Human airway epithelia express catalytically active NEU3 sialidase

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1Department of Pediatrics, 2Department of Medicine, 3Center for Vaccine Development, 4Department of Microbiology and Immunology, 5Department of Pathology, 6Department of Anatomy and Neurobiology, 7Institute of Human Virology, 8Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, Maryland; and 9Department of Veterans Affairs, Baltimore VA Medical Center, Baltimore, Maryland

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Lillehoj EP, Hyun SW, Feng C, Zhang L, Liu A, Guang W, Nguyen C, Sun W, Luzina IG, Webb TJ, Atamas SP, Passaniti A, Twaddell WS, Puché AC, Wang L, Cross AS, Goldblum SE. Human airway epithelia express catalytically active NEU3 sialidase. Am J Physiol Lung Cell Mol Physiol 306: L876–L886, 2014. First published March 21, 2014; doi:10.1152/ajplung.00322.2013.—Sialic acids on glycoconjugates play a pivotal role in many biological processes. In the airways, sialylated glycoproteins and glycolipids are strategically positioned on the plasma membranes of epithelia to regulate receptor-ligand, cell-cell, and host-pathogen interactions at the molecular level. We now demonstrate, for the first time, sialidase activity for gangliosides in human airway epithelia. Of the four known mammalian sialidases, NEU3 has a substrate preference for gangliosides and is expressed at mRNA and protein levels at comparable abundance in epithelia derived from human trachea, bronchi, small airways, and alveoli. In small airway and alveolar epithelia, NEU3 protein was immunolocalized to the plasma membrane, cytosolic, and nuclear subcellular fractions. Small interfering RNA-induced silencing of NEU3 expression diminished sialidase activity for gangliosides by >70%. NEU3 immunostaining of intact human lung tissue could be localized to the superficial epithelia, including the ciliated brush border, as well as to nuclei. However, NEU3 was reduced in subepithelial tissues. These results indicate that human airway epithelia express catalytically active NEU3 sialidase.

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Since the sialylation state of a glycoconjugate can influence its function, and is dictated, in part, by sialidase activity, we initially asked whether airway epithelia express one or more catalytically active sialidases. In recent studies in human airway epithelia, we found heat-labile sialidase activity for the fluorogenic substrate, 2-(4-methylumbelliferyl)-α-D-Neu5Ac (4-MU-Neu5Ac), that was dose dependently inhibited by the sialidase inhibitor, 2-deoxy-Neu5Ac, but not its negative control, 2-keto-3-deoxyoctulosonic acid (31). These airway epithelia expressed predominantly NEU1 sialidase at the mRNA and protein levels, and small interfering (si)RNA-induced silencing of NEU1 diminished sialidase activity for the 4-MU-Neu5Ac substrate >70% compared with control siRNA-treated ECs. NEU3 was the second most abundant EC sialidase found at the mRNA level. When airway epithelia were assayed for sialidase activity for ganglioside substrates, SA release was evident (31), suggesting that the ~30% residual sialidase activity detected in airway epithelia after NEU1 silencing might be explained, in part, through NEU3 enzymatic activity. Accordingly, in the present study we surveyed a diverse collection of human airway ECs for sialidase activity against ganglioside substrates, identified NEU3 mRNA and protein in these same epithelia, as well as in intact human airway tissues, and correlated siRNA-induced knockdown of NEU3 expression with reduced ganglioside-directed sialidase activity.

**MATERIALS AND METHODS**

**Materials.** Unless otherwise stated, all chemical reagents were from Sigma (St. Louis, MO). Neu5Ac was from Research Products International (Mt. Prospect, IL). NEU1- and NEU3-targeting siRNAs, and control siRNAs, were from Dharmaco (Lafayette, CO). Lipofectamine and protein G-agarose were from Invitrogen (Carlsbad, CA). Precast sodium dodecyl sulfate (SDS)-polyacrylamide gels were from Novex (San Diego, CA). Polyvinylidene fluoride (PVDF) membrane was from Millipore (Bedford, MA). Enhanced chemiluminescence (ECL) reagents and prestained protein molecular weight markers were from Amersham (Piscataway, NJ). Rabbit anti-human NEU3 antibody was from BD Biosciences (San Jose, CA). Mouse anti-HRP β-tubulin antibody was from Boehringer-Mannheim (Indianapolis, IN). Mouse anti-FLAG and rabbit anti-hemagglutinin (HA) antibodies were from Cell Signaling Technology (Danvers, MA). Cy3-conjugated goat anti-rabbit secondary antibody was from Jackson ImmunoResearch (West Grove, PA).

