TSG-6 Potentiates the Antitissue Kallikrein Activity of Inter- α -inhibitor through Bikunin Release

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TSG-6 (the protein product of TNF-stimulated gene-6), an inflammation-associated protein, forms covalent complexes with heavy chains (HCs) from inter- α -inhibitor and pre- α -inhibitor and associates noncovalently with their common bikunin chain, potentiating the antiplasmin activity of this serine protease inhibitor. We show that TSG-6 and TSG-6-HC complexes are present in bronchoalveolar lavage fluid from patients with asthma and increase after allergen challenge. Immunodetection demonstrated elevated TSG-6 in the airway tissue and secretions of smokers. Experiments conducted *in vitro* with purified components revealed that bikunin-HC complexes (byproducts of TSG-6-HC formation) release bikunin. Immunoprecipitation revealed that bikunin accounts for a significant proportion of tissue kallikrein inhibition in bronchoalveolar lavage after allergen challenge but not in baseline conditions, confirming that bikunin in its free state, but not when associated with HCs, is a relevant protease inhibitor in airway secretions. In primary cultures of differentiated human airway epithelial and submucosal gland cells, TSG-6 is induced by TNF- α and IL-1 β , which suggests that these cells are responsible for TSG-6 release *in vivo*. Bikunin and HC3 (i.e., pre- α -inhibitor) were also induced by TNF- α in primary cultures. Our results suggest that TSG-6 may play an important protective role in bronchial epithelium by increasing the antiprotease screen on the airway lumen.

Keywords: TSG-6; I α I; tissue kallikrein; bikunin; airway

Tissue kallikrein (TK) is a serine protease involved in airway inflammatory responses toward a variety of stimuli, such as allergens, bacterial products, neutrophil elastase, and ozone exposure (1–5). All of these insults are associated with oxidative stress; thus, inhibition of TK activity and scavenging of reactive oxygen species (ROS) have been shown to prevent the resulting airway hyper-responsiveness and/or bronchoconstriction (1–6). TK cleaves high- and low-molecular-weight kininogen to yield lysyl-bradykinin (kallidin), which is converted by a neutral endopeptidase into bradykinin. Kallidin and bradykinin cause vasodilatation (7, 8), increase vascular permeability (9), and mediate bronchoconstriction and airway hyperresponsiveness (10, 11), all hallmarks of airway inflammation in diseases such as asthma and chronic bronchitis.

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CLINICAL RELEVANCE

This original work describes a previously unrecognized antiprotease system operating in human airways. The findings of this study may provide new tools for the prevention or/and treatment of airway diseases.

In normal conditions, TK is bound and inhibited by hyaluronan (HA) (12), a nonsulfated glycosaminoglycan present in the apical pole of airway epithelium (13). Depolymerization of HA by ROS or hyaluronidase results in the release and activation of TK (14), a finding further supported by the ability of aerosol-delivered HA in preventing ROS and TK-mediated responses (15, 16). Because protease inhibitors in the airway lumen are weak or inefficient against TK (17), during ROS-induced HA depolymerization, additional mechanisms are likely to exist to control TK catalytic activity and thus resolve inflammation.

TSG-6, the protein product of TNF-stimulated gene-6 (18), also referred to as TNFIP6 (19), is differentially induced by inflammatory cytokines (e.g., TNF- α , IL-1, IL-17), growth factors (e.g., transforming growth factor- β), lipopolysaccharide, and prostaglandin E2 in various cell types, including fibroblasts, vascular/cervical smooth muscle cells, monocytes, neutrophils, vascular endothelial cells, proximal tubular epithelial cells, synovial cells, chondrocytes, and cumulus cells (20–22). TSG-6 protein has been detected at high levels in inflammatory conditions—for example, in synovial fluids and joint tissues from individuals with rheumatoid arthritis and osteoarthritis (23–25), and in the ovaries of various mammals during cumulus oocyte complex expansion before ovulation (26, 27).

The induction of TSG-6 serves a variety of structural and antiinflammatory functions, such as the inhibition of neutrophil chemotaxis (25, 28, 29) and the stabilization of HA-rich extracellular matrices (e.g., in the cumulus oocyte complex) (19) via its interactions with inter- α -inhibitor (I α I) (19, 27) and pentraxin-3 (30); TSG-6 has been found to be essential for female fertility in mice (19, 22) and to protect joint tissues (e.g., cartilage) from degradation in animal models of arthritis (31).

I α I, a member of a family of human plasma serine-protease inhibitors, consists of three polypeptide chains that are covalently linked by chondroitin sulfate: two heavy chains (HC1 and HC2) and a light chain termed bikunin, which contains two Kunitz domains responsible for its antiprotease activity (32, 33). TSG-6 has been found to form covalent complexes with HC1 and HC2 (34, 35), which act as intermediates in the covalent transfer of these heavy chains onto HA (27) where TSG-6 acts as an essential cofactor and catalyst in these transfer reactions (27). Studies on the Tsg-6^{-/-} mouse (19) and recent biochemical experiments (27) reveal that TSG-6 is involved in the covalent transfer of HC3 onto HA from the related molecule pre- α -inhibitor (P α I), which consists of a single heavy chain (HC3)

linked to bikunin via chondroitin sulfate. Although IαI circulates at high concentrations in plasma (36), its antiprotease activity is weak (37), suggesting that additional functions unrelated to protease inhibition and/or mechanisms for increasing its inhibitory activity operate in tissues where IαI “leaks” during inflammatory responses. In this regard, TSG-6 has been shown to potentiate the antiplasmin activity of IαI (25, 28) as the result of an interaction between the Link module domain of TSG-6 and the bikunin chain of IαI (38).

Because TSG-6 is induced by cytokines that are upregulated in airway inflammation (39–41), we hypothesized that TSG-6 might increase IαI antiprotease activity against TK in the bronchial lumen. The present work was aimed at determining whether TSG-6 and IαI/PαI are induced during inflammation in human airways and, if so, whether they regulate TK activity. We tested this hypothesis *in vitro* using primary cultures of normal human epithelial (NHE) and submucosal gland (SMG) cells and *in vivo* by comparing samples obtained from healthy volunteers with those from patients with asthma and cigarette smokers (i.e., two conditions associated with airway inflammation).

