Anti-inflammatory effect of MUC1 during respiratory syncytial virus infection of lung epithelial cells in vitro

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Submitted 10 July 2009; accepted in final form 12 January 2010

Li Y, Dinwiddie DL, Harrod KS, Jiang Y, Kim KC. Anti-inflammatory effect of MUC1 during respiratory syncytial virus infection of lung epithelial cells in vitro. Am J Physiol Lung Cell Mol Physiol 298: L558–L563, 2010. First published January 15, 2010; doi:10.1152/ajplung.00225.2009.—MUC1 is a transmembrane glycoprotein expressed on the apical surface of airway epithelial cells and plays an anti-inflammatory role during airway bacterial infection. In this study, we determined whether the anti-inflammatory effect of MUC1 is also operative during the respiratory syncytial virus (RSV) infection. The lung epithelial cell line A549 was treated with RSV, and the production of TNFα and the levels of MUC1 protein were monitored temporally during the course of infection by ELISA and Western blot analysis. Small inhibitory RNA (siRNA) transfection was utilized to assess the role of MUC1 in regulating RSV-mediated inflammatory responses by lung epithelial cells. Our results revealed that: 1) following RSV infection, an increase in MUC1 level was preceded by an increase in TNFα production and completely inhibited by soluble TNF receptor (TNFR); and 2) knockdown of MUC1 using MUC1 siRNA resulted in a greater increase in TNFα level following RSV infection compared with control siRNA treatment. We conclude that the RSV-induced increase in the TNFα levels upregulates MUC1 through its interaction with TNFR, which in turn suppresses further increase in TNFα by RSV, thus forming a negative feedback loop in the control of RSV-induced inflammation. This is the first demonstration showing that MUC1 can suppress the virus-induced inflammatory responses.

HUMAN RESPIRATORY SYNCYTIAL VIRUS (RSV) was first characterized in 1957 as chimpanzee coryza agent (11). Since then, RSV has been considered the leading cause of viral bronchiolitis and pneumonia accompanied with a major health and economic burden worldwide (15, 33, 38). Epidemiological data show that more than 70% of children in the first year of life and 100% of children by the age of 2 years were infected with RSV (31). In the United States alone, an estimated 75,000–125,000 children aged <1 year are hospitalized with RSV each year (38). Although common RSV infection in adults is a kind of self-limiting disease, RSV does not induce an effective immunological memory, and repeated infections are therefore very frequent (8, 10). On the other hand, severe RSV infection is frequently observed in both premature infants and persons of any age with compromised respiratory, cardiac, and immune systems (33, 42). Furthermore, it has been proven that exposure to RSV infection early in life can lead to an increased susceptibility to suffer from recurrent allergic wheezing and asthma (18). Unfortunately, until now, neither an effective clinical treatment nor any useful vaccine is available.

RSV, which belongs to the Paramyxoviridae family, is an enveloped, single-stranded negative RNA virus (15). The RSV genome contains 15,200 nucleotides encoding 11 proteins (15). Entering the airways, RSV first infects airway epithelial cells, which normally respond by activation of innate immunity. They produce opsonins and collectins as well as a range of cytokines such as IL-6 (4, 6), IL-8 (4, 6), regulation upon activation normal T cell-expressed and secreted (RANTES) (5), and TNFα (6, 16). These cytokines have been shown to initiate the chemotaxis of neutrophils, CD4+ T helper cells, and eosinophils, which reduce pulmonary shedding of the virus but also cause increased pulmonary damage (3, 34, 36). This is one of the reasons why RSV infection can also cause severe symptoms.

Although the antiviral activity of TNFα during virus infection is generally well established (7, 27), it is also believed that TNFα is the primary perpetrator of T cell-mediated lung injury after RSV infection (34, 36). Thus, it is reasonable to speculate that there must be a mechanism to inhibit excess secretion of TNFα, which would exacerbate the illness after RSV infection. Recently, we showed that MUC1 is an anti-inflammatory molecule (22, 23, 41) and is upregulated by TNFα (23). Based on these findings, we hypothesized that the level of TNFα during RSV infection is controlled by MUC1, thus preventing the harmful effects of excessive TNFα during the RSV infection.

MATERIALS AND METHODS

Materials. All the chemicals and reagents used in this study were purchased from Sigma (St. Louis, MO) unless otherwise stated.

Cell culture. A549 cells [human lung adenocarcinoma cell line; American Type Culture Collection (ATCC) no. CCL-185] and HEP-2 cells (human laryngeal carcinoma cell line; ATCC no. CCL-23) were obtained from ATCC (Manassas, VA) and maintained in Opti-MEM medium supplemented with 5% heat-inactivated FBS (Gibco-BRL, Gaithersburg, MD), 50 U/ml penicillin, and 50 μg/ml streptomycin (Cellgro; Mediatech, Herndon, VA). Primary mouse tracheal surface epithelial (MTSE) cells were prepared from 10- to 12-wk-old, male FVB mice and cultured as previously described (28, 29). Muc1+/− mice (39) were a gift from Dr. Sandra Gendler (Mayo Clinic, Scottsdale, AZ). All animal procedures were approved by the IACUC of Temple University.

