Transforming Growth Factor β Enhances Respiratory Syncytial Virus Replication and Tumor Necrosis Factor Alpha Induction in Human Epithelial Cells

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Asthma is characterized as a chronic inflammatory disease associated with significant tissue remodeling. Patients with asthma are more susceptible to virus-induced exacerbation, which subsequently can lead to increased rates of hospitalization and mortality. While the most common cause of asthma-related deaths is respiratory viral infections, the underlying factors in the lung environment which render asthmatic subjects more susceptible to viral exacerbation are not yet identified. Since transforming growth factor β (TGF-β) is a critical cytokine for lung tissue remodeling and asthma phenotype, we have focused on the effects of TGF-β on viral replication and virus-induced inflammation. Treatment of human epithelial cells with TGF-β increased respiratory syncytial virus (RSV) replication by approximately fourfold. Tumor necrosis factor alpha (TNF-α) mRNA and protein expression were also significantly increased above levels with RSV infection alone. The increase in RSV replication and TNF-α expression after TGF-β treatment was concomitant with an increase in virus-induced p38 mitogen-activated protein kinase activation. Our data reveal a novel effect for TGF-β on RSV replication and provide a potential mechanism for the exaggerated inflammatory response observed in asthmatic subjects during respiratory viral infections.

Infection with many different viruses, such as human rhinovirus, adenovirus, parainfluenza virus, influenza virus, and respiratory syncytial virus (RSV), can trigger severe asthma exacerbation and hospitalization, but these infections are generally well tolerated in normal subjects (22, 23). The exact reason for this difference between normal and asthmatic responses to respiratory virus infections is not yet clear. Recent evidence by Wark et al. and Contoli et al. (13, 46) suggests that the lack of an efficient innate immune response, as detected by evidence by Wark et al. and Contoli et al. (13, 46) suggests that the lack of an efficient innate immune response, as detected by lower levels of beta interferon (IFN-β) and lambda interferon (IFN-λ) produced by asthmatic epithelial cells, may be responsible for higher levels of viral replication, which can lead to an exaggerated asthmatic response.

A cytokine that is pivotal in acute and chronic inflammation is tumor necrosis factor alpha (TNF-α). This pleiotropic cytokine has been implicated in many inflammatory diseases, such as rheumatoid arthritis, ankylosing spondylitis, Crohn’s disease, and psoriasis (6, 7, 18). In addition to proinflammatory and immunomodulatory functions, deleterious effects of TNF-α are associated with the proapoptotic consequences of this cytokine, which contribute to tissue damage (9, 14). TNF-α is also increased in asthmatic bronchoalveolar fluid, with higher levels detected in severe asthmatics (16, 38). In fact, recent evidence from clinical trials and animal studies showed that anti-TNF-α therapy was beneficial for asthma patients (3, 20, 25). Although it is accepted that respiratory viral infections induce TNF-α, it is not yet known if the level of this cytokine is differentially modulated by any factor in the asthmatic lung environment compared to the lung environment of normal subjects.

In addition to the immunoregulatory functions, transforming growth factor β (TGF-β) has diverse effects in the lung environment, such as inhibition and induction of cell proliferation, induction of mucus secretion, and increasing cell migration (4, 11, 12, 21, 24, 32, 37, 42). It is also well accepted that TGF-β expression is increased in asthmatic lungs, and it directly correlates with chronic asthma phenotype and severity (2, 5, 36). TGF-β is a critical regulatory cytokine for chronic lung fibrosis and tissue remodeling through myofibroblast differentiation and fibroblast activation, which is a hallmark of chronic asthma (12). Therefore, TGF-β is considered a key mediator of asthmatic phenotype; however, the role of this cytokine in virus-induced lung inflammation is not well studied.