MATERIALS AND METHODS

Human Tracheal Aspirates

Following a protocol approved by the Institutional Review Board at the University of Miami, human tracheal aspirates (HTA) were collected from patients (9 women and 6 men; age range, 17–62 yr [SEM 37 ± 8 yr]) undergoing general anesthesia for elective surgery indicated for nonpulmonary reasons as described previously (42). These patients had no symptoms of acute exacerbations or infection. Active smokers ($n = 5$, at least 10 packs/yr) fulfilled clinical and histologic criteria for chronic bronchitis. Ex-smokers ($n = 5$) did not smoke for at least 2 yr, and nonsmokers ($n = 5$) reported that they never had smoked. Bronchial secretions were collected by instilling 4 ml saline through a suction catheter that was advanced through an endotracheal tube into the trachea followed by immediate suctioning. The samples were centrifuged at $500 \times g$ for 5 min to remove cells, and the supernatant was centrifuged at $16,000 \times g$ for 20 min at 4°C. The second supernatant was stored at –20°C until use.

BAL

BAL samples were obtained from four patients with allergic asthma and four healthy subjects before and 24 h after segmental challenge with ragweed antigen. These samples were provided by Drs. A. Hastie and S. Peters (Thomas Jefferson University, Philadelphia, PA). The chosen individuals with asthma had positive skin test and known sensitivity to ragweed. To confirm that the patients with asthma developed an inflammatory reaction to allergen challenge, samples were assayed for TK activity (11) as described below. Results are expressed in $\mu\text{U}/\text{mg}$ protein, a Unit being defined as 1 mol of substrate degraded per minute at 37°C. Eosinophil-like peroxidase activity was determined using a colorimetric assay as previously reported (43). This assay is performed in the presence of Dapsone (10^{-4} M), and although it does not distinguish between lactoperoxidase and eosinophil peroxidase activities, it excludes myeloperoxidase activity at the used concentration. Results are expressed as ng/mg of protein in BAL fluid (BALF) using lactoperoxidase as standard.

Full-Length Human TSG-6 and IαI

Full-length TSG-6 protein (Q allotype; TSG-6Q) was expressed in *Drosophila* Schneider 2 cells and purified by ion exchange chromatography and reverse-phase HPLC (44). The protein concentration was determined by amino acid analysis as described previously (44). IαI was purified from human serum (45), and its concentration was determined as described previously (46). Human PαI was a generous gift from Dr. Mizon (Faculté de Pharmacie, Lille, France) and was purified as described (47).

Generation of Bikunin and HC3 Antibodies

These polyclonal antibodies were raised in rabbits by Mimotopes Pty Ltd. (Clayton, Australia) using peptides corresponding to the N-terminal 10 amino acids of human bikunin (i.e., AVLPEEEEGSC-NH₂) or HC3 (SLPEGVANGIC-NH₂), which were coupled to a Diphtheria toxoid carrier protein via the nonauthentic cysteine residue at their C-termini. The anti-bikunin and anti-HC3 antibodies were characterized by Western blot analysis using purified preparations of IαI, PαI, and TSG-6/IαI complexes (27) formed by incubating TSG-6 (80 $\mu\text{g}/\text{ml}$) and IαI (320 $\mu\text{g}/\text{ml}$) in 20 mM HEPES-HCl (pH 7.5), 150 mM NaCl, and 5 mM MgCl₂ for 4 h at 37°C. Briefly, IαI, PαI, and TSG-6/IαI reaction mixtures were run untreated or after treatment with chondroitinase ABC lyase or NaOH (27) under reducing conditions on 10% (wt/vol) Tris-Tricine SDS-PAGE (at 800 ng/lane IαI/PαI) and electroblotted onto Hybond-P membranes as described (46). The blots were incubated with the antisera at 1:10,000 dilution and developed (46). The identity of the released bikunin was further confirmed by protein sequence analysis as described previously (27).

Immunoblotting

For the visualization of TSG-6 and IαI in the BALF of patients with asthma or in the *in vitro* generated TSG-6-HC complexes, samples containing equal amounts of protein were electrophoresed on 4–15% (wt/vol) polyacrylamide minigels (BioRad, Hercules, CA) and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). After blocking with 1% (wt/vol) gelatin in Tris-buffered saline containing 0.05% (vol/vol) Tween-20, the membranes were probed with the appropriate antibody. In the case of the BAL samples, rabbit anti-human TSG-6 (1/10,000) (48) or rabbit anti-human IαI (1/10,000; DAKO) were used. For visualization of bikunin in the TSG-6-HC reaction mixes, samples for each time point were analyzed by Western blotting as described (44) using a 1 in 10,000 dilution of the rabbit polyclonal antiserum specific for bikunin as described previously.

Quantitative Analysis of TSG-6

TSG-6 protein levels in bronchial secretions from nonsmokers ($n = 5$), smokers ($n = 5$), and ex-smokers ($n = 5$) were determined by ELISA. Nunc Maxisorp 96-well plates (Nunc, Rochester, NY) were coated overnight with 50 μl of 10 $\mu\text{g}/\text{ml}$ mAb A38 (49) specific for TSG-6 in bicarbonate buffer (pH 9.2). Plates were washed with PBS and blocked with 0.25% (wt/vol) BSA and 0.05% (vol/vol) Tween-20 in PBS for 30 min at room temperature. Plates were again washed with PBS and incubated with 50 μl of HTA or TSG-6Q standards diluted in blocking buffer. After 2 h at room temperature, wells were washed with PBS followed by 50 $\mu\text{l}/\text{well}$ of 0.5 $\mu\text{g}/\text{ml}$ Q75 anti-TSG-6 antibody (49), which had been conjugated using the Versalinx Alkaline phosphatase-protein conjugation kit (Calbiochem, San Diego, CA) to give Q75-AP. After 2 h, plates were washed with PBS. Color was developed with p-nitrophenylphosphate and stopped with 3N NaOH. Absorbance was read at 410 nm using the SPECTRAMax plus microplate reader, and TSG-6 concentrations were determined by interpolation from the standard curves using TSG-6Q with the SOFTmax software, both from Molecular Devices (Sunnyvale, CA). Because mAb A38 recognizes an epitope within the TSG-6 Link module domain that overlaps with its HA-binding site (49), experiments were done to determine if HA/TSG-6 complexes that are likely to be present in these samples were equally recognized by these antibodies. Aliquots from four of these samples (two smokers and two nonsmokers) were treated with 10 mU of hyaluronidase from *Streptomyces* (Seikagaku Corp., Tokyo, Japan) for 3 h at 37°C and compared with nondigested samples by dot-blot. Membranes were photographed using the Chemidoc XRS system and analyzed using the Quantity One software (both from BioRad).