**Human airway EC cultures.** Human respiratory ECs derived from distinct regions of the airway, including the trachea (1Haeo, CFTEB290 cells), bronchus (16HBE140 and BEAS-2B cells), terminal bronchioles (small airway ECs (SAECs)), and alveolus (A549 cells), were studied. A549 cells are an alveolar type II cell line derived from a lung adenocarcinoma (American Type Culture Collection, Manassas, VA). 16HBE140, CFTEB290, and HAEo are SV40 T antigen-transfected cell lines provided by Dr. Dietert Gruenert (California Pacific Medical Center Research Institute, San Francisco, CA). BEAS-2B is a SV40-transformed cell line that was provided by Dr. Sekhar Reddy (University of Illinois at Chicago, Chicago, IL). Cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 50 U/ml penicillin, and 50 μg/ml streptomycin. Human primary SAECs (Lanza, Walkersville, MD) were cultured in predefined small airway growth medium (Lanza) containing hydrocortisone, human EGF, epinephrine, transferrin, insulin, retinoic acid, triiodothyronine, and fatty acid-free bovine serum albumin as described (22, 31). Only SAEC passages 2–4 were studied.

**Sialidase activity for ganglioside substrates.** Since NEU3 preferentially hydrolyzes SA linkages within gangliosides (36, 63), sialidase activity for a bovine brain mixed ganglioside substrate was assayed (11, 31). The ganglioside mixture contained 55% GD1a, 18% GM1, 15% GD1b, 10% GT1b, and 2% other gangliosides (Calbiochem, La Jolla, CA). SAECs and A549 cells, or isolated nuclei of A549 cells, were suspended in 200 μl of 50 mM sodium acetate, pH 4.4, containing 0.1% Triton X-100 and protease inhibitor cocktail. The cell suspensions were mixed with 25 μl of gangliosides (2.0 mg/ml) and incubated for 1 h at 37°C. The reaction was terminated by addition of 25 μl of 1.33 M glycine, pH 10.3, 0.6 M NaCl, 0.42 M Na2SO4. Released SA was quantified by high pH anion-exchange chromatography with pulsed amperometric detection as described (11, 31). For each assay, serial dilutions of known concentrations of pure Neu5Ac were measured and a standard curve was generated. The SA concentration in each sample was interpolated from the standard curve by use of GraphPad Prism 4 (GraphPad, La Jolla, CA). The background concentration of SA spontaneously released from the simultaneous cell-free ganglioside control was subtracted from each value.

