ORIGINAL RESEARCH

IL-13 Augments Compressive Stress–Induced Tissue Factor Expression in Human Airway Epithelial Cells

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Abstract

Tissue factor (TF) is best known as a cellular initiator of coagulation, but it is also a multifunctional protein that has been implicated in multiple pathophysiologic conditions, including asthma. In the lung, airway epithelial cells express TF, but it is unknown how TF expression is regulated by asthma-associated mediators. We investigated the role of IL-13, a type 2 cytokine, alone and in combination with compressive stress, which mimics asthmatic bronchoconstriction, on TF expression and release of TF-positive extracellular vesicles from primary normal human bronchial epithelial cells. Well-differentiated normal human bronchial epithelial cells were treated with IL-13 and compressive stress, alone and in combination. TF mRNA, protein and activity were measured in the cells and conditioned media. TF was also measured in the bronchoalveolar lavage (BAL) fluid of allergen-challenged mice and patients with asthma. IL-13 and compressive stress increased TF expression, but only compressive stress induced TF-positive extracellular vesicle release. Pretreatment with IL-13 augmented compressive stress–induced TF expression and release. TF protein and activity in BAL fluid were increased in allergen-sensitized and -challenged mice. TF was elevated in the BAL fluid of patients with mild asthma after an allergen challenge. Our *in vitro* and *in vivo* data indicate close cooperation between mechanical and inflammatory stimuli on TF expression and release of TF-positive extracellular vesicles in the lungs, which may contribute to pathophysiology of asthma.

Keywords: asthma; bronchoconstriction; mechanotransduction; coagulation; airway epithelium

Clinical Relevance

Two critical components of asthma–IL-13 and bronchoconstriction–cooperatively work to induce tissue factor (TF) expression and release of TF-positive extracellular vesicles from well-differentiated human bronchial epithelial cells. Furthermore, in both mice and humans, allergen challenge increases the release of TF into bronchoalveolar lavage fluid.

Asthma is characterized by chronic airway inflammation and airway hyperresponsiveness (1, 2). Although the immune system is certainly implicated in asthma, a growing body of evidence suggests that airway epithelial cells also initiate and perpetuate disease (3, 4). During asthma exacerbations, airway smooth muscle contracts excessively and the airway becomes narrowed, resulting in buckling of the airway epithelium (5, 6). Epithelial cells in such buckled airways are subjected to a compressive mechanical stress of approximately 30 cm H_2O (7). Using an *in vitro* system mimicking the

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buckled epithelium of constricted airways, we previously showed that compressive mechanical stress initiates mechanotransduction signals in airway epithelial cells (8, 9) and contributes to airway remodeling in asthma (10, 11). Importantly, the role of bronchoconstriction in airway remodeling was validated in humans (12). The evidence from these studies suggests that bronchoconstriction itself can play a major role in asthma.

A well known feature of asthma is a procoagulant environment that is induced by the leakage of plasma proteins into the airway lumen (13). Although coagulation is classically thought to happen in blood vessels, coagulation also occurs on the luminal surface of the airway epithelium (14). Compared with control subjects, coagulation activity and the concentrations of coagulation-associated mediators, including thrombin, thrombin–antithrombin complex, and tissue factor (TF) are elevated in sputum and bronchoalveolar lavage (BAL) fluid from patients with asthma (15–17). TF is a 47-kD transmembrane protein that functions as the primary cellular initiator of blood coagulation by binding Factor VII/Factor VIIa (FVII/FVIIa) (18, 19). It is expressed in a variety of cell types, including airway epithelial cells (20). Previously, we showed that TF expression is increased in the airway epithelium of patients with asthma and that bronchial epithelial cells are a source of TF (21). In a mouse model of asthma, mice with a severe deficiency of FVII have attenuation of airway hyperresponsiveness and mucus production induced by allergen challenge (22). Together, these studies suggest that TF-dependent activation of coagulation may contribute to asthmatic disease presentation. Therefore, we need a better understanding of how TF expression is regulated in asthma.