TSG-6-HC Complex Formation

TSG-6-HC complexes were formed by incubating recombinant full-length human TSG-6Q (80 $\mu\text{g}/\text{ml}$) with purified human IαI (320 $\mu\text{g}/\text{ml}$) in 20 mM HEPES-HCl (pH 7.5), 150 mM NaCl, and 5 mM MgCl₂ at 37°C for 0.5, 5, 30, 120, or 240 min as described (27, 44). At the 240-min time point, 5 μl of the reaction mix was treated with 1 μl chondroitinase ABC lyase (10 U/ μl) and incubated for 2 h at 37°C.

Recombinant TK

Recombinant tissue kallikrein (rTK) was produced in *Pichia pastoris* as described (14, 50). Briefly, *Pichia pastoris* (strain GS115) were transfected with a plasmid encoding rTK (pPIC9pro-TK) generously donated by Dr. Hedy Chan (Axys Pharmaceuticals) using a *Pichia* Expression kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The plasmid contains the full coding sequence of human salivary pro-TK, including a signal sequence to allow secretion into the media, where expression is driven by the alcohol dehydrogenase promoter. The secreted rTK was purified by chromatography as previously reported (14, 50) and lyophilized before use.

Determination of K_m and K_i Values

The enzymatic activity of TK, including its kinetic constants, was determined using a chromogenic substrate as described previously (12, 51). Briefly, triplicate samples containing rTK (10 nM) were incubated in an ultralow-binding 96-well plate in the presence of increasing concentrations of I α I (50 nM to 50 μ M) and TSG-6Q (750 nM to 750 μ M) individually or preincubated for 30 min at 37°C (to generate TSG-6-HC complexes) in 20 mM HEPES-HCl (pH 7.5) 150 mM NaCl, 5 mM MgCl₂, and 0.02% (vol/vol) Tween-20 for 2 h at 37°C (final volume 100 μ l). After these incubations, 100 μ l of the peptide substrate DL-Val-Leu-Arg-pNA (Bachem, Torrance, CA) in 200 mM Tris (pH 8.2) and 0.02% (vol/vol) Tween 20 was added at final concentrations ranging from 0.05 to 4 mM, and absorbance was monitored at 405 nm using a microplate reader (Molecular Devices). Kinetic constants (K_m and V_{max}) were calculated from the initial rates recorded (20 min) at different substrate concentrations (0.1 μ M to 200 mM). Inhibition constants (K_i) were determined by measuring TK activities at various inhibitor and substrate concentrations and calculated by nonlinear regression analysis of the activities using equations for different types of inhibition (Plowman and Cornish-Bowden) using SOFTmax software (Molecular Devices). Data were fitted using the Lineweaver-Burk double-reciprocal plot.

Bikunin Immunoprecipitation

A total of 300 μ l of BALF ($n = 4$) or normal human bronchial epithelial cells (NHBE) apical washes ($n = 2$) were incubated with 5 μ l of rabbit anti bikunin antipeptide or rabbit serum control during 12 h at 4°C. Protein A-sepharose beads (Amersham Biosciences) were added and incubated for 1 h. After centrifugation for 5 min at 300 $\times g$, pellets were used for Western blot, and the enzymatic activity of TK was assessed in the supernatants as described below.

Cell Cultures

Samples from human lung donors who had been rejected for transplantation were obtained through the University of Miami Life Alliance Organ Recovery Agency with approval from the local Institutional Review Board.

Primary Cultures of NHE Cells at the Air-Liquid Interface

NHE cells at the air-liquid interface were grown as described previously (52, 53) in an incubator at 37°C in ambient air supplemented with 5% (vol/vol) CO₂. Their apical surface was exposed to air as soon as they reached confluence, and cells were used for experiments when they reached full redifferentiation (~ 3 wk) as assessed by positive labeling with antibodies to acetylated tubulin (that specifically label ciliated cells) and the visual confirmation of beating cilia and mucus.

Primary Cultures of SMG Cells

SMG cells were grown as described (12, 14). After 10 d of growth, cultures showed positive labeling with antibodies against TK and lysozyme (specific serous cell products) and Muc 5AC (specific mucous cell product) that accounted for $89 \pm 18\%$ of cells ($n = 5$ cultures, 100 cells counted per well; mean $\pm$ SEM).

Protocols

NHBE and SMG cultures were exposed at their basolateral compartments to TNF- α (20 ng/ml) or IL-1 β (50 ng/ml).

RT-PCR

RNA (2 μ g) was extracted from NHE and SMG cell cultures using Trizol (Invitrogen, Carlsbad, CA), and cDNA was obtained using a SuperScript First Strand Synthesis System for RT/PCR kit (Invitrogen). TSG-6 specific oligonucleotide primers were designed according to Kehlen and colleagues (21): sense (s), 5'TTGATTGGAAACCTCCAGC3', antisense (as) 5'CCAGGCTTCCCAAATGAGTA3'. Primers specific for the individual chains of I α I and P α I were designed according to Bourguignon and colleagues (54): bikunin (s) 5'GTCCGGAGGGCTGTGCTACC3', (as) 5'GATGAAGGCTCGGCAGGGGC3'; HC1 (s) 5'CCACCC ATCGGTTTTGAAGTGTC3', (as) 5'TGCCACGGGTCCTTGCTGTAGTCT3'; HC2 (s) 5'ATGAAAAGACTCACGTGCTTTTTC3', (as) 5'ATTTGCCTGGGGCCAGT3'; and HC3 (s) 5'TGAGGAGGTGGCCAACCCACT3', (as) 5'CGCTTCTCCAGCAGCTGCT3'. The final reaction mixture (0.025 μ g/ μ l oligodT, 0.5 mM dNTPs, 1 $\times$ RT buffer, 5 mM MgCl₂, 0.01 M DTT, and RNase inhibitor) was incubated with SuperScript II reverse transcriptase (2.5 U/ μ l) for 50 min at 42°C. The reaction was terminated at 37°C for 20 min with RNase H. To control for genomic DNA contamination, additional RNA samples were processed without reverse transcriptase. First-strand cDNAs were amplified by PCR using Taq polymerase (2.5 U/ μ l) and the oligonucleotide primers described below. Initial denaturation at 95°C for 300 s was followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and elongation at 72°C for 20 s using a thermal cycler (Brinkmann Instruments Inc., Westbury, NY).