**Quantitative RT-PCR for NEU3.** Total cellular RNA was extracted from cells by using TRIzol reagent (Invitrogen) following the manufacturer’s recommendations (11, 31). RNA purity was established with the 260 nm/280 nm absorption ratio (>1.90). Total RNA (1.0 μg) was additionally cleaned of possible contamination with genomic DNA and reverse transcribed by using the RT2 SYBR Green qPCR Master Mix (SA Biosciences) in the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA), with all samples tested in duplicate for each of the targets. The levels of NEU3 transcripts were normalized to 18S rRNA transcripts by the 2**−ΔΔCT** method (31). Immunoblotting for NEU3. Cells were thoroughly rinsed with ice-cold HEPES buffer and lysed with ice-cold 50 mM Tris-HCl, pH 8.0, 1.0% Nonidet P-40, 0.5% SDS, 150 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 5.0 μg/ml leupeptin, 1.0 μg/ml pepstatin A, 1.0 mg/ml aprotinin, 1.0 mM vanadate, 1.0 mM sodium fluoride, 10 mM disodium pyrophosphate, 500 μM p-nitrophenol, and 1.0 mM phenylarsine oxide as described (11, 31). The cell lysates were assayed for protein concentration with the Protein Assay Dye Reagent (Bio-Rad, Richmond, CA). Equal amounts of protein were resolved by electrophoresis on 8–16% SDS-polyacrylamide gels and transferred to PVDF membranes. In some experiments, the blots were blocked for 1 h using 5.0% nonfat milk in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.01% Tween-20 (TBS-T), probed with rabbit anti-human NEU3 antibody followed by HRP-conjugated goat anti-rabbit antibody, and developed with ECL reagents (11, 31). To confirm equivalent protein loading and transfer, blots were stripped with 62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoethanol, 2.0% SDS, washed with TBS-T, reprobed with mouse anti-Physarum β-tubulin antibody, followed by HRP-conjugated goat anti-mouse antibody, and developed with ECL reagents. Quantitative densitometry of each NEU3 signal was normalized to densitometry of β-tubulin signal in the same lane in the same blot. In selected experiments, airway ECs were cultured overnight in the absence or presence of 1.0 μg/ml of Streptomyces tunicamycin (Sigma), after which the ECs were lysed and the lysates were processed for NEU3 immunoblotting.

**Adenoviral constructs encoding HA-tagged NEU3 and FLAG-tagged NEU1.** To regulate NEU3 and NEU1 expression in SAECs and A549 cells, recombinant adenovirus (Ad) encoding HA-tagged human

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NEU3 (Ad-NEU3) and FLAG-tagged human NEU1 (Ad-NEU1) were generated, as described (11, 31). The human NEU3 (GenBank accession number NM_006656.5) and NEU1 (NM_000434.3) sequences were cloned by RT-PCR using PCR primers synthesized by Promega Biotech (Cambridge, MA), after which the HA tag and 3 × FLAG tag sequences were inserted prior to the stop codon at the 3’ end of the NEU3 and NEU1 sequences, respectively. SAECs and A549 cells were transiently infected with packaged Ad-NEU3 or Ad-NEU1 at increasing multiplicities of infection (MOIs) and, after 24 h, were lysed, and the lysates were processed for HA or FLAG immunoblotting. In selected experiments, A549 cells infected with increasing MOIs of Ad-NEU3 were assayed for sialidase activity for the mixed ganglioside substrate as described above.

**NEU3 cell surface biotinylation.** Since NEU3 is associated with the plasma membrane (40, 67, 68), the ability of the ectopically expressed HA-tagged NEU3 to localize there was tested by membrane-impermeable biotinylation. A549 cells infected with Ad-NEU3-HA were washed with ice-cold PBS and incubated with gentle agitation for 30 min with sulfo-NHS-SS-biotin (Pierce Cell Surface Protein Isolation Kit, Thermo Fisher Scientific, Waltham, MA), after which quenching solution was introduced. The cells were centrifuged (500 g, 5 min) and the pellet was washed with PBS, fixed with 4% paraformaldehyde for 10 min at 4°C, and the membrane was fixed for 10 min at 4°C with nuclear extraction buffer. The membrane extract was centrifuged (5,000 g, 5 min) and the pellet was extracted with 10 min at 4°C with nuclear extraction buffer. The membrane extract was centrifuged (5,000 g, 5 min) and the pellet was extracted with 10 min at 4°C with nuclear extraction buffer. The nuclear extract was centrifuged (3,000 g, 5 min) and the pellet was extracted with 10 min at 4°C with nuclear extraction buffer. The nuclear extract was centrifuged (5,000 g, 5 min) and the nuclear proteins in the supernatant were resolved by SDS-PAGE and transferred to PVDF membrane, and the membranes were processed for NEU3 immunoblotting. To verify subcellular fractionation, the blot was stripped and reprobed for the cytoplasmic marker protein IκBα and the nuclear marker protein lamin B1.