Various inflammatory mediators and cytokines regulate the level of TF expression in nonepithelial cells (23), but their effect on TF production in bronchial epithelial cells is unknown. Here, we investigated the effects of IL-13, a type 2 cytokine, on TF expression and release of TF-positive extracellular vesicles from airway epithelial cells. IL-13 is elevated in the lungs of patients with allergic inflammation, IL-13 expression is associated with the severity of asthma (24, 25), and IL-13 regulates asthma-associated genes in airway epithelial cells (26). Though IL-13 has the capacity to induce airway hyperresponsiveness (27, 28), its cooperative effects with bronchospasm on airway epithelial cells are not known. We tested the hypothesis that IL-13 enhances compressive stress–induced TF expression and release of TF-positive extracellular vesicles. We also determined the epithelial cell type expressing TF in mouse lung, and determined whether allergic inflammatory conditions alter the level of TF in BAL fluid from mice. Finally, we evaluated TF levels in BAL fluid from patients with mild asthma after an allergen challenge.

Materials and Methods

A detailed description of the methods is provided in the online supplement.

Air–Liquid Interface Culture of Primary Normal Human Bronchial Epithelial Cells

Normal human bronchial epithelial (NHBE) cells were obtained at passage 1 from the Marsico Lung Institute/Cystic Fibrosis Center at the University of North Carolina, Chapel Hill (Chapel Hill, NC). The cells used were obtained from five donors. Passage 2 cells were cultured and maintained in air–liquid interface (ALI) culture for 14–17 days, as previously described (11).

In Vitro Exposure of NHBE Cells to IL-13 or Compressive Stress

To examine the effect of IL-13 on TF expression, NHBE cells were incubated with recombinant IL-13 (Cell Signaling Technology, Danvers, MA) either acutely or chronically, at the concentration described. For the acute exposure, cells were incubated with IL-13 for 24 hours. For the chronic exposure, cells were incubated with media containing IL-13 from ALI Days 0–14. During chronic treatment, fresh medium with or without IL-13 was fed every other day.

To examine the effect of IL-13 on compressive stress–induced TF expression, NHBE cells were incubated with IL-13 for 21 hours before the application of compressive stress. Cells were then exposed to 30 cm H_2O pressure for 3 hours, as previously described (11). Cells and basolateral conditioned media (CM) were collected immediately or at the indicated time relative to the start of pressure. Control cells were handled identically to experimental samples, but were not exposed to pressure.

Measurement of Coagulation Activity

Coagulation activity of CM and of BAL fluid was measured using a single-stage clotting assay with normal plasma from humans or mice, as described previously (29). Lipidated recombinant human TF (Innovin; Dade Behring, Marburg, Germany) was used to generate a standard curve. To determine the contribution of TF to the procoagulant activity, we used TF-blocking antibodies (clones HTF-1 or 1H1 at 10 μg/ml) to block human or mouse TF, respectively, as previously described (30).

Allergen-Sensitized and -Challenged Mice

All experiments were approved by the Harvard Medical Area Standing Committee on Animals (Harvard T. H. School of Public Health, Boston, MA). Male mice (C57BL/6) were purchased from Jackson laboratory (Bar Harbor, ME). Sensitization and challenge to ovalbumin (OVA) were performed as previously described (31). BAL fluid was collected 24 hours after the last OVA inhalation, as previously described (31).

Collection of BAL Fluid from Allergen-Challenged Patients

The protocol was reviewed by, and was in accordance with the ethical standards of the Partners Human Research Committee at Brigham and Women's Hospital (Boston, MA). Written informed consent was obtained by a physician from each patient. BAL fluid was collected, as described previously (32, 33), from patients who had previously been diagnosed with mild asthma. In each patient, BAL fluid was collected immediately before a cat or house dust mite allergen challenge, and at 48 hours after the challenge. Demographic information and degree of eosinophil induction after allergen challenge are shown in Table E1 in the online supplement. Two donors were excluded *post hoc* due to a less than 1% increase in eosinophils after the allergen challenge.

Statistical Analysis

Statistical analysis was performed in Matlab (R2013; Mathworks, Natick, MA). A two-tailed Student's *t* test was used for datasets with two groups. A one-way ANOVA followed by a *post hoc* multiple comparison test with the Tukey-Kramer correction was

used to compare within datasets of three or more groups. Statistical significance is reported at a P value less than 0.05.

Results

IL-13 Induces TF mRNA and Protein Expression in NHBE Cells

We examined the effect of IL-13 on TF mRNA expression and cellular TF protein in NHBE cells. Cells were exposed to IL-13 acutely (24 hours) or chronically (14 days). Compared with vehicle control (0 ng/ml), acute exposure to IL-13 at 10 and 50 ng/ml significantly increased TF mRNA expression by 2.1-fold and 3.1-fold, respectively (Figure 1A). Acute exposure to IL-13 (10 ng/ml) also increased TF protein expression (Figure 1B). Compared with the vehicle control, chronic exposure to IL-13 at 10 ng/ml for 14 days significantly increased TF mRNA expression by 1.9-fold (Figure 1C). Correspondingly, chronic exposure to IL-13 increased TF protein (Figure 1D). The increase in TF by chronic IL-13 treatment was accompanied by an increase in the number of goblet cells (data not shown), as reported previously (34).