Products of the RT-PCR reaction were subjected to sequence analysis performed by the DNA Core at the University of Miami School of Medicine. Actin gene expression was used to normalize the amplified products. Gels were photographed using the Chemidoc XRS system and analyzed using the Quantity One software (BioRad).

Immunofluorescence

Formalin-fixed sections of human trachea were deparaffinized, hydrated, and incubated in acetone (-20°C) for 15 min. Sections were rinsed with PBS and incubated in a blocking solution consisting of 1% (wt/vol) BSA in PBS for 1 h. Endogenous Biotin Blocking kit was used according to the manufacturer's instructions (Molecular Probes, Eugene, OR). For colocalization studies, sections were incubated with rabbit anti-human I α I (Dako, 1:1,000) or bikunin (1:1,000) antibodies diluted in blocking solution overnight in a humidity chamber at 4°C. After labeling with Alexa 555-conjugated goat anti-rabbit IgG (Molecular Probes), the sections were washed with PBS and incubated for 2 h with rat anti-TSG-6 Q75 (49) (1 μ g/ml) followed by a biotinylated anti-rat IgG antibody (Molecular Probes) and FITC-avidin-DCS (Vector Laboratories, Burlingame, CA) to visualize TSG-6. Detection of HC3 was achieved by using the HC3-specific antibody described previously and FITC-conjugated anti-rabbit IgG (Vector) as second antibody. Nuclei were stained using DAPI (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD), and images were obtained using a Zeiss LSM-510 confocal laser-scanning microscope (Zeiss, Germany) at the University of Miami Analytical Imaging Core Facility.

Statistical Analysis

Data were expressed as mean $\pm$ SEM. Differences between multiple groups were tested for significance using a one-way ANOVA followed by the Tukey-Kramer honestly significant difference test. The Levene test was used to analyze the homogeneity of variances.

RESULTS

TSG-6, TSG-6-HC, and HC-HA Complexes Are Present in the BALF of Individuals with Asthma

To determine if TSG-6 is induced in the airways and to evaluate if it is associated with heavy chains of I α I/P α I, which are expected to "leak" from the vasculature during inflammatory responses, Western blots of samples of BALF obtained from four healthy control subjects and four patients with asthma before and 24 h after allergen challenge were analyzed using antibodies to TSG-6

Figure 1. TSG-6 and TSG-6-HC complexes are present in the BALF of individuals with asthma. BALF obtained from two representative patients with asthma (denoted 1 and 2) were analyzed by Western blotting using polyclonal antibodies to TSG-6 and IαI as described in MATERIALS AND METHODS. All wells were loaded with 250 μ g of total protein. BAL samples taken before antigen challenge are labeled 1a and 2a; BAL samples taken 24 h after challenge are labeled 1b and 2b. The image was inverted (black to white) to highlight bands.

and IαI. As can be seen in Figure 1 (where two representative BALF samples are shown), a band of ~ 120 kDa was recognized by both antibodies likely corresponding to covalent complexes of TSG-6 with the heavy chains (i.e., TSG-6-HC) (27). In addition, a band of ~ 35 kDa was seen with the anti-TSG-6 antiserum, corresponding to free (i.e., uncoupled) TSG-6. This signal was much more intense in the BALF of patients with asthma after allergen challenge (Samples 1b and 2b), suggesting that TSG-6 levels are increased after allergen challenge. With the anti-IαI antiserum, a band of ~ 180 kDa is visible, and above this there was a smear of immunoreactivity up to very high molecular weights. These species are likely to correspond to intact IαI and heavy chains covalently linked to HA (i.e., HC-HA), respectively. TSG-6 and TSG-6-HC complexes were not observed in the BALF obtained from normal individuals (data not shown), suggesting that their concentrations were below our detection limit of 1 ng. To confirm that allergen challenge induced an inflammatory response in these individuals, TK activity and eosinophil peroxidase activity were measured. TK activity increased in patients with asthma after allergen challenge from 45.6 ± 22.1 to 457.9 ± 144.3 μ U/ml (mean $\pm$ SEM; $P = 0.003$), whereas in healthy volunteers this did not change significantly (28.2 ± 11.3 to 44.4 ± 10.8 μ U/ml [mean $\pm$ SEM]; $P > 0.05$) (Figure 2, top). Likewise, peroxidase activity (Figure 2, bottom) did not change after allergen challenge in normal individuals (55.9 ± 24.1 to 194 ± 180 ng/ml [mean $\pm$ SEM]; $P > 0.05$) but significantly increased in individuals with asthma from 62.5 ± 50.8 to 758.4 ± 260.8 ng/ml ($P = 0.0002$).

Characterization of Bikunin and HC3 Antipeptide Antibodies

Because the antibikunin and anti-HC3 antibodies used here are newly generated reagents that have not been used previously, they were fully characterized on Western blots (Figure 3). This analysis with the antibikunin antiserum (Figure 3, top) indicates that this antibody can detect free bikunin (e.g., as released from IαI, PαI, or TSG-6/IαI reaction mixtures by chondroitinase treatment) (lanes 3, 6, and 8, respectively). In addition, it detects bikunin-CS (i.e., bikunin linked to chondroitin sulfate) as formed

Figure 2. Eosinophil-like peroxidase and TK activities are elevated after ragweed allergen challenge in patients with asthma but not in healthy volunteers. BALF from healthy volunteers had no significant changes after allergen ($P > 0.05$), whereas patients with asthma responded with increases in peroxidase activity and TK activity. * $P < 0.05$. Error bars are SEM.

by NaOH-treatment of IαI (lane 2), which is also seen due to the breakdown of the bikunin-HC1/bikunin-HC2 by-products (lane 7) generated during the formation of TSG-6-HC2/TSG-6-HC1 complexes (27). Bikunin-HC complexes (lanes 1, 2, 4, 5, and 7) and intact IαI (lanes 1 and 7) are also detected by this antiserum. In the case of bikunin-HC complexes, these can correspond to bikunin-HC1/bikunin-HC2, which are present as contaminants in IαI preparations (lane 1) and also formed as by-products of TSG-6-HC complexes (lane 7) or as bikunin-HC3 (i.e., intact PαI; lane 4). Thus, it is clear that the antibikunin antibody raised here can detect a range of bikunin-containing species, including free bikunin (with or without a CS chain attached), bikunin HCs, and intact IαI and PαI. Of these species, the intact IαI is the most poorly recognized, suggesting that when bikunin is linked to two heavy chains, this may hinder the binding of the antibody.