**Flow cytometry for NEU3 surface expression.** Nonpermeabilized unstimulated 1HAEo+, CFTE29o−, 16HBE14o−, and BEAS-2B cells, SAECs, and A549 cells were stained with rabbit polyclonal anti-human NEU3 antibody (Novus Biologicals, Littleton, CO) or control nonimmune rabbit IgG followed by phyceroerythrin-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch). Cellular fluorescence was measured with an Accuri C6 Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ) and data were analyzed with FlowJo Software (Tree Star, Ashland, OR) (11, 31).

**Immunostaining of NEU3 in human tissues.** Human trachea, main stem bronchus, segmental bronchus, and expanded alveoli were each obtained from 2 ± normal subjects through an institutional review board-approved protocol at the University of Maryland, Baltimore. The tissues were embedded in paraffin and 5-μm sections were prepared. The sections were deparaffinized in xylene and rehydrated in graded series of ethanol (11, 31). Sections were pretreated for heat-induced epitope retrieval by using a pressure cooker and Target Retrieval solution, pH 6.1 (Dako, Carpinteria, CA), followed by endogenous peroxidase blocking for 5 min with 0.3% hydrogen peroxide. The sections were incubated overnight with anti-NEU3 antibody at 1:250 dilution at 4°C in a hydration chamber. Antibody detection was performed by incubation with biotinylated goat anti-rabbit secondary antibody for 30 min at room temperature. Slides were developed for 5 min with diaminobenzidine as the chromagen (Dako) and were counterstained with hematoxylin. As a negative control, tissue sections were reacted with nonimmune rabbit IgG plus the secondary antibody. Staining was performed on an automatic stainer with EnVision+ (Dako), a biotin-free detection system that consists of a secondary antibody covalently linked to peroxidase coated dextrase polymers (11, 31).

**Statistical analysis.** All values were expressed as means ± SE. Differences between means were compared by the Student’s t-test or ANOVA and were considered significant at P < 0.05.

**RESULTS**

**Airway EC sialidase activity.** Respiratory epithelial surfaces express multiple glycoconjugates that are terminally sialylated (12, 31). We asked whether airway epithelia might also express sialidase activity. Increasing primary SAEC and alveolar A549 cell numbers expressed increasing sialidase activity for the ganglioside mixture (Fig. 1A). This sialidase activity was destroyed by boiling (Fig. 1B). ECs derived from human trachea, bronchus, small airways, and the alveolus, each contained sialidase activity for the ganglioside mixture (Fig. 1C). These data indicate that airway epithelia derived from along the entire respiratory tract express sialidase catalytic activity for ganglioside substrate.

**NEU3 expression in airway epithelia.** Since ECs derived from the trachea, bronchus, small airways, and the alveolus contain sialidase activity for ganglioside substrates (Fig. 1, A and C), we asked whether NEU3, a mammalian sialidase with
preferential activity for SA linkages within gangliosides (36, 63), might be expressed in these same cells. In airway epithelia, a quantitative (q)RT-PCR approach was adopted to detect mRNA for NEU3. NEU3 mRNA was identified in all ECs studied, including primary, nontransformed SAECs (Fig. 2A). NEU3 mRNA expression was highest in trachea-derived cells, intermediate in bronchus-derived cells, and lowest in cells derived from the alveolus. We then used an antibody specific for the NEU3 protein to probe lysates of these same airway epithelia (Fig. 2B). In all airway ECs examined, including primary SAECs, NEU3 was detected as a 55.0-kDa band that corresponded to the predicted molecular size of NEU3 protein based on its translated cDNA sequence, 51.7 kDa (40). When NEU3 signal was normalized to -tubulin signal, all analyzed airway epithelia expressed comparable levels of NEU3 protein (Fig. 2C). In 1HAEo, CFTE29o, and BEAS-2B cells, prob- ing for NEU3 revealed a second band of higher molecular mass, ≈55.5 kDa (Fig. 2B and D, arrow). When 1HAEo− and BEAS-2B cells were cultured in the presence of the N-linked glycosylation inhibitor, tunicamycin, the higher molecular weight band either was not detected (Fig. 2D, lane 2) or profoundly diminished (lane 4), while the lower band was unchanged. These combined data suggest that NEU3 protein is expressed along the entire respiratory tract and, in some ECs, exhibits glycosylation isoforms.

