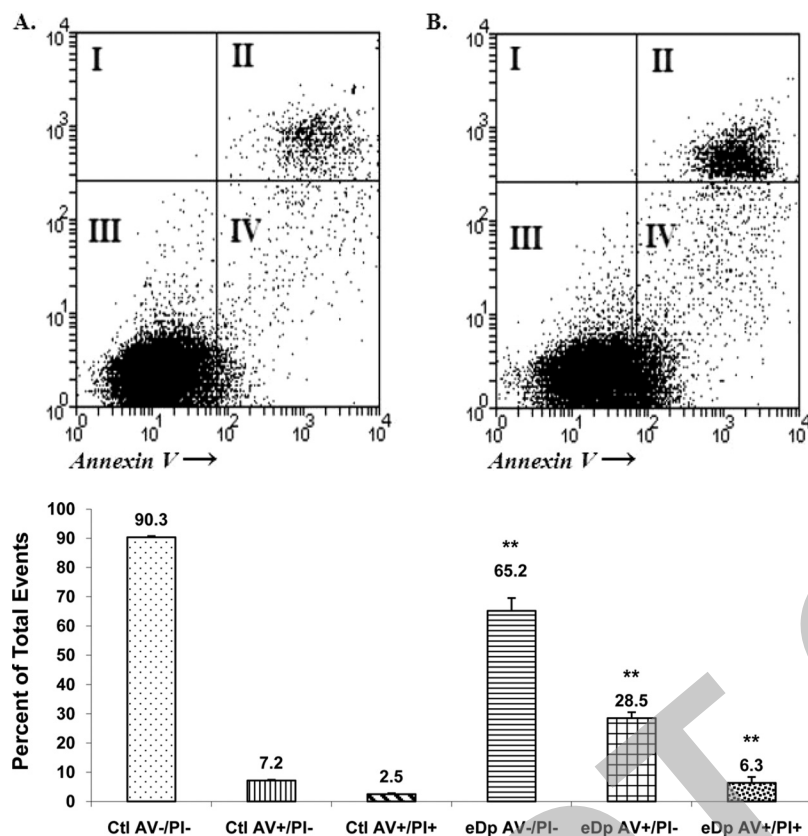


Figure 1. FL1 versus FL3 plot of (A) control (Ctl) and (B) Dermatophagoides pteronyssinus immunotherapy extract (eDp)-treated groups. Quadrant I consists of AV⁻/PI⁺ cells; quadrant II are annexin V-FITC/propidium iodide (AV⁺/PI⁺) cells; quadrant III are AV⁻/PI⁻ cells; quadrant IV are AV⁺/PI⁻ cells. Horizontal and vertical cursors were set for zero AV⁻/PI⁺ events in the quadrant. Merged plot of all experiments.

Figure 2. Bar graph representation of FL1 versus FL3 plot for all events registered in respect to control (Ctl) and Dermatophagoides pteronyssinus immunotherapy extract (eDp)-treated groups (see Fig. 1). Cells were harvested and stained after 24-hour incubation with eDp or Ctl and then analyzed by flow cytometry. eDp had a 4-fold increase in early apoptotic (annexin V-FITC/propidium iodide [AV⁺/PI⁻]) cells, a 2.5-fold increase in late apoptotic (AV⁺/PI⁺) cells, and diminished viability (AV⁻/PI⁻). ***p* < 0.005.

There has been increased attention placed on the airway epithelium in relation to asthma.^{4,5,25} Allergens in the environment can disrupt the epithelium that diminishes its barrier function causing leaky airways.²⁶ One study showed how certain environmental pollutants can induce apoptosis in airway epithelium.²⁷ In our study, we examined the effect of dialyzed *Dermatophagoides pteronyssinus* extract (eDp) on A549 cell line, a transformed type II epithelial line that possess characteristics of normal alveolar cells at confluency, as an *in vitro* model for the initial insult to the EMTU. TGF- β and IL-8 blockade has been shown to efficiently prevent neutrophil infiltrates from A549 cells.²⁸ We had previously shown that human nasal epithelial cells can also release IL-8 that is blocked by antihistamines.²⁹ We evaluated cell viability with respect to apoptotic events using flow cytometry and if eDp-stimulated A549 cells can increase TGF- β 1 secretion, all independent of any immunologic factors.

MATERIALS AND METHODS

Dialysis of eDp

Standardized *D. pteronyssinus*, 10,000 AU/mL, immunotherapy extract in 50% glycerin (a gift from Nelco, Deer Park, NY) was dialyzed using Spectra/Por Biotech RC Irradiated Membrane with 5-kDa cut-off (Spectra Lab, Inc., Rancho Dominguez, CA) in a 4°C cold room with three to four passages of 1× phosphate-buffered saline (PBS) for 72 hours. A Ctl dialysate was used to screen for toxicity of dialysate buffer using a lactate dehydrogenase colorimetric cytotoxicity kit (Roche, Indianapolis, IN).