In the case of the anti-HC3 antisera (Figure 3, bottom), this recognizes free HC3, released from PαI by NaOH or chondroitinase treatment (lanes 5 and 6, respectively) and from the intact PαI protein (lane 4). Although the intact PαI is only weakly detected by this antiserum compared with free HC3, there is no cross-reactivity with IαI (lanes 1–3) or TSG-6/IαI complexes (lanes 7–8). Furthermore, in lane 4, which corresponds to purified PαI, the major band detected is that of the intact protein; a diffuse high-molecular-weight species is also present that seems to be detected with the antibikunin antiserum. Additional experiments using antibodies preadsorbed with the antigenic peptides for immunofluorescence showed no positive labeling (data not shown) confirming their specificity.

These data show that these antibodies are suitable for specific recognition of bikunin and HC3 in human biological samples.

TSG-6 Is Present in Human Airway Secretions and Is Elevated in Smokers

To test whether TSG-6 is induced in association to other inflammatory conditions, HTA obtained from smokers were compared with HTA obtained from nonsmokers and ex-smokers (see MATERIALS AND METHODS). Analysis of these aspirates showed a significant increase in TSG-6 protein content in the bronchial secretions of smokers compared with nonsmokers (i.e., 123.3 ± 32.8 ng/mg protein [$n = 5$] versus 40.4 ± 8.3 ng/mg protein [$n = 5$], respectively) (Figure 4). This elevation was likely associated with smoke-induced inflammation because ex-smokers ($n = 5$) showed values that were not significantly different from normal individuals (i.e., 34.2 ± 12.4 ng/mg protein).

Analysis of samples treated with hyaluronidase by dot-blot demonstrated that TSG-6 and TSG-6/HA complexes were equally assessed using this assay (data not shown). The result

Figure 3. Characterization of antibikunin and anti-HC3 antibodies by Western blot analysis. Blots containing I α I (lanes 1–3), P α I (lanes 4–6), and TSG-6-I α I complexes (lanes 7–8), untreated (lanes 1, 4, and 7) or treated with NaOH (lanes 2 and 5) or chondroitin ABC lyase (Ch'ase; lanes 3, 6, and 8), were probed with antibikunin (top) and anti-HC3 (bottom) antisera, both at 1:10,000 dilution, where these were developed for 10 s and 2 min, respectively; no bands were seen with equivalent dilutions of preimmune sera (data not shown). In lane 2 (top), the NaOH-mediated release of bikunin-CS from I α I (via degradation of the ester bond that links the HCs to CS) has clearly not gone to completion because bikunin-HC complexes (i.e., HCs linked to bikunin via a CS chain) are present; this is in contrast to chondroitinase (Ch'ase) digestion of I α I, which leads to complete degradation of I α I to yield free bikunin (lane 3). An ~ 45 kDa band in lane 6 (top panel, labeled A) is present in P α I after chondroitinase digest, indicating that this bikunin-related species (which is also present in untreated P α I preparations [lane 4] and is larger than bikunin-CS formed from I α I) may correspond to bikunin linked to a different glycosaminoglycan or potentially another protein. In lane 6 (lower panel), the identity of the high-molecular-weight species formed on chondroitinase digestion of P α I (labeled B) is unknown.

that HA did not interfere with the binding of TSG-6 antibodies was expected because A38 can effectively compete for HA binding, where this antibody is believed to have a much higher affinity for TSG-6 relative its affinity for HA (49). This indicates that this ELISA is suitable for assessing TSG-6 concentration in biological samples where HA is likely to be present.

TSG-6 Potentiates I α I Antiprotease Activity

Given that TSG-6 is released into the airway lumen during inflammation and that I α I is also present, as shown by the presence of TSG-6-HC complexes, we thought that TSG-6 could potentiate the antiprotease activity of I α I against TK as it does with plasmin (25, 28, 38). To test this hypothesis, the effects of I α I and TSG-6 (and mixtures of these proteins) on TK activity were

Figure 4. TSG-6 levels in tracheal aspirates. TSG-6 is elevated in the airway secretions of smokers. HTA were obtained from nonsmokers, smokers, and ex-smokers ($n = 5$ for each group), and TSG-6 levels were measured by ELISA as described in MATERIALS AND METHODS. Results are expressed as ng/mg of protein. * $P < 0.05$ compared with nonsmoker. # $P > 0.05$ compared with nonsmoker. Error bars are SEM.

investigated in an *in vitro* assay. The inhibitory activities of these purified proteins alone or in complexes against TK are summarized in Table 1, where the inhibition constants were determined as described in MATERIALS AND METHODS. As expected, I α I alone was found to be a weak inhibitor of TK activity with a K_i of 24 mM. However, this was greatly enhanced after chondroitinase treatment, leading to a K_i value of 1.2 nM and suggesting that bikunin, as part of I α I, is not an effective inhibitor of TK but can become a potent inhibitor via its release from the intact protein. Incubation of TSG-6 with I α I, under conditions that are optimal for TSG-6-HC complex formation, increased the anti-TK activity of I α I lowering the K_i to 12.1 μ M, whereas TSG-6 alone did not have any inhibitory activity ($K_i > 200$ mM).

TSG-6-HC Complex Formation Results in the Release of Free Bikunin

Free bikunin was detected in samples in which TSG-6 and I α I are incubated together, where there was an increasing amount of the free protein seen over the time course of the reaction (Figure 5). No bikunin was present after 0.5 or 5 min, even though bikunin-HC species, which are by-products of TSG-6-HC complex formation (27), were clearly detected in these samples. Free bikunin was present in the 30-, 120-, and 240-min time points. This is consistent with our previous data indicating that free bikunin results from the breakdown of the bikunin-HC by-products rather than being released as part of the mechanism of TSG-6-HC complex formation (27). The bikunin resulting from chondroitinase digestion of TSG-6/I α I reaction mixtures (Figure 4) is of lower molecular weight than the bikunin released during complex formation, indicating that in the latter case there is no fission of the chondroitin sulfate chain. It is most likely, therefore, that the release of bikunin-CS from bikunin-HC results from the hydrolysis of the ester bond linking the heavy chain

TABLE 1. KINETIC CONSTANT OF INHIBITION OF TISSUE KALLIKREIN

Protein Sample	K_i
I α I	24 mM
TSG-6	> 200 mM
I α I/TSG-6	12.2 μ M
I α I + chondroitinase	1.2 nM

Definition of abbreviations: K_i , inhibition constant; TSG-6, TNF-stimulated gene 6.