**NEU3 sialidase activity.** On the basis of qRT-PCR (Fig. 2A) and Western analyses (Fig. 2, B and C), we asked whether EC-associated sialidase activity might be, in part, explained through NEU3. HA-tagged NEU3 was ectopically expressed in A549 cells through Ad infection (Fig. 3A). Whether the cellular proteins probed for HA or NEU3, NEU3 protein expression was clearly dose dependently increased (Fig. 3A). To establish that the ectopically expressed HA-tagged NEU3 was enzymatically active, A549 cells infected with increasing MOIs of Ad-NEU3 were assayed for sialidase activity for ganglioside substrates (Fig. 3B). Infection with MOIs of 100 dose dependently increased sialidase activity. To establish that the ectopically expressed HA-tagged NEU3 could be detected in the plasma membrane, surface biotinylated proteins were harvested and processed for immunoblotting with both anti-HA and anti-NEU3 antibodies (Fig. 3C). To control for loading and transfer of the plasma membrane preparations, the blots were stripped and reprobed with anti-pan-cadherin antibody. The HA-tagged NEU3 was detected in the cadherin-containing

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**Fig. 1.** Airway epithelial cell (EC) sialidase activity. *A:* increasing primary small airway EC (SAEC; *n* = 2) and A549 (*n* = 3) cell numbers were assayed for sialidase activity for a ganglioside mixture. *B:* A549 cells (1.0 × 10⁶) were assayed for sialidase activity prior to and after boiling (*n* = 2). *C:* equal numbers of airway ECs (1.0 × 10⁶) derived from the trachea (1HAEo−, CFTE29o−), bronchus (16HBE14o−, BEAS-2B), small airways (SAECs), and alveolus (A549), or PBS as a negative control, were assayed for sialidase activity for a ganglioside mixture (*n* = 2). Vertical bars represent mean ± SE sialidase activity, expressed as sialic acid (SA) release in μM. The results are representative of ≥2 independent experiments.
plasma membrane preparations. To unambiguously establish siRNA-induced knockdown of NEU3, A549 cells overexpressing HA-tagged NEU3 were transfected with NEU3-targeting or control siRNAs, after which the cells were lysed and the lysates were processed for HA immunoblotting (Fig. 3D). At 24, 48, and 72 h, NEU3 levels were reduced by 97.6, 96.0, and 82.8%, respectively, relative to control siRNA-transfected cells (Fig. 3D, lanes 2 vs. 1, 4 vs. 3 and 6 vs. 5). To exclude off-target effects, A549 cells overexpressing FLAG-tagged NEU1 were transfected with NEU3-targeting, NEU1-targeting, or control siRNAs and lysed, and the lysates processed for FLAG immunoblotting (Fig. 3E). At 48 h, NEU1 in cells transfected with NEU1-targeting siRNAs was reduced by >80% (Fig. 3E, lane 2 vs. 1), whereas in cells transfected with NEU3-targeting siRNAs, NEU1 protein levels were unchanged (lane 3 vs. 1), each relative to control siRNA-transfected cells. Therefore, NEU3-targeting siRNA did not diminish NEU1 protein, indicating efficient and selective siRNA-induced depletion of NEU3 in A549 cells. Finally, to establish the contribution of NEU3 to sialidase activity in airway ECs, A549 cells transfected with NEU3-targeting, NEU1-targeting, or control siRNAs, after 48 h, were assayed for sialidase activity for the ganglioside mixture (Fig. 3F). siRNA-mediated knockdown of NEU3 decreased sialidase activity by >70% compared with control siRNA-transfected cells. In contrast, knockdown of NEU1 had no such effect. Whether the residual sialidase activity for the ganglioside substrate detected after NEU3 depletion can be explained through NEU1, 2, and/or 4 activities remains to be determined. We have recently reported that in SAECs, at the mRNA level, NEU2 and 4 are expressed at 7.7 and 13.8%, respectively, relative to NEU3 (31). Furthermore, compared with NEU3 (36, 63), the catalytic activities of NEU1, 2, and 4 for gangliosides are less clear (19, 56, 65).