Based on the accumulated evidence, it is reasonable to hypothesize that a factor in the asthmatic lung environment affects epithelial cells in such a way as to render them more susceptible to viral replication and the virus-induced inflammation. To identify the underlying causes of exaggerated inflammatory responses by asthmatic subjects during viral infections, we have tested the role of TGF-β in RSV replication and virus-induced epithelial inflammatory responses. For these experiments, we used primary human bronchial epithelial (PHBE) cells from normal donors and the human alveolar epithelial cell line A549. We investigated the effect of TGF-β treatment on replication of a common respiratory virus, RSV, and expression of TNF-α in these cells. We further examined the effects of TGF-β on the virus-induced activation of a key signaling pathway, namely, the p38 mitogen-activated protein kinase (MAPK).

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MATERIALS AND METHODS

Cell culture conditions, viral infections, and reagents. PHBE cells were purchased from Cambrex BioScience (Walkersville, MD) and were grown as a monolayer in serum-free medium BEBM-2 (Cambrex BioScience). The human epithelial cell line A549 was grown as a monolayer in Dulbecco's modified Eagle's medium and Ham's F-12 medium supplemented with 5% fetal calf serum and 10 μg/ml penicillin-streptomycin at 37°C in a 5%-CO2 humidified chamber. The recombinant human TGF-β was purchased from RDI-Fitzgerald (Concord, MA) and was diluted in phosphate-buffered saline (PBS) with 2% bovine serum albumin. PHBE and A549 cells were plated at 8 × 10^5 cells and allowed to grow overnight. At 70% confluence, the cells were treated with TGF-β at various concentrations. The cells were then incubated for 2 h prior to infection with RSV (human A2 strain) at various multiplicities of infection (MOIs). To determine the presence of contaminating cytokines and the necessity for virus replication in the induction of proinflammatory cytokines, the RSV stocks were tested by UV light inactivation using a Stratagene (Cedar Creek, TX) Stratalink 1800 UV crosslinker.

RNA extraction and RT-PCR. RNA was isolated using the TRIzol total RNA isolation reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized using 0.5 μg purified RNA and SuperScript reverse transcriptase (Invitrogen, Carlsbad, CA). The cDNA was then amplified by reverse transcription-PCR (RT-PCR) in the presence of 2 μg/ml primers and iQ Sybr Green supermix (Bio-Rad, Hercules, CA).

The sequences of primers used in RT-PCR were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense, GGACCCTGACTGCTGCTC TAG; GAPDH antisense, TACGCCAGATGCCCTGAG; G-TNF-α sense, CGATCTACCTACAGACCAAG; G-TNF-α antisense, CAGCAGTTCCAGAAGGAGGA.

**Western blot and enzyme-linked immunosorbent assay (ELISA)**. For isolation of cellular proteins, after each treatment, cells were washed two times in PBS, and equal numbers of cells were lysed using 1× sodium dodecyl sulfate (SDS) sample buffer containing 2.5% β-mercaptoethanol. The proteins were denatured and reduced by heating the samples at 95°C for 3 min. The chromosomal DNA was then removed by passing the samples through a 26-gauge needle several times. The proteins were resolved by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and were electrophoretically transferred onto nitrocellulose membranes. Polyclonal rabbit anti-β38 MAPK and anti-phospho-β38 MAPK (Cell Signaling, Beverly, MA) were used according to the manufacturer's instructions. Polyclonal goat anti-RSV was purchased from Chemicon International (Temecula, CA). The concentration of antibody used to probe blots was optimized relative to protein concentrations. The immunoblotted proteins were visualized by UV light inactivation using a Stratagene (Cedar Creek, TX) Stratalink 1800 UV crosslinker.

**RESULTS**

TGF-β treatment enhances RSV replication. To address our hypothesis that TGF-β plays a role in exaggerated responses in asthmatics during viral infections, we used PHBE cells and the human epithelial cells line A549. Initially, we investigated the effect of TGF-β on viral replication. PHBE and A549 cells (Fig. 1A and B) were treated with TGF-β, and after 2 h, the cells were infected with RSV at a MOI of 0.5 PFU/cell. The low MOI was chosen to allow a detectable change in the accumulation of viral proteins. Total cellular proteins were extracted after 16 h of infection, and RSV proteins were visualized by SDS-PAGE followed by Western blot analysis using anti-RSV goat serum (Fig. 1A and B). The viral proteins were identified by molecular weight mobility patterns as described by Teng and Collins (43). The data showed that viral protein accumulation increased with TGF-β treatment.