Compressive Stress Increases TF mRNA Expression in NHBE Cells and TF Activity in the Basolateral Media

We previously showed that compressive stress at 30 cm H_2O increases TF mRNA expression in NHBE cells (21). Here, we examined if TF mRNA expression is dependent on the magnitude of pressure. Compressive stress induced TF mRNA expression in a pressure-dependent manner (Figure 2A). Compressive stress with 20 or 30 cm H_2O pressure significantly increased TF mRNA expression by 2-fold or 2.3-fold, respectively, compared with no pressure.

We previously showed that TF-positive extracellular vesicles are released into the basolateral CM from compressed NHBE cells in ALI culture (21). We measured the level of TF activity in the CM using a clotting assay (30). Compressive stress increased TF activity in the CM (Figure 2B).

We next examined the localization of TF within extracellular vesicles using transmission electron microscopy after immunogold labeling of the pellet obtained via ultracentrifugation. TF is a transmembrane protein, and, as expected, was present in the membra1ne of extracellular vesicles (Figure 2C).

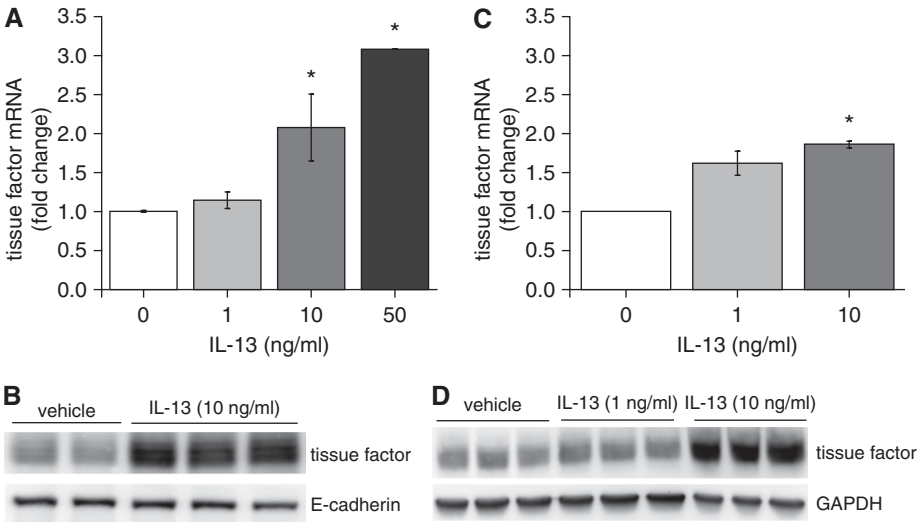

Figure 1. IL-13 induces tissue factor (TF) expression in normal human bronchial epithelial (NHBE) cells. TF expression was determined in NHBE cells after acute (*A* and *B*; at 10 or 50 ng/ml for 24 hours) or chronic (*C* and *D*; at 10 ng/ml for 14 days) exposure to IL-13. (*A* and *C*) TF mRNA expression (*A*, acute, four donors; *C*, chronic, one donor) was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression (mean ± SD). (*B* and *D*) Representative blots of cellular TF detected by Western blot analysis (*B*, acute; *D*, chronic). E-cadherin was used as a loading control in *B*, whereas GAPDH was used as a loading control in *D*. *Grayscale shading* corresponds to amount of cytokine used (*A* and *C*). *$P < 0.05$ versus 0 ng/ml.

Pretreatment with IL-13 Augments Compressive Stress–Induced TF mRNA Expression and Release of TF-Positive Extracellular Vesicles

As shown previously here, both IL-13 and compressive stress individually increase TF mRNA and protein expression. We next examined if IL-13 and compressive stress cooperatively increase TF expression. We pretreated cells with IL-13 (10ng/ml) for 21 hours followed by compressive stress for 3 hours, and then measured TF mRNA expression. Compared with vehicle control, IL-13 and compression alone each significantly induced TF mRNA expression by 1.7-fold and by 4.1-fold, respectively. The combination of IL-13 and compression increased TF mRNA expression by 5.5-fold, which was significantly higher than either stimulus alone (Figure 3A).