Experimental Procedures

A549 cells (ATCC, Manassas, VA) were grown to 100% confluence in 6-well plates using Eagle's minimal essential media with 5% heat inactivated fetal bovine serum; penicillin G, 100U/mL;

streptomycin, 100 μ g/mL; and glutamine, 5 mM, at standard cell culture conditions, which were in a cell culture incubator with humidified air at 37°C and 5% carbon dioxide. Experimental media (eDp) consisted of 600 AU/mL of dialyzed eDp in serum-free media with penicillin G, 100 U/mL, and streptomycin, 100 μ g/mL; control media (Ctl) consisted of serum-free media plus penicillin, G 100 U/mL, and streptomycin, 100 μ g/mL. Cells were gently washed with cell culture grade 1× PBS before addition of eDp or Ctl and incubated for 24 hours at standard cell culture conditions. Assays were conducted as follows.

Apoptosis Assay

Cells and supernatant were collected, washed with cold 1× PBS, and stained with 10 μ L of Annexin V-fluorescein isothiocyanate (AV) and propidium iodide (PI), respectively (Apoptosis Detection Kit II; BD Pharmingen, Franklin Lakes, NJ), and then as per manufacturer protocol. PI binds to DNA when the cell membrane is disrupted and AV binds to translocated phosphatidylserine on the outer surface of the cell membrane (early apoptosis signal). Stained cells were analyzed by Epics XL-MCL flow cytometer (Biocompare for Beckman, San Francisco, CA) for the first 10,000 events counted and scatterplots were analyzed using Weasel 2.6.1 software. Parameters were set for zero AV⁻/PI⁺ events because cells with a disrupted membrane stain with both AV and PI. Gates were constructed based on Ctl's representing zones of viable, nonviable, and early apoptotic cells. This was accomplished based on FL1 (AV signal) versus FL3 (PI signal), side light scatter (SS) versus forward light scatter (FS) and FL1 versus FS plots analysis.

TGF- β 1 Measurement

TGF- β 1 was measured using Quantikine ELISA Kit (R & D Systems, Minneapolis, MN). Supernatants were used to measure

Figure 3. Early apoptotic cells determined by annexin V-FITC (AV) staining and cell size using FL1 versus forward light scatter (FS) parameters. This gate represented baseline control (Ctl) cell size with increased AV signal, representing early apoptosis. For both groups, 97% of all cells in the gate were AV⁺/propidium iodide (PI)⁻ and 3% were AV⁺/PI⁺. This gate represented 24.5% of all the events registered for the (B) eDp group and (A) 5.3% for Ctl. Merged plot of all experiments.

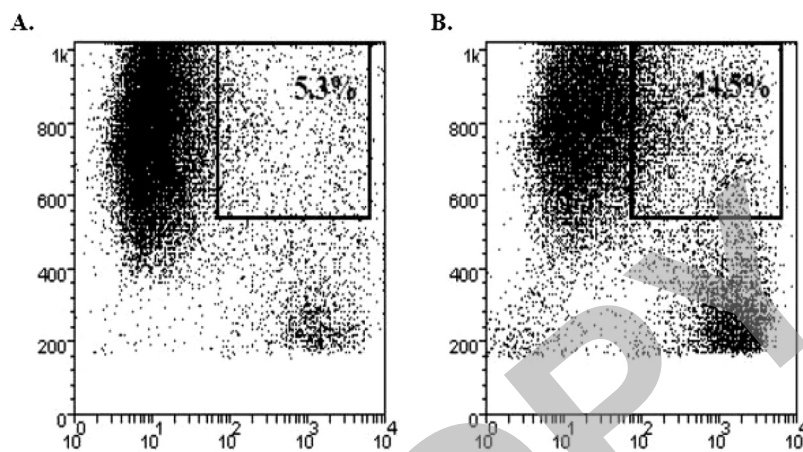


Figure 4. Side light scatter (SS) versus forward light scatter (FS) plot of flow cytometry data with gated regions for (A) control (Ctl) and (B) Dermatophagoides pteronyssinus immunotherapy extract (eDp)-treated groups. Gate 1 was constructed in a high viability region based on high FS and low annexin V-FITC/propidium iodide (AV⁺/PI⁺)-stained cells. This gate represented 44.5% of all events for the eDp group and 63.2% for the Ctl group. Gate 2 was constructed in a zone of low viability based on low FS and high AV⁺/PI⁺-stained cells. This region contained 10.6 and 5.0% of all eDp and Ctl events, respectively. Merged plot of all experiments.