Figure 5. TSG-6-I α I complex formation results in the release of free bikunin. TSG-6-I α I complexes were formed by incubating full-length human TSG-6 with purified human I α I for various time points and analyzed by Western blotting with an antibikunin antibody. This antibody recognizes the bikunin-HC complexes produced as by-products during TSG-6-HC complex formation in addition to free bikunin, which results from the breakdown over time of the bikunin-HC species. Chondroitinase treatment of the 240-min time point gives rise to a bikunin species lacking its chondroitin sulfate moiety, which is of lower molecular weight than the bikunin released during TSG-6-HC complex formation that still has its CS chain attached. This demonstrates that the release of free bikunin-CS does not involve fission of the chondroitin sulfate chain. After chondroitinase treatment, only a single species is seen with the antibikunin antibody, which corresponds to free bikunin. This indicates that all the immunoreactive species seen in the other lanes are likely to contain bikunin.

to the CS. These experiments provide evidence that TSG-6 can enhance the anti-TK activity of I α I via the release of bikunin.

Released Bikunin Inhibits TK Activity in the Airway Lumen

To determine if bikunin plays a role as a TK inhibitor *in vivo*, BALF obtained from patients with asthma ($n = 2$) before and after allergen challenge was subjected to immunoprecipitation with antibikunin antibodies as described in MATERIALS AND METHODS. Plotting increasing concentration of sample against TK activity values (expressed as V_{max}) revealed that there were no significant changes in TK activity in baseline BALF after immunoprecipitation. In contrast, after allergen challenge, where TSG-6 is increased and complex formation occurs, bikunin immunoprecipitation resulted in an increase of TK activity of $\sim 38\%$ (Figure 6, top), consistent with the hypothesis that TSG-6-mediated bikunin release from I α I proteins significantly contributes to inhibition of TK catalytic activity *in vivo*.

To mimic the proposed mechanism in a cell culture system, I α I and TSG-6 were added to apical washes of NHBE cells ($n = 2$) previously digested with hyaluronidase to disrupt TK-HA complexes as described in MATERIALS AND METHODS. Consistent with the experiments performed with purified proteins, addition of I α I had no significant inhibitory effect on TK catalytic activity. In contrast, supplementing the washes with TSG-6 resulted in $\sim 37\%$ inhibition. This inhibitory effect was reversed by bikunin immunoprecipitation, confirming that bikunin was responsible for the antiprotease activity induced by TSG-6. These results are summarized in Figure 6 (bottom).

Figure 6. Top: Bikunin inhibits TK activity in the airway lumen after allergen challenge. Bikunin from BALF before and after allergen challenge was removed by immunoprecipitation as described in MATERIALS AND METHODS. TK activity in baseline BALF (inverted triangle) was not affected by bikunin immunoprecipitation (square), whereas after allergen challenge (triangle) TK activity increased by 38% (diamond). Bottom: Bikunin release and TK inhibition can be mimicked by adding I α I and TSG-6 to hyaluronidase-treated apical washes of NHBE cells. TK activity in washes (circle) did not change after exogenous I α I supplementation (inverted triangle) but decreased after the addition of TSG-6 (square). Bikunin immunoprecipitation reversed the TSG-6-induced TK inhibition (diamond).

TSG-6, HC3, and Bikunin Are Induced in Human Airway Epithelial and Submucosal Gland Cells

To determine the cellular sources of TSG-6, primary cultures of submucosal and epithelial cells were used. RT-PCR of RNA extracted from SMG cells show amplification of a band of the expected size (283 bp) for TSG-6 that, when sequenced, was confirmed to be the product of TSG-6 mRNA amplification (Figure 7). In the case of the NHE cells, only a faint band was seen in nonstimulated conditions. However, treatment of the NHE and SMG cultures (each obtained from three different lung donors) with TNF- α (20 ng/ml) or IL-1 β (50 ng/ml) resulted in an increase of TSG-6 expression of 2.9 ± 0.4 -fold in SMG and 10.5 ± 1.1 in NHE with TNF- α and 6.0 ± 2.0 in NHBE cells with IL-1 β treatment when normalized with respect to β -actin ($P < 0.05$; indicated by * on Figure 7). SMG cells did not show significant changes in TSG-6 expression after IL-1 β exposure (0.7 ± 0.5 -fold, $P > 0.05$).

To determine whether the various gene products of I α I/P α I were expressed in our cultures and could potentially be synthesized locally in human airways, RT-PCR specific for bikunin, HC1, HC2, and HC3 was performed as described in MATERIALS AND METHODS. Although no products for HC1 or HC2 were present (data not shown), bands of the expected size for bikunin (294 bp) and HC3 (318 bp) were observed (Figure 8); the identities of the RT-PCR products were confirmed by sequence analysis. In the absence of stimulation, there were low levels of expression for these gene products, particularly in the SMG cells, with modest increases after TNF- α stimulation (i.e., bikunin increased by 1.5 ± 0.2 -fold in SMG cells and by 1.4 ± 0.5 -fold in NHE cells, whereas HC3 expression increased by 2.5 ± 0.3 -fold in SMG cells and by 1.8 ± 0.2 -fold in NHE cells (Figure 8). Overall, these data indicate that TNF- α can upregulate gene expression above the constitutively expressed levels of P α I (i.e., bikunin and HC3) and TSG-6 in these cultures.

TSG-6, I α I, Bikunin, and H3 (P α I) Are Localized in Surface Epithelium and Submucosal Glands in Human Airways

To determine if gene expression results in protein synthesis in the airways and to visualize the cell populations expressing TSG-6, bikunin, and HC3, we labeled human tracheal tissue sections obtained from the lungs of nonsmokers ($n = 3$) and smokers

Figure 7. TSG-6 expression is induced by TNF-α and IL-1β in primary cultures. Human SMG cells and NHE cells were grown at the air-liquid interface in control conditions in RPMI (R) or after treatment with TNF-α or IL-1β. (Top) TSG-6 induced by TNF-α. (Bottom) IL-1β treatment. Relative expressions are normalized with respect to α-actin.