We then examined the effect of IL-13 on compressive stress–induced release of TF, measured 24 hours after the initiation of pressure. As shown in Figure 3B, TF was undetectable in the CM from cells incubated with either vehicle or IL-13 alone, but was present in the CM from cells exposed to compressive stress. Compared with compressive stress alone, pretreatment of cells with IL-13 augmented the release of TF into the basolateral CM (Figure 3B). The level of TF released by compressive stress in the absence or presence of IL-13 was quantified by densitometry analysis (Figure 3B) on triplicate wells in three independent experiments using primary cells from three normal donors. Pretreatment with IL-13 augmented compressive stress–induced release of TF by 2.7-fold over compressive stress alone.

TF released from compressive stress–treated cells was exclusively detected in the pellet after ultracentrifugation of CM whether treated with vehicle or IL-13, (Figure 3C). Actin was used as a marker for the extracellular vesicle–positive fraction (21, 35).

TF Is Elevated in the BAL Fluid of OVA-Challenged Mice

We previously reported that TF expression is significantly higher in the airway epithelium and BAL fluid from subjects with asthma versus normal subjects (21). Here, we examined if the increase in TF expression in patients with asthma can be recapitulated in an allergic mouse model of asthma. First, we examined differential TF expression in major airway and lung

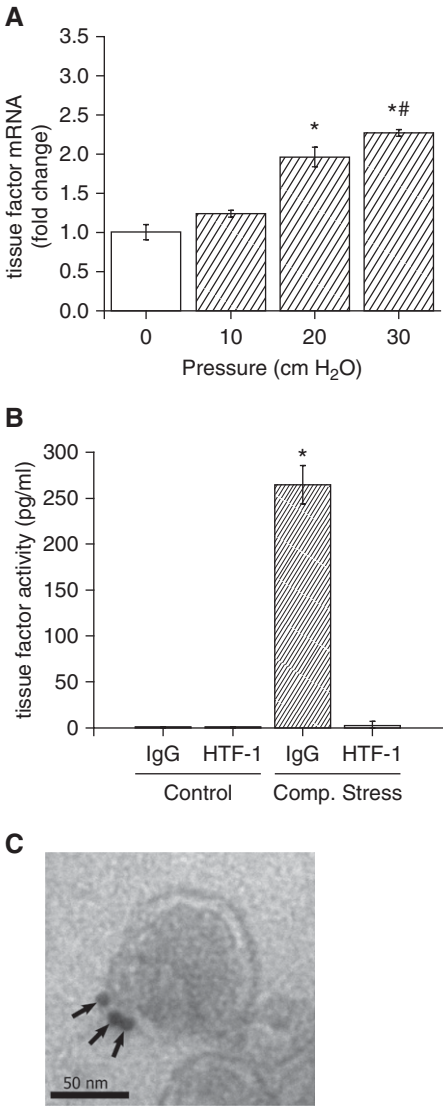

Figure 2. Compressive stress (Comp. Stress) induces TF mRNA expression and TF-dependent coagulation activity. (*A*) TF mRNA expression at 3 hours was normalized to GAPDH expression (mean ± SD, one donor). (*B*) Coagulation activity from conditioned media (CM) collected at 24 hours was measured in the presence or absence of HTF-1, a TF-blocking antibody (mean ± SEM, five donors). (*C*) A representative image of immunogold-labeled TF (*arrows*) in an extracellular vesicle by TEM. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus other conditions. *Diagonal stripes* indicate the application of compressive stress (*A* and *B*). *Scale bar,* 50 nm.

epithelial cells to determine which is the major cell type expressing TF. Among lung epithelial cells that we examined, basal cells showed the highest level of TF mRNA expression, over club and type 2 epithelial cells (Figure 4A). To confirm TF protein expression in basal cells, we analyzed TF protein by immunofluorescence staining in mouse trachea. TF was abundantly expressed in the layer of cells that costained with an antibody against p63, a basal cell marker (Figure 4B). TF staining was also detected on the luminal surface of the epithelium.