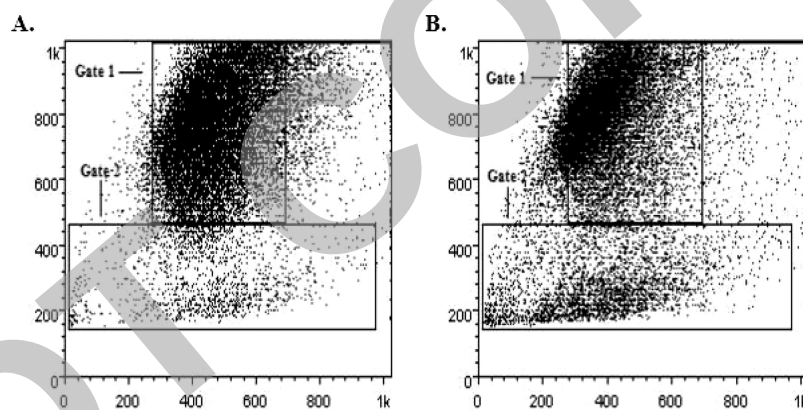
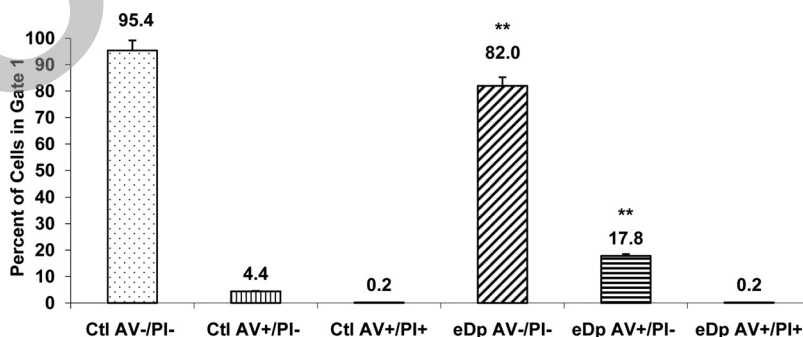


Figure 5. Bar graph representation for FL1 versus FL3 plot of gate 1. Compared with the control (Ctl) group, Dermatophagoides pteronyssinus immunotherapy extract (eDp) had a fourfold increase in early apoptotic cells with a decrease in viable cell events. The gated region consisted of high cell viability, as determined by low annexin V-FITC/propidium iodide (AV⁺/PI⁺) cells and high forward light scatter (FS). ***p* < 0.005.



secreted TGF- β 1 as per manufacturer's protocol. This assay recognizes both natural and recombinant TGF- β 1 and no significant cross-reactivity or interference was observed with other recombinant protein. Values were normalized based on Ctl and viable cell populations.

Statistical Analysis

Statistical analysis was performed using two-tailed *t*-test, ANOVA, and Scheffe *post hoc* group analysis. Results are expressed as mean and error bars represent 1 SD of the mean.

RESULTS

Merged FL1 versus FL3 plot (Fig. 1) analysis of all experiments in the eDp group showed a fourfold increase in early apoptotic (AV⁺/PI⁻) cells (*p* < 0.005), with a twofold increase in late apoptotic (AV⁺/PI⁺) cells (*p* < 0.005), versus the Ctl group; 65.2% of cells were viable in the eDp group and 90.3% were viable in the Ctl group (Fig. 2). Early apoptotic cells were further confirmed by FL1 versus FS

plot analysis, taking into account the cell size of AV⁺/PI⁻ cells (Fig. 3). A gate was constructed using baseline Ctl FS (cell size) and increased FL1 signal (AV⁺/PI⁻) for the parameters. For both groups, 97% of all cells in the gate were AV⁺/PI⁻ and 3% were AV⁺/PI⁺, indicating a zone of early apoptosis. This gate contained 24.5% of all AV⁺/PI⁻ cells in eDp group and 5.3% in the Ctl group (*p* < 0.005). Gate 1 of the SS versus FS plot represented 44.5% of all events for the eDp group and 63.2% of all events for the Ctl group (Fig. 4). FL1 versus FL3 analysis of this gate showed a fourfold increase in the eDp group versus Ctl (Fig. 5). This gate contained <1% of AV⁺/PI⁺ cells. Gate 2 contained an increased number of events that were AV⁺/PI⁺ and had small cell morphology, based on FS, FL1, and FL3 (Fig. 4). In the gate, eDp showed a twofold increase in events registered along with an increase of both AV⁺/PI⁻ and AV⁺/PI⁺ cells compared with Ctl (Fig. 6). Quantitative ELISA showed that A549 cells stimulated with eDp had increased TGF- β 1 secretion versus Ctl. Groups were normalized to Ctl and on viable cell population; this revealed the eDp group having a 1.65-fold increase in TGF- β 1 versus the Ctl group (Fig. 7).