RSV. RSV A2 was propagated in HEP-2 cells and titrated by standard plaque assay techniques. Immunofoci staining to verify RSV was performed using anti-RSV antibodies (Chemikon International, Temecula, CA) (9).

Real-time RT-PCR. Confluent A549 cells were treated with RSV [multiplicity of infection (MOI) = 0, 1, 5]. In an experiment designed to see the effect of MUC1 on mRNA levels of TNFα, ~90% confluent
cultures were transiently transfected with pMUC1 or its empty vector (pcDNA3.1) before RSV infection. Twenty-four hours posttransfection, total RNA was isolated using the Mini RNA Isolation II Kit (Zymo Research, Orange, CA). One-hundred nanogram total RNA was used for real-time RT-PCR using iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. MUC1 forward primer: 5′-TCAGCTTCTACTCTG-GTGCAACA-3′; reverse primer: 5′-ATTGGAATGGAGTGCTCTTGCT-3′; GAPDH forward primer: 5′-AGCCTCAAGATCATCACTAGCAATG-3′; reverse primer: 5′-GGTTGCTAGGATGACCTTGCC-3′; TNFα forward primer: 5′-GCTGCACTTTGGAGTGAT-3′; reverse primer: 5′-AGGTACAGGCCCTCTGAT-3′; GAPDH forward primer: 5′-AGCCTCAAGATCATCACTAGCAATG-3′; reverse primer: 5′-GTTGTCATGGATGACCTTGGC-3′; TNFα forward primer: 5′-GCTGCACTTTGGAGTGAT-3′; reverse primer: 5′-AGGTACAGGCCCTCTGAT-3′ (IDT, Coralville, IA). The levels of MUC1 or TNFα transcripts were normalized to GAPDH transcripts using the 2^ΔΔCt method.

**MUC1 immunoblotting.** Confluent A549 cells were treated with RSV (MOI = 5) or PBS for varying time periods. The cells were washed with PBS, pH 7.4, at 4°C and then treated 45 min on ice with a lysing buffer (RIPA buffer: 50 mM Tris · HCl, pH 7.5, 150 mM NaCl, 1.0% Nonidet P-40, 0.1% sodium deoxycholate, and 1.0% protease inhibitor cocktail) while stirring every 10 min. The resulting lysates were centrifuged at 16,000 g for 20 min at 4°C to remove the cell debris. The amount of proteins in the lysate was measured using BCA Protein Assay Kit (Thermo Scientific, Waltham, MA) with BSA as a standard. Aliquots of the lysate containing 30 μg of total protein were resuspended in SDS-PAGE Laemmli buffer (0.05 M Tris · HCl, pH 6.8, 2.5% 2-mercaptoethanol, 1.0% SDS, 5% glycerol, and 0.1% bromophenol blue), boiled for 5 min, and resolved on 15% acrylamide gels. Resolved proteins were transferred to PVDF membrane (Bio-Rad), and the membranes were blocked for 1.5 h at room temperature with 5% skim milk (BD Biosciences, San Jose, CA)/TBS-T (0.01 M Tris · HCl, pH 7.4, 0.15 M NaCl, and 0.1% Tween 20). After being washed three times with TBS-T, the membranes were incubated overnight at 4°C with CT2 monoclonal antibody (1:500, TBS-T) that reacts with the COOH-terminal 17 residues of the MUC1 CT subunit (12) or antitubulin antibody (1:20,000, in 5% skim milk/TBS-T solution). Following incubation, the membranes were washed three times with TBS-T, reacted 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody (peroxidase-labeled goat anti-Armenian hamster IgG, Jackson ImmunoResearch Laboratories, West Grove, PA or goat anti-mouse IgG, KPL, Gaithersburg, MD) in 5% skim milk/TBS-T solution. The membranes were washed again three times with TBS-T before developing with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). The band corresponding to the smaller subunit of MUC1 containing the cytoplasmic tail (CT) domain was identified by comigration of prestained protein size markers (Bio-Rad).