Since viral protein accumulation may not reflect an increase in infectious viral particles, we assessed the role of TGF-β in viral replication by titration using plaque assays (Fig. 1C). PHBEs were treated with TGF-β at 5 ng/ml, and after 2 h, the cells were infected with RSV at a MOI of 0.5 PFU/cell (total initial inoculum, 8 × 10^5 PFU). The cells were harvested after 24 h and were lysed, and the cleared lysates were used in standard plaque assays (Fig. 1C). The titer of virus in the untreated cells increased by approximately 11-fold after 24 h (9.85 × 10^5 PFU). TGF-β treatment enhanced RSV titers by an additional 3.9-fold, which is a total of an approximately 40-fold increase over the initial inoculum. Collectively, our data obtained by Western blots and titration assays showed that TGF-β caused a significant increase in viral replication.

**TGF-β enhances RSV-induced TNF-α expression.** Since exaggerated inflammation is associated with viral exacerbation of asthma, we tested whether TGF-β treatment increased RSV induction of TNF-α expression in epithelial cells.

First, to determine the dose-response curve of RSV induction of TNF-α, we infected A549 cells with RSV at increasing MOIs (Fig. 2A). After 24 h, the level of TNF-α mRNA was determined by RT-PCR. The data showed that a MOI of 1 PFU/cell was sufficient to cause a detectable increase in TNF-α mRNA. Therefore, we used this MOI as a starting point in our experiments examining the effect of TGF-β on cytokine expression.

A549 (Fig. 2B) and PHBE (Fig. 2C) cells were treated with increasing concentrations of TGF-β, and after 2 h, cells were infected with RSV at a MOI of 1.0 PFU/cell. Culture supernatants and cells were harvested at 24 h postinfection. Total RNA was isolated from the cells and was used in RT-PCR to determine TNF-α and IFN-β levels. TGF-β increased the RSV-induced TNF-α mRNA by 3.2-fold above the level with RSV alone in A549 cells (Fig. 1B) and by 4.3-fold in PHBE cells (Fig. 2B and C). On the other hand, there was a decrease, albeit not significant, in IFN-β expression (Fig. 2B).

To measure cytokine protein release, we performed a specific ELISA with the culture supernatants. Since in our experience cell lines do not produce significant TNF-α protein, we focused on culture supernatants obtained from PHBE cells (Fig. 2D). TGF-β treatment enhanced TNF-α release by 1.9-fold over levels with RSV alone (Fig. 2D).
TGF-β enhancement of TNF-α is not due to enhanced RNA stability. It is known that a regulatory step in TNF-α expression is at the level of RNA stability (8). To test the possibility that the enhancement of RSV-induced TNF-α expression with TGF-β was due to enhanced RNA stability, we used actinomycin D to inhibit de novo transcription (Fig. 3). Cells were treated with TGF-β at 5 ng/ml for 2 h and were then infected with RSV at a MOI of 5 PFU/cell. After 24 h of incubation, Actinomycin D was added at 1 μg/ml, total cellular RNA was harvested at various time points, and TNF-α mRNA was measured by real-time RT-PCR. The data showed that TGF-β treatment did not alter the stability of TNF-α mRNA compared to results with untreated cells, suggesting that the increase in TNF-α mRNA was due to a transcriptional activation (Fig. 3).

TGF-β enhances RSV activation of p38 MAPK. A key signaling molecule for inflammatory responses and virus-induced TNF-α expression is p38 MAPK. We therefore tested the effect of TGF-β on RSV activation of p38 MAPK by Western blot analysis. First, we determined the dose-response effect of RSV infection on p38 activation (Fig. 4A). A549 cells were infected with increasing concentrations of RSV, and after 24 h, total cellular protein was extracted and used in Western blot assays. RSV activated p38 MAPK in a dose-response manner.