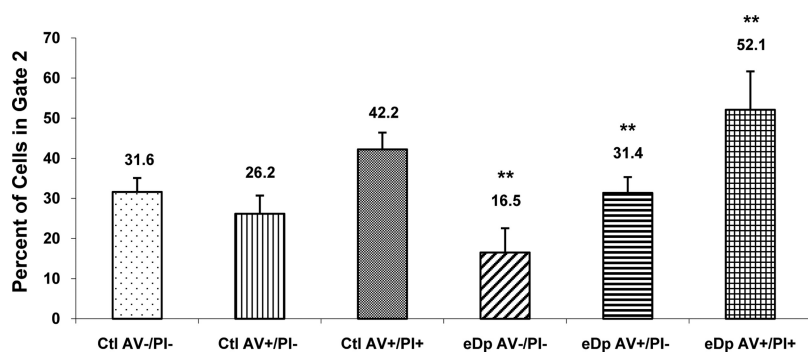


Figure 6. Bar graph representation for FL1 versus FL3 plot of gate 2. Gate 2 contained an increase in both early and late apoptotic cells with a decrease in viable events in the *Dermatophagoides pteronyssinus* immunotherapy extract (eDp) group. A twofold increase in events was seen compared with control (Ctl). This gate region consisted of low forward light scatter (FS) and high AV⁺/PI⁺ cells. ***p* < 0.005.

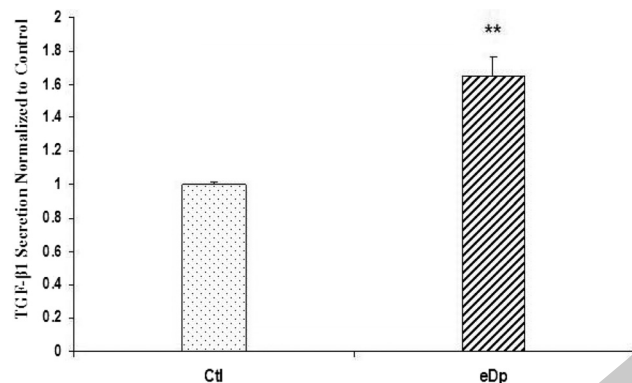


Figure 7. Transforming growth factor (TGF) β 1 secretion of control (Ctl) and *Dermatophagoides pteronyssinus* immunotherapy extract (eDp)-treated groups normalized based on Ctl and viable cell population. eDp-treated A549 cells had a 1.65-fold increase in TGF- β 1 (*n* = 5) secretion compared with Ctl (*n* = 9)-treated cells. ***p* < 0.005.

of the airway and not being detrimental to the organ system. We showed *D. pteronyssinus* allergen inducing apoptosis in airway epithelium and in response increased the secretion of TGF- β 1, a known stress response component. This can also occur in rhinitis and possibly sinusitis. Additional studies are needed to further understand the mechanisms of this process and to examine alternative airway, nasal models, and additional allergens. We believe this study can offer a new perspective in the interaction between environmental factors and the airway epithelium, which links with the upper airway in allergic rhinitis and asthma.

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DISCUSSION

The results of this study show the effect of *D. pteronyssinus* allergen from dialyzed standardized immunotherapy extract caused viable A549 cells to undergo apoptosis and stimulate the increased secretion of TGF- β 1, independent of any immunologic factor. Apoptotic cells were determined by morphologic changes and presence of early apoptotic markers. TGF- β 1 secretion was significantly increased compared with Ctl when stimulated by eDp, possibly as part of a stress response.^{22–24} This effect was not seen when the extract was dialyzed in a nonphysiological pH buffer and as per the manufacturer, the extract was produced under strict observance of Good Manufacturing Practices and Food and Drug Administration guidelines to be free of endotoxin or any other potentially harmful contaminant. We used an A549 cell line model to elucidate the effect of *D. pteronyssinus* allergen on the alveolar epithelium and gain further insight on its role in EMTU and asthma. Of interest, TGF- β and IL-8 blockade has been shown to efficiently prevent neutrophil infiltrates from A549 cells.²⁸ This cell line possesses characteristics of normal type II alveolar cells at full confluence and when stimulated by eDp underwent apoptosis. In addition, the concomitant increase in TGF- β 1 secretion leads us to theorize cytokines having a prosurvival role in response to the initial insult, reactivating the EMTU resulting in smooth muscle hypertrophy and mucosal hyperplasia. Previous studies have reported the increased release of prosurvival growth factors in response to dust-mite allergens.^{12,16,22–24} We argue that the initial release of these cytokines and consequential activation of the EMTU are in response to the damaged epithelium alone, which are exacerbated by the immune response. In addition, there is no direct correlation between airway remodeling leading to loss of lung function.³⁰ This potential trigger for both allergic rhinitis and airway remodeling, as shown here, provides a perspective to viewing remodeling as prosurvival function

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