Using Western blot analysis, we detected TF in BAL fluid collected from mice (21). Compared with BAL fluid from PBS-challenged mice, BAL fluid from OVA-challenged mice had significantly elevated levels of TF, confirmed by densitometry analysis (Figure 5A). We also examined TF activity of the BAL

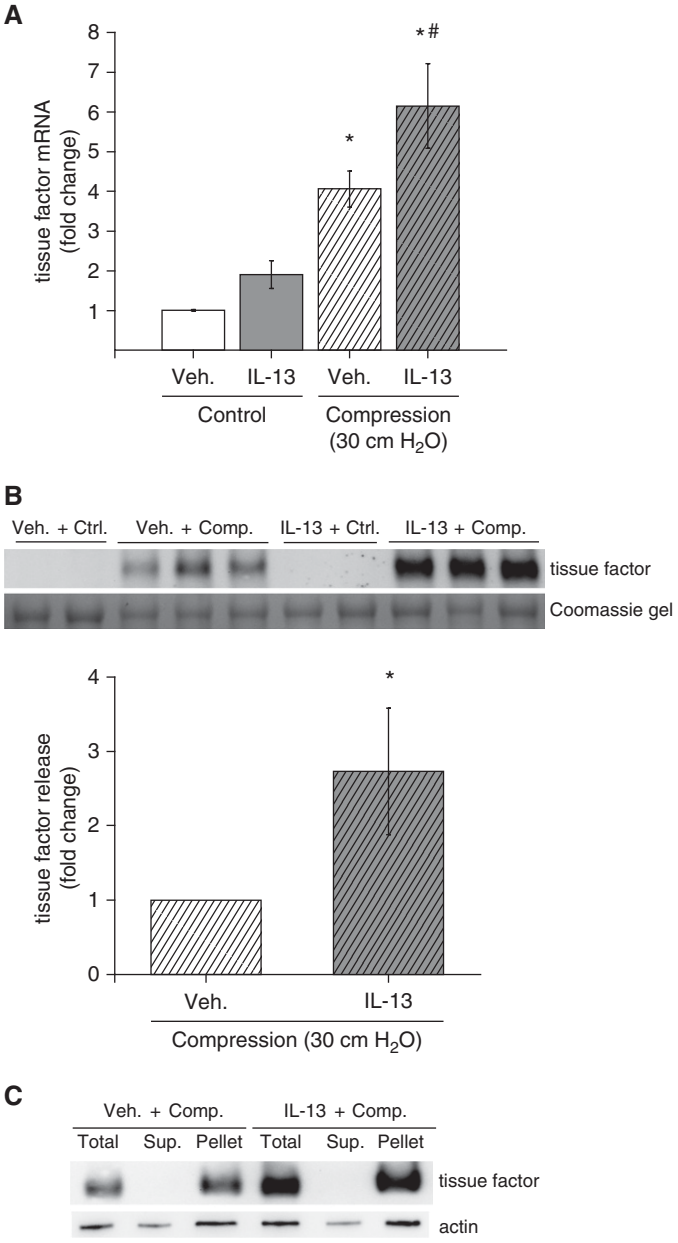

Figure 3. Pretreatment of IL-13 augments TF production in NHBE cells. TF mRNA expression (*A*, mean ± SEM, two donors) and released TF were determined (*B*, mean ± SEM, three donors) in the presence or absence of IL-13 (at 10 ng/ml for 24 hours). In the representative Western blot detections of TF in the whole CM (*B* and *C*) and in the extracellular vesicle fraction (*C*), Coomassie staining of SDS-PAGE (*B*) or actin (*C*) was used as a loading control. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus compressive stress alone. Comp., compressive stress; Ctrl., control; Pellet, extracellular vesicle fraction; Sup., supernatant; Veh., vehicle. *Grayscale shading* corresponds to amount of cytokine used, whereas *diagonal stripes* indicate the application of compressive stress (*A* and *B*).