($n = 3$) as described in MATERIALS AND METHODS. TSG-6 was detected at the apical pole of epithelial cells (Figure 9, Panels 1C and 2C) and in the submucosal glands (Figure 10, Panels 1C and 2C) in nonsmokers, and labeling was clearly increased in the epithelium (Figure 9, Panels 4C and 5C) and glands (Figure 10, Panels 4C and 5C) of smokers. TSG-6 in the epithelium of smokers showed staining that was more widely distributed. Anti-bikunin antibodies gave no visible staining in control glands (Figure 9, Panel 1B), but in smokers staining colocalized with TSG-6 at the apical pole of cells and at the ciliary border (Figures 9 and 10, Panels 4D and 5D). IαI immunoreactivity was observed as expected in the interstitial space and was not associated with TSG-6 in the inflamed subepithelial tissue of smokers (Figure 9, Panels 4B, 4C, and 4D). There was no expression of bikunin in control airways (Figures 9 and 10, Panel 1B), which is consistent with the low level of PCR products seen in Figure 8, but in smokers it was prominently visible at the ciliary border and in submucosal glands and seemed not to be associated with TSG-6 (Figures 9 and 10, Panels 5B and 5D).

To verify that the HC3 gene expressed in NHE and SMG cell cultures results in protein expression *in vivo* and to assess

Figure 8. TNF-α induces bikunin and HC3 in SMGs and NHE cells. Amplified RT-PCR products of bikunin (top) and HC3 (bottom) are visualized, and relative expressions are normalized with respect to α-actin.

its localization (because anti-IαI antibodies recognize HC1, HC2, and bikunin), we labeled tracheal tissue using a specific antibody to HC3 as described in MATERIALS AND METHODS. HC3 protein is expressed intracellularly in SMG cells and surface epithelium in the airways of smokers, whereas in noninflamed tissues HC3 is limited to vessels (Figure 11). These data provide evidence that the HC3 gene expression observed in culture conditions correlates reasonably well with protein synthesis *in vivo*, which is also the case for TSG-6 and bikunin, as evidenced by positive immunostaining in tracheal tissue sections. In addition, the upregulation of gene expression observed after TNF-α treatment is mirrored by an increase in tissue labeling in the inflamed lungs.

DISCUSSION

We report that TSG-6 enhances the antiprotease activity of IαI against TK and that this is due to the release of bikunin resulting from TSG-6-HC complex formation. We found that TSG-6 is present in airway secretions where its levels are upregulated in two conditions associated with airway inflammation: asthma and chronic exposure to cigarette smoke. We identified submucosal glands and surface epithelial cells as sources of TSG-6 in airway secretions.

When the BALF of individuals with asthma was examined, free TSG-6 and 120-kD TSG-6-HC complexes were observed,

Figure 9. Immunolocalization of TSG-6, I α I, and bikunin in tracheal tissue sections. Confocal micrographs depict airway epithelium from a nonsmoker (Panels 1, 2, and 3) and a smoker (Panels 4, 5, and 6). Pictures in Column A correspond to DIC images; Column B: green channel; Column C: red channel; and Column D: merged images that include nuclei (blue) stained with DAPI. Panels 1 and 4 are labeled with antibodies to I α I (green) and TSG-6 (red); Panels 2 and 5 show immunolocalization of bikunin (green) and TSG-6 (red), whereas Panels 3 and 6 are nonimmune control subjects. Arrow in D4 (smoker) points toward TSG-6 and I α I colocalization (yellow) at surface epithelium but not in subepithelial tissue.

and the amount of the free TSG-6 seemed to be significantly increased after allergen challenge. Furthermore, high-molecular-weight species were detected with the anti-I α I antibody (running as a smear on Figure 1), and these are likely to correspond to HC:HA complexes, as seen in the cumulus matrix from murine cumulus oocyte complexes (34). It is probable therefore that a significant amount of TSG-6:HC complexes had been formed in these patients because TSG-6:HC can act as intermediates in the production of HC:HA (27) and that this is likely to be accompanied by the release of bikunin, as demonstrated here *in vitro*. We show that TSG-6 is able to increase the inhibitory activity of I α I against TK, a serine protease that plays a key role in asthma pathophysiology (10, 11, 17). Our data show that the TSG-6-induced increase in I α I antiprotease activity is due to the release of free bikunin after the formation of TSG-6:HC complexes. Immunoprecipitation of bikunin resulted in increased TK activity in BALF obtained from patients with asthma after allergen challenge but not in baseline conditions, confirming that released bikunin plays an important role in controlling TK catalytic activity as described (55). This phenomenon was mimicked *in vitro* by adding I α I and TSG-6 to hyaluronidase-

treated apical cell washes on NHBE cell cultures. In fact, bikunin immunoprecipitation seemed to have resulted in higher activity than before I α I and TSG-6 supplementation, suggesting that a fraction of TK was already being inhibited by bikunin in these cell cultures. Bikunin is also an effective inhibitor of other serine proteases such as prostatic (56), which is involved in the regulation of the epithelial Na⁺ channel (57, 58) and has been linked to the loss of periciliary fluid on the airway epithelium in cystic fibrosis (14); therefore, TSG-6-induced bikunin release may have wider implications in airway homeostasis. TSG-6 potentiates the antiplasmin activity of I α I (25, 28), contributing to its antiinflammatory and chondroprotective effect in arthritic joints (31). This enhancement of I α I inhibitory activity toward TK occurs via a different mechanism from that which we have recently described for the potentiation of the antiplasmin activity of I α I, which involves the formation of a noncovalent complex between the TSG-6 Link module and the bikunin chain (38). Bikunin is an effective inhibitor of TK only when it is in a free form (i.e., not part of the intact I α I molecule).

We induced TSG-6 gene expression in NHE and SMG cells by TNF- α and IL-1 β , as has been reported for most cell

Figure 10. Immunolocalization of TSG-6, I α I, and bikunin in submucosal glands. Tracheal tissue sections obtained from a nonsmoker (Panels 1, 2, and 3) and a smoker (Panels 4, 5, and 6) were stained as described in MATERIALS AND METHODS. Confocal micrographs in Column A correspond to DIC images; Column B; green channel; Column C: red channel; Column D: merged images that include nuclei (blue) stained with DAPI. Panels 1 and 4 are labeled with antibodies to I α I (green) and TSG-6 (red), Panels 2 and 5 are labeled with antibodies to bikunin (green) and TSG-6 (red), and Panels 3 and 6 are nonimmune control subjects. Arrows in D4 (smoker) point toward TSG-6 and I α I labeling.