Next, to test if TGF-β enhanced RSV activation of p38 MAPK, A549 cells were treated with increasing concentrations of TGF-β and were then infected with RSV at a MOI of 1.0 PFU/cell (Fig. 4B). After 24 h, cells were harvested, total cellular proteins were isolated, and the phosphorylation state of p38 MAPK was determined by using a specific antibody. TGF-β enhanced RSV activation of p38 MAPK in a dose-response manner.

To verify these data with primary cells, we treated PHBE cells with TGF-β at 5 ng/ml for 2 h prior to infection with RSV at a MOI of 1.0 PFU/cell (Fig. 4C). After an additional 24 h, total cellular proteins were harvested and used in Western blot analysis. Similar to results with A549 cells, TGF-β enhanced RSV activation of p38 MAPK in PHBE cells. Collectively, these data suggest that an increase of p38 MAPK activation is a potential mechanism of TGF-β enhancement of TNF-α expression.

TGF-β reduces cellular metabolism but not cellular replication. Viruses are intracellular parasites; therefore, they rely on cellular machinery for replication. To test the possibility that TGF-β affected viral replication and TNF-α expression through a change in cellular metabolism or replication, we used two different colorimetric assays (Fig. 5). First, to test cellular replication, cells were treated with increasing concentrations of TGF-β for 24 h, and then a BrdU DNA labeling reagent was added. After 24 h, BrdU incorporation was colorimetrically measured on an ELISA plate reader. TGF-β did not have any significant effect on epithelial cell replication. Under identical conditions, staurosporin, which is a known inducer of apoptosis, caused a dramatic decrease in cell proliferation.

Next, to examine the effect of TGF-β on the metabolic rate of epithelial cells, we used a WST-1 cell viability assay. A549 cells were treated with various concentrations of TGF-β for 24 h, and cell viability was then assessed by using a WST-1 assay. There was a significant decrease in the metabolic rate of the cells after TGF-β treatment (Fig. 5).
DISCUSSION

To examine the mechanisms of viral induction of asthma exacerbation, we have investigated the role of TGF-β as the basis for the enhanced virus-induced inflammatory effects in human bronchial epithelial cells. In this report we showed that TGF-β enhanced RSV replication and viral induction of TNF-α expression in human epithelial cells. This suggests that by increasing the viral load and TNF-α levels, the presence of TGF-β in asthmatic lungs may contribute to the exaggerated responses observed during respiratory infections in these patients.

While exaggerated lung inflammation is a frequent occurrence during common respiratory viral infections in asthmatic lungs, the underlying events leading to these responses have remained largely unknown. To study these events, we exam-
ined the role of TGF-β in RSV replication and inflammatory cytokine expression. TGF-β is a pleiotropic cytokine which possesses both pro- and antiproliferative effects and has been clearly linked with asthma phenotype (5). TGF-β enhances fibrosis and remodeling, cell migration, and mucus secretion (4, 11, 12, 21, 24, 32, 37, 42).

Bronchial biopsy samples from asthmatics show a higher level of TGF-β immunoreactivity than samples from normal subjects (2, 36). There is also significant correlation between basement membrane thickness and TGF-β expression in epithelial and submucosal cells (26, 45). Alveolar macrophages and eosinophils from asthmatics also show higher levels of TGF-β than macrophages from normal subjects (29, 45).

Interestingly, a polymorphism within the TGF-β gene promoter (position −509 C/T) is associated with asthma phenotype (17, 40). Further evidence for the TGF-β gene polymorphism in asthma was reported by Pulleyn et al., showing that homozygosity of this allele (−509 C/T) was positively correlated with the severe asthma phenotype (35).

Recently, Wark et al. and Contoli et al. reported that bronchial epithelial cells from asthmatic subjects had a defect in innate immune responses and in IFN-β and IFN-λ expression, and therefore, rhinovirus replication was more robust in asthmatics (13, 46). It is not clear, however, whether asthmatic subjects have any other deficiency in antiviral responses in other organs. Therefore, their data suggest that underlying events of asthmatic exacerbation during viral infections must be an organ-specific effect. Also, Wark et al. showed that epithelial cells from asthmatics had a delayed apoptotic response which may be a part of virus-induced exacerbation in these patients. In our experiments, TGF-β did not have any effect on cellular apoptosis in infected or noninfected cells (data not shown). At this point, we cannot account for this discrepancy, but it could be due to usage of different experimental conditions.