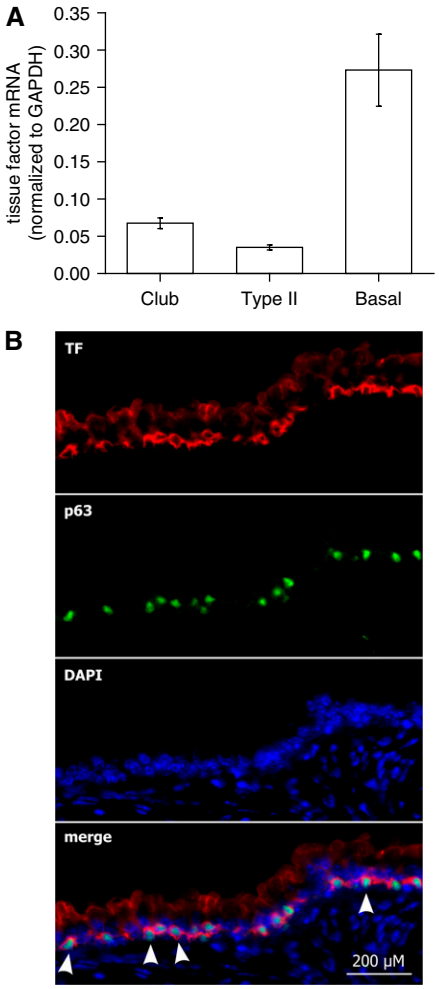

Figure 4. TF is predominantly expressed in basal cells. (*A*) TF mRNA expression in mouse lung epithelial cells was normalized to GAPDH. (*B*) Representative immunofluorescence staining of TF in the mouse trachea. P63 was stained as a marker for basal cells. *Arrowheads* indicate cells costained for TF and P63. DAPI, 4′,6-diamidino-2-phenylindole.

fluid and found that OVA-challenged mice had significantly greater TF activity compared with controls (Figure 5B). Last, we isolated extracellular vesicles using ultracentrifugation to determine if TF released into BAL fluid is contained in extracellular vesicles. TF was detected in the pellet, not exclusively, but predominantly, in both PBS-challenged and OVA-challenged mice (Figure 5C).

TF Is Elevated after Allergen Challenge in Patients with Asthma

To determine if TF is released into the airways of patients with asthma during asthma exacerbations, we detected TF in the BAL fluid of patients before and after exposure to an allergen challenge. The BAL fluid was collected from patients immediately before instillation of an allergen into the lungs, and collected again from the same location 48 hours later. Compared with the preallergen samples, TF protein levels were significantly higher in the postallergen samples in all patients (Figure 6).

Discussion

The mechanotransduction paradigm (36) of asthma pathogenesis hypothesizes that, even in the absence of inflammatory cells, compressive mechanical stimulation of airway epithelial cells initiates signaling cascades causing airway remodeling (9). This hypothesis has been tested *in vitro* (10, 11) and in humans (12). However, during asthmatic disease progression, we cannot separate the effects of bronchoconstriction from inflammation, as both occur. In fact, our data suggest that the production of TF is augmented in the inflamed airways as a result of the cooperative actions of pre-existing inflammatory mediators and bronchoconstriction. To test our