Figure 11. Immunolocalization of HC3 in tracheal tissue sections. Pictures depict airway epithelium (a–d) and submucosal glands (e–h) from a nonsmoker (Panels a, b, e, and f) and a smoker (Panels c, d, g, and h). Nonimmune control subjects are in Panels a, c, e, and g. Although HC3 is visible in normal tissue (Panels a, b, e, and f), it is clearly increased in the tissues obtained from the smoker (Panels d and h).

types expressing this gene (20). In contrast to reports that TSG-6 expression does not occur unless induced by inflammatory mediators, constitutive expression of TSG-6 mRNA was seen in our cell cultures (particularly SMG cells), as has been observed in renal epithelial cells (59) and chondrocytes (60). This is unlikely to be only an *in vitro* phenomenon because bronchial secretions obtained from normal volunteers contained low but measurable amounts of TSG-6. These findings are in agreement with a recent report where Lilly and colleagues (61), using microarray analysis of airway epithelial cells, found basal TSG-6 gene expression that was increased in subjects with asthma after allergen challenge.

In addition to TSG-6, we detected constitutive gene expression of HC3 and bikunin (i.e., components of P α I) that could be further increased by TNF- α . The HC3 protein was detected by immunostaining with an anti-HC3 antibody in SMG and surface epithelial cells in the airways of smokers, which is consistent with the local synthesis of P α I. These findings are not unique to airway epithelium; in fact, Janssen and colleagues (59) reported that human renal proximal tubular epithelial cells also

constitutively express HC3 and bikunin. The presence of locally synthesized P α I, in addition to TSG-6, in inflammatory conditions supports the notion that airway epithelium is able to generate local responses to limit protease activity without necessarily relying on plasma proteins that become available during inflammatory responses. It is therefore tempting to hypothesize that the constitutive expression of P α I and TSG-6 could be part of a homeostatic mechanism aimed at providing antiprotease protection to the airways under normal conditions, as has been suggested in the kidney (59). Positive immunostaining of TSG-6 in tracheal tissue sections obtained from nonsmokers and smoker lung donors confirmed that TSG-6 gene expression results in increased protein expression; TSG-6 mRNA was present in SMG cells in control individuals and smokers but was clearly upregulated in the latter. In normal control subjects, faint staining was also observed in the apical pole of epithelial cells, in contrast to smokers, in whom protein expression was widely distributed (e.g., in the surface epithelium, submucosal glands, and at the ciliary border). Additionally, in control subjects we observed faint TSG-6 staining not associated with I α I, whereas in smokers TSG-6 labeling was increased (in agreement with the results on gene expression), confirming that enhanced TSG-6 protein expression is associated with airway inflammation. These findings are in agreement with the observation that cigarette smoke induces TNF- α gene and protein expression (62–64). I α I was not seen in normal tissues but was present in the subepithelial and interstitial tissues of smokers (likely due to vascular leakage), where it is not associated with TSG-6. However, colocalization of TSG-6 and I α I staining was observed at the ciliary border, suggesting that this could be the site of TSG-6-HC complex formation. This notion is supported by the increase in soluble TSG-6 in the bronchial secretions of smokers when compared with nonsmokers.

The roles that TSG-6 may play in human airways during inflammation are likely to be diverse. For example, TSG-6 mediates the transfer of the heavy chains from I α I/P α I onto HA (19, 27), which is believed to lead to HA cross-linking, increasing its level of aggregation, as seen in the synovial fluids of patients with arthritis (65). TSG-6-mediated cross-linking of HA is likely to be a common feature of inflammation (26, 66). In this regard, it is possible that free HA could act as a water reservoir in airway secretions and that the cross-linking of HA with HC, due to the increased production of TSG-6 during inflammation, may decrease its water-retaining capacity (i.e., due to a reduction in the HA domain size). This could directly affect the viscoelastic properties of mucus, thus contributing to the mucus “dehydration” seen in asthma and other airway diseases. It has also been suggested that cross-linked HA may be more resistant to depolymerization by ROS (27), suggesting that increases in TSG-6 and P α I expression (and leakage of serum-derived I α I) could play a role in protecting the epithelial surface against oxidative insults.

We have previously shown that soluble HA is increased in the BALF of patients with asthma, coinciding with a decrease in its average molecular mass (55) and suggesting that HA depolymerization occurs in the asthmatic airways. The fact that TSG-6 and I α I show colocalization at the luminal pole of epithelial cells but are dissociated in subepithelial tissue and in glands in inflamed lungs further supports the hypothesis that TSG-6-HC complex formation and the release of bikunin occurs extracellularly, after secretion, and is consistent with the detection of free TSG-6 and TSG-6-HC complexes in the BALF of subjects with asthma and the presence of HC-HA complexes, including some of low molecular weight that are likely to correspond to heavy chains attached to relatively short HA chains.

Because TSG-6 is expressed in a variety of inflammatory

conditions (20, 67), we did not limit our observations to patients with asthma. We looked also at the levels of TSG-6 in the bronchial secretions of smokers, where an increased amount of soluble TSG-6 was observed in smokers compared with nonsmokers and ex-smokers, supporting the notion that TSG-6 upregulation is a common component of a broad range of airway inflammatory responses.

The present study identifies a novel function of TSG-6 (i.e., enhancement of the anti-TK activity of I α I) by a distinct mechanism (i.e., bikunin release after TSG-6-HC complex formation) and provides a rationale for this activity within the context of inflamed airways.

In summary, we report a previously unrecognized antiprotease system regulated by TSG-6, which is induced in inflammatory responses. TSG-6, HC3, and bikunin are expressed in airway epithelial cells in culture where their mRNA levels are increased by TNF- α and IL-1 β . These findings correlate with the observed increases in protein expression in tracheal tissue sections and elevated protein concentrations in the bronchial secretions of smokers and in the BALF of patients with asthma, where TNF- α is upregulated (62–64, 68, 69). The interactions of TSG-6 with other molecules are also potentially important to the pathophysiologic aspects of inflammatory airway diseases that remain to be elucidated.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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