It is clearly established that the presence of TGF-β in the respiratory system is significantly associated with the asthmatic condition; therefore, our data provide an alternative explanation for the organ-specific events during viral infections of the asthmatic respiratory system. It is also possible that TGF-β, which is an anti-inflammatory cytokine, could reduce IFN-β levels in the lungs and allow more-efficient viral replication. Since treatment of cells with TGF-β alone enhanced IFN-β levels, albeit not significantly, at this point we favor a direct effect of TGF-β on cells and viral replication.

This assertion is strengthened by our data from cell viability...
and DNA replication assays showing that TGF-β treatment did not affect DNA replication but did significantly reduce cellular metabolic activity (Fig. 5). Since viral replication machinery is in competition with cellular machinery for availability of substrates, it is plausible that a reduction in cellular metabolic substrate competition would allow more-efficient virus replication. Alternatively, TGF-β may induce an as yet unknown factor that can be advantageous to RSV replication.

Current research has identified TGF-β as a critical molecule for immunoregulation and Th1/Th2 differentiation, but the role of TGF-β in viral replication is not well studied. Previous work by Subauste and Proud showed that TGF-α did not affect rhinovirus replication in the epithelial cell line BEAS-2B (41). However, TGF-β has been reported to either enhance or suppress replication of human immunodeficiency virus depending on the experimental conditions (27, 34), although the exact mechanisms are not yet clear. TGF-β can also suppress replication of hepatitis C and the human T-cell leukemia virus type I (31, 33).

Surprisingly, our data showed that TGF-β enhancement of RSV replication was concomitant with a significant increase in RSV-induced TNF-α mRNA and protein expression. TNF-α is a pivotal regulatory cytokine in innate and adaptive immune responses. Furthermore, TNF-α has been clearly associated with asthma (3, 30, 44). At a genetic level, two different polymorphisms in the TNF-α promoter were reported to correlate with asthma phenotype (1, 10, 39). Importantly, TNF-α is correlated with severe refractory asthma, and treatments aimed at neutralizing TNF-α are gaining acceptance as a novel therapeutic regimen. This is interesting because during respiratory viral infections, asthmatic exacerbation is also refractory to glucocorticoid anti-inflammatory therapies (15, 19). Our data showing that TGF-β enhanced RSV induction of TNF-α are in agreement with these clinical data in that exaggerated production of TNF-α in asthmatic subjects during viral infections may be the underlying event leading to refractory inflammation, exacerbation, and hospitalization.

Our signal transduction data showed that TGF-β enhanced RSV activation of p38 MAPK, a critical molecule in the inflammatory response pathway. We previously reported that viral induction of TNF-α in epithelial cells was mediated by the activation of p38 MAPK (28). It is possible that an increase in viral titers can lead to an upregulation of p38 MAPK activation and hence an increase in TNF-α expression.

Although our data were generated using human epithelial cells from normal subjects, we believe that our observations are pertinent to asthmatic conditions. TGF-β is known to be significantly associated with an asthmatic lung environment. Further experiments are needed to more clearly delineate the effects of TGF-β on epithelial cells which render them more capable of supporting viral replication and TNF-α expression. Our future experiments will be aimed at addressing these questions.

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REFERENCES


FIG. 5. TGF-β caused a reduction in the cellular metabolic rate. The effect of TGF-β on cell proliferation and the metabolic rate was determined by BrdU and WST-1 assays, respectively. A549 cells were treated with vehicle control (PBS [mock]) or TGF-β at the indicated concentrations. After 24 h, cell proliferation and metabolic rates were determined (n = 3). The error bars indicate the standard errors of the means; *, P < 0.01. Staur, staurosporin.