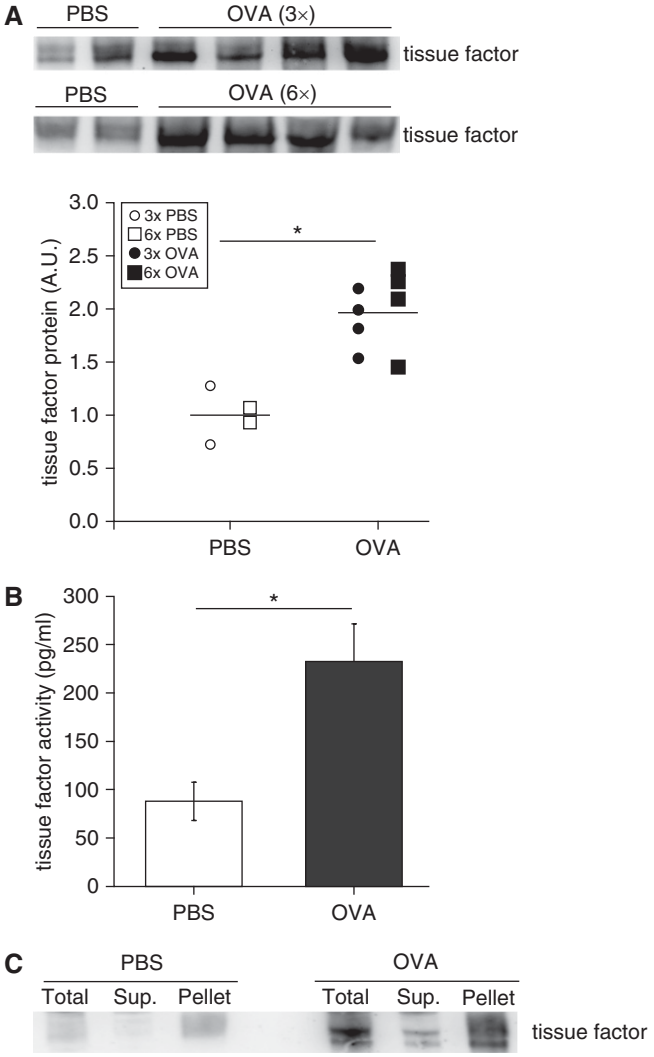

Figure 5. TF is elevated in bronchoalveolar lavage (BAL) fluid from the ovalbumin (OVA)-challenged mice. (*A*) Western blot analysis of TF in BAL fluid from mice. A.U., arbitrary units. (*B*) TF activity was measured in BAL fluid from mice (mean ± SEM; $n = 4$ PBS, $n = 8$ OVA). *$P < 0.05$ versus PBS control. *Open bar/open symbols*, control/PBS mice; *solid bar/solid symbols*, OVA-challenged mice (*A* and *B*). (*C*) Western blot analysis of fractionated BAL fluid from PBS and OVA-challenged mice. TF is present predominantly in the extracellular vesicle (pellet) fraction.

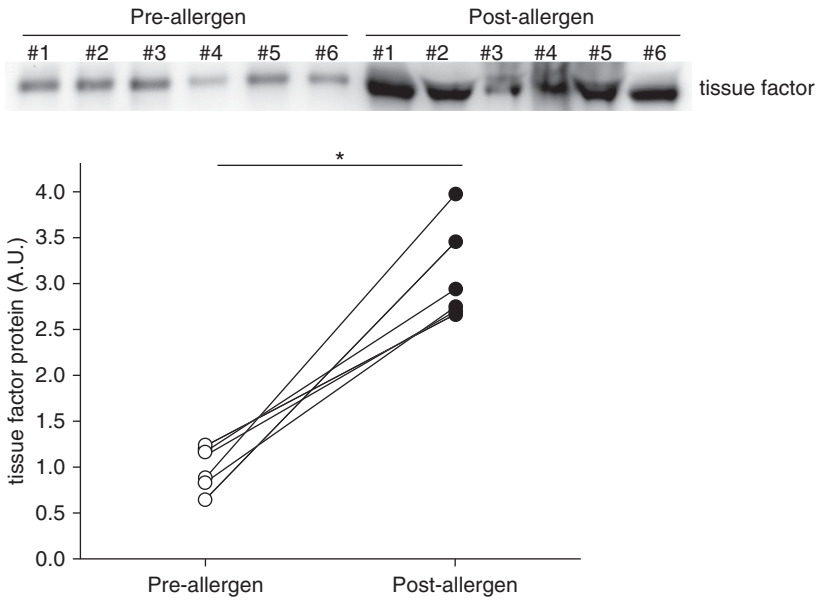

Figure 6. TF is elevated after allergen challenge in patients. Western blot detection of TF in BAL fluid from patients immediately before an allergen challenge and at 48 hours after the challenge. BAL fluid was collected from the same location of the airway in both procedures. *$P < 0.05$ preallergen versus postallergen groups.

hypothesis, we used an *in vitro* model of bronchoconstriction and a mouse model of allergic inflammation. Finally, to validate our findings, we used BAL fluid collected from patients with mild asthma after an allergen challenge.

From the multiple inflammatory mediators implicated in dysregulation of the airway epithelium in asthma, we chose to examine the type 2 cytokine, IL-13 (37, 38). *In vitro* and *in vivo* experiments have shown that IL-13 induces airway hyperresponsiveness (27, 28) and reduces β-adrenoreceptor–induced smooth muscle relaxation (39). The concentration of IL-13 is elevated in BAL fluid from many patients with asthma (40), and, therefore, IL-13 and compressive mechanical stress may act in concert in the asthmatic airway. Exposure of airway epithelial cells to IL-13 is well recognized to modify the expression of a variety of genes and proteins that play roles in asthma (26), although the effect of IL-13 on TF expression was unknown. We found that both acute and chronic exposure to IL-13 induced TF mRNA expression and cellular TF protein.

In our previous study, we reported that compressive stress at a magnitude of 30 cm H_2O pressure, which is the estimated compression in a maximally constricted airway (7), induces both mRNA expression and release of TF-positive extracellular vesicles from NHBE cells. We showed that TF mRNA expression is induced maximally at 3 hours and returns to baseline by 24 hours. Here, we find that compressive stress induced TF mRNA expression in a magnitude-of-pressure–dependent manner, rather than as a threshold effect. Therefore, we can speculate that the degree of TF production in the airway may depend on the severity of bronchoconstriction in patients with asthma, and suggest that bronchodilators not only relax constricted airways, but may also reduce the abnormal presence of TF.