**ELISA.** At the end of incubation, the supernatant was collected, and both the cells and the cell debris in the supernatant were removed by centrifugation at 200 g for 5 min and at 16,000 g for 10 min at 4°C, respectively. A549 cells were lysed by RIPA buffer. Human TNFα
and MUC1 were quantified by ELISA using antibodies: anti-human TNFα antibody pair (eBioscience, San Diego, CA), CT2 anti-MUC1 monoclonal antibody, peroxidase-labeled goat anti-Armenian hamster IgG (Jackson ImmunoResearch Laboratories), recombinant human TNFα (eBioscience), and peroxidase-labeled streptavidin antibody (KPL). The XTT assay (Roche Applied Science, Indianapolis, IN) was performed to normalize the variations in cell density among the culture wells. A standard ELISA curve was generated for each plate.

**Transfection.** A549 cells were transfected with pcDNA3-MUC1 (a gift from Dr. Sandra Gendler), pcDNA3.1 (Invitrogen, Carlsbad, CA), MUC1-siRNA, and control-siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. MUC1-siRNAs were designed to target the MUC1 sequence 5’-AACGATTCAGT-GCCCATGTCTTCAT-3’ and synthesized commercially (Dharmacon Research, Waltham, MA); 5’-GUUCAGUGCCCAGCUACUCdA-3’ (sense) and 5’-GUUCAGUGCCCAGCUACUCdTdT-3’ (antisense). Sequences of a nonspecific control siRNA (control-siRNA) were 5’-CCAAUGUUCUACAAAAGGCGGdA-3’ (sense) and 5’-GGCGCCUUGUUAGCAUCUdA-3’ (antisense).

**Statistical analysis.** Differences among the groups were assessed by comparing their means using either Student’s t-test or ANOVA and were considered significant if P < 0.05.

**RESULTS**

**RSV induces both TNFα release and MUC1 mRNA expression in both A549 cells and primary mouse airway epithelial cells.** Because the objective of this study was to determine whether MUC1 could control inflammation induced by RSV infection, we sought to identify a human lung epithelial cell line that produces a minimum amount of MUC1 that could be induced by RSV infection. The cell lines that were tested included HAEo-, 16HBE 14o-, BEAS-2B, NCI-H292, and A549 cells. A549 cells expressed the lowest levels of MUC1 without RSV treatment (data not shown). A549 cells treated with RSV for 24 h drastically increased both MUC1 expression and TNFα release in a dose-dependent manner, about an eightfold increase in MUC1 mRNA (Fig. 1A) and a 10-fold increase in TNFα release with MOI = 5 (Fig. 1B). RSV doses equal to or greater than MOI = 10 were found to be lethal when observed 24 h postinfection (data not shown). To test whether or not the stimulatory effects of RSV on the expression of MUC1 and TNFα as observed in a lung epithelial cell line are also present in a primary lung epithelial cell culture, we repeated the same experiment using primary MTSE cells. Treatment of MTSE cells with RSV (MOI = 5) resulted in a time-dependent increase in TNFα release (Fig. 1C) and Muc1 protein expression (Fig. 1D). The effect of RSV on TNFα release at 48 h was significantly greater in MTSE cells of Muc1−/− mice than that of their wild-type (WT) littermates (Fig. 1C) suggesting that the results from A549 cells are not likely an artifact from a cell line. Based on these results, we decided to use A549 cells treated with MOI = 5 for 24 h to study the role of MUC1 during RSV infection.

**Time course study of MUC1 expression and TNFα release by RSV.** In our recent report (23), we showed that TNFα stimulates MUC1 production in A549 cells mainly through transcriptional regulation. Since RSV increased both MUC1 expression and TNFα release, we sought to determine whether the increased MUC1 levels were due to the increased TNFα production. We took two different experimental approaches to...
answer this question: 1) a time course of MUC1 expression and TNFα release following RSV infection; and 2) inhibition of TNFα. Figure 2 shows that MUC1 protein levels increased even at 12 h following RSV treatment, reaching a peak at 24 h and remaining for at least 24 h before slowly leveling off at 72 h. In contrast, TNFα levels in the spent media reached a peak even at 6 h and started decreasing between 24 and 48 h (Fig. 2). Thus, the increased levels of TNFα in the culture media preceded the increase in MUC1 expression, suggesting that the increased TNFα levels may be responsible for the increased production of MUC1 as predicted from our recent report (23).

Soluble TNFR inhibits RSV-induced MUC1 production. To confirm the above notion that TNFα induced by RSV is responsible for an increase in MUC1 production, we elected to use soluble TNF receptor (TNFR; Prospec, Rehovot, Israel) to block the interaction of TNFα with TNFR. Figure 3 shows that pretreatment with soluble TNFR almost completely inhibited MUC1 protein expression despite high levels of TNFα produced by RSV. Together, these results (Figs. 2 and 3) strongly suggest that RSV infection of A549 cells results in the production of TNFα, which in turn stimulates the production of MUC1.