Subcellular localization of NEU3. Since NEU3 has been previously immunolocalized in nonairway cells to specific subcellular compartments (40, 67, 70), we utilized confocal fluorescence microscopy to probe for NEU3 protein (Fig. 4A). A549 cells revealed punctuate immunostaining for NEU3, uniformly throughout the cytosol (Fig. 4Ai). Of note, NEU3 was detected within nuclei (Fig. 4Aii, arrows). To confirm nuclear expression of NEU3, the nuclear fraction of A549 cells was probed with anti-NEU3 antibody and NEU3 immunoreactivity was seen (Fig. 4B). Finally, A549 cells and nuclei isolated from an equal number of A549 cells were assayed for sialidase activity with the ganglioside mixture used as substrate. Isolated airway EC nuclei expressed 59.8% of the sialidase activity with the ganglioside mixture used as substrate. Isolated airway EC nuclei expressed 59.8% of the sialidase activity with the ganglioside mixture used as substrate. Isolated airway EC nuclei expressed 59.8% of the sialidase activity with the ganglioside mixture used as substrate.
the surface expression levels of NEU3, with CFTE29o showing strong expression, followed by 16HBE14o, BEAS-2B, and 1HAEo. NEU3 surface expression was less pronounced on SAEC and A549 cells. Although NEU3 surface expression in each type of airway EC correlated with levels of NEU3 mRNA expressed in the same cell type (Fig. 2A), it did not correlate as well with total NEU3 protein expressed in the same cell type (Fig. 2C). These combined data indicate that epithelia derived from along the human respiratory tract all surface express NEU3, although at varying levels.

NEU3 immunostaining in human airway epithelial tissues. Sialidase activity can be detected in cultured ECs derived from various portions of the human airway (Fig. 1, A and C) and NEU3 protein is expressed in these same epithelia (Fig. 2B and C). We asked whether NEU3 protein expression could be extended to normal intact human tissues. Immunohistochemical techniques were used to establish whether NEU3 protein could be detected in human trachea, main stem bronchus, segmental bronchus, and the alveolus (Fig. 6). Each airway epithelium was photographed at 400 and 1,000 (see insets). NEU3 immunostaining appears brown and is indicated by arrows. In the trachea, intense NEU3 staining was evident at the mucosal surface (Fig. 6A). At higher magnification, NEU3 staining was clearly detected within the ciliated brush border (Fig. 6A, inset, arrow). The subepithelial mesenchymal cells displayed diminished staining relative to epithelia. In the main stem bronchus, NEU3 staining was most intense in the superficial portion of the epithelial cytoplasm, particularly the ciliated brush border (Fig. 6B). Here, the subepithelial mesenchymal cells were weakly stained, whereas goblet cell mucus granules were completely devoid of staining. In the segmental bronchi, strong cytoplasmic staining was evident in the EC
layer (Fig. 6C). Here, the subepithelial mesenchyma exhibited more NEU3 immunostaining than did subepithelial regions in the sections of trachea (Fig. 6A) and main stem bronchus (Fig. 6B). In the expanded alveoli, the epithelial cells contained cytoplasmic and nuclear staining (Fig. 6D, inset, arrow). Sections probed with a species- and isotype-matched, nonimmune control IgG were completely nonreactive (Fig. 6E). In those hematoxylin-stained tissues where NEU3 staining appeared to localize to the nucleus, whether this represented true nuclear staining vs. cytoplasmic staining superimposed over the nucleus, or both, was unclear. However, our confocal microscopic and immunoblotting studies in A549 cells immunolocalized NEU3 to the plasma membrane (Figs. 3C and 5) as well as to the cytosolic and nuclear subcellular compartments (Fig. 4). NEU3 protein was detected in comparable abundance