In a recent study, Sebag and colleagues (41) found that mechanical stretch reduces LPS-induced TF mRNA expression, although mechanical stretch alone did not change TF mRNA expression. The discrepancy between our studies in TF mRNA expression may be due to differences in the type or duration of mechanical stimulation applied, or the use of different cell types. In particular, Sebag and colleagues used MLE12, alveolar type II–derived cells, from murine lungs, whereas we used primary NHBE cells from human airways. Despite the apparent contradiction in mRNA expression after mechanical stimuli, both studies support the conclusion that mechanical stimuli can alter TF-derived coagulation activity in the lung.

Coagulation activity in the airway lumen is critical for fibrin accumulation and asthma pathogenesis (42–44), and coagulant and fibrinolytic mediators can directly mediate inflammatory responses in the lung (45). Therefore, we determined the coagulation activity of CM. The coagulation activity of CM from compressed NHBE cells was totally abrogated by a TF-blocking antibody, indicating that the coagulation activity was TF dependent. These findings show that bronchial epithelial cells release active TF in response to compressive stress. We confirmed that TF is localized to the membrane of released extracellular vesicles.

Both IL-13– and bronchoconstriction-induced mechanical stress are important in asthma, but their cooperative role has never been studied. Here, we tested if priming airway epithelial cells with IL-13 can exaggerate TF production. Pretreatment of NHBE cells with IL-13 augmented the compressive stress–induced responses, mRNA expression, and release of TF-positive extracellular vesicles. Regardless of pretreatment, TF was released in extracellular vesicles. Although the function of TF released from bronchial epithelial cells remains unknown, TF contained in extracellular vesicles has the capacity to activate neighboring cells and the local microenvironment (18, 46). Together with the coagulation activity of CM from compressed cells, these data suggest that constriction of airway epithelial cells directly modulates their biochemical properties, and also indirectly modulates the airway microenvironment.

To validate our *in vitro* findings in a biologically relevant asthma model, we used a mouse model of allergic inflammation using OVA. Our data are the first to show enhanced TF expression under allergic inflammatory conditions in mice, indicating that this model will be useful for studying the function of TF in asthma. The distribution of TF in mouse lung epithelial cells was unknown, although, in humans, it is considered a basal cell marker (47, 48). We show that, as in humans, TF is abundantly expressed in basal epithelial cells in the mouse airway. Compared with mice exposed to PBS, TF and coagulation activity were significantly elevated in the BAL fluid collected from sensitized and challenged mice. These data recapitulate our previous observation that the airway epithelium of patients with asthma expresses a higher level of TF than that of control subjects (21).

To highlight our findings *in vitro* and in mouse studies, we used materials collected from patients with asthma who had been challenged with allergen. In all patients tested, the level of TF was elevated in the BAL fluid at 48 hours after a single challenge compared with BAL fluid collected from the same subjects before allergen challenge. Schouten and colleagues (49) previously showed that exposure to allergens increases the availability of TF in BAL fluid from patients with asthma at 4 hours after exposure. Here, we see that the effect of allergen challenge on available TF can be persistent over 2 days, potentially contributing to generation of an abnormally procoagulant microenvironment and chronic inflammation (13, 50) and aspects of airway remodeling (18, 51).

Although the role of TF has not yet been unequivocally established, our data suggest that TF is implicated in asthma pathogenesis. In addition to its well established role as a cellular initiator of coagulation, TF also plays a pathological role in a variety of diseases, including thrombosis, cancer, diabetes, and acute lung respiratory syndrome (52). As a pathologic mediator, TF contributes to coagulation, inflammation, cell migration, and angiogenesis, all of which occur during tissue remodeling (18, 53). Although approaches using TF-deficient mice will clearly elucidate the role of TF in asthma, we speculate here that TF plays a role in the remodeling process that occurs in asthmatic airways.

This is the first study to examine the combined action of biochemical and mechanical stimuli that could act cooperatively in the asthmatic airway epithelium. The responsiveness of epithelial cells to compressive stress can be exaggerated by the presence of a pre-existing asthma mediator. We tested our hypothesis that IL-13 enhances compressive stress–induced TF expression and release of TF-positive extracellular vesicles. The *in vitro* data show that compressive stress and IL-13 both act at the transcriptional level to increase mRNA expression of TF in NHBE cells, whereas only compressive stress acts to cause the release of membrane-bound TF into extracellular vesicles. The increased level of TF in BAL fluid from mice and humans challenged with allergen also supports the possible role of TF in asthma. Taken together, our results show that the availability of active TF in the lung was substantially increased by conditions associated with asthma. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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