Overexpressed MUC1 suppresses TNFα release. Interestingly, TNFα levels started to decline when the levels of MUC1 reached a peak during RSV infection (Fig. 2). In light of the anti-inflammatory role of MUC1 during *Pseudomonas* infection (22, 28), we wanted to see whether the increased MUC1 level suppressed TNFα production induced by RSV infection. Overexpression of MUC1 was achieved by transiently transfecting MUC1 cDNA plasmid (pMUC1) (Fig. 4A). Cells transfected with pMUC1 expressed significantly lower levels of TNFα mRNA (52 ± 4%) and released significantly lower amounts of TNFα (40 ± 0.3%) compared with cells transfected with empty vector control (pcDNA3.1) (Fig. 4B). As an alternative approach to test the anti-inflammatory effect of MUC1 during RSV infection, we also knocked down MUC1 expression using siRNA. Our result showed that inhibition of MUC1 levels by siRNA (Fig. 5A) results in a significant increase in TNFα production compared with cells treated with either control siRNA or vehicle alone (Fig. 5B). A similar
result was obtained with primary MTSE cell cultures prepared from Muc1−/− mice (Fig. 1C).

DISCUSSION

The results from the present in vitro study show that RSV induces TNFα release (inflammatory response) from lung epithelial cells, which in turn upregulates MUC1, which then suppresses the release of TNFα, thus bringing the inflammation to an end. These results suggest that the anti-inflammatory function of MUC1 is operative not only during the bacterial infection but also during the viral infection, suggesting the importance of MUC1 levels during inflammation. This is the first report clearly demonstrating the presence of a negative feedback loop in controlling the inflammation by MUC1 as illustrated in Fig. 6.

The production of TNFα by RSV is well known (6, 16). Upregulation of MUC1 is mediated mainly through transcriptional stimulation (25, 26). In our recent publication, we elucidated a signaling pathway involved in TNFα-induced MUC1 transcription as TNFα → TNFRI → ERK → Sp1 (23). How TNFα-induced increase in the MUC1 level suppresses RSV-induced TNFα level is not known. The possible mechanism(s) would be the interruption with the viral entry and/or the signal transduction leading to TNFα release. How RSV enters the mammalian cell was reviewed by Harris and Werling (19). Recently, a small hydrophobic (SH) protein, an integral membrane protein encoded by RSV, has been shown to suppress TNFα signaling (14). Thus, it might also be possible that the SH protein is involved in the suppression of TNFα by MUC1. However, based on our recent report that TNFα and/or IL-8 release (i.e., inflammatory responses) by flagellin, a specific ligand of Toll-like receptor (TLR) 5, can be suppressed by overexpression of MUC1 and enhanced by knockdown of MUC1 (21, 41), it seems quite possible that suppression by MUC1 of RSV-induced TNFα release is mediated via suppression of a specific TLR(s) responsible for mediating TNFα release. Recently, Ahmad et al. (1, 2) showed in breast cancer cells that overexpression of MUC1 is associated with constitutive activation of NF-κB and that TNFα recruits MUC1 to the TNFR1 complex leading to a direct binding between MUC1 CT and NF-κB and resulting in activation of NF-κB. However, our recent study with HEK-293 cells revealed that MUC1 suppresses the NF-κB activity induced by TNFα but not by TNFα (unpublished data). In addition, Thompson et al. (40) first demonstrated the potential involvement of MUC1 in the regulation of the basal level of NF-κB activity in COS-7 cells in which overexpression of MUC1 had no effect while mutation of seven tyrosine moieties in MUC1 CT resulted in downregulation of the basal level of NF-κB activity. Thus, it seems noteworthy to mention that the effect of MUC1 on TNFα and NF-κB may depend on the cell type, tyrosine phosphorylation status, and/or subcellular localization of MUC CT.

TLRs are thought to be directly involved in activating innate immunity against various kinds of viruses including RSV following the recognition of certain conserved viral motifs (24, 35, 37). Early inflammatory signals generated via virus-TLR interactions can contribute to the recruitment of additional inflammatory mediators, including neutrophils and NK cells, into the lung that are thought to be important for clearing RSV-infected cells (6, 30). TLRs that have been shown to be associated with RSV-induced inflammation are TRL2 (32), TLR4 (13, 17, 20, 24), TLR7, and TLR9 (37). Investigation is currently under way in our laboratory to determine whether the anti-inflammatory effect of MUC1 during RSV infection involves the interaction with a specific TLR(s).

ACKNOWLEDGMENTS

We thank Dr. Erik Lilleshøj (Dept. of Pediatrics, Univ. of Maryland Baltimore, School of Medicine, Baltimore, MD) and Dr. Kosuke Kato (Dept. of Physiology, Temple Univ. School of Medicine, Philadelphia, PA) for editing the manuscript.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grants ROI HL-47125 and ROI HL-81852.

DISCLOSURES

No conflicts of interest are declared by the author(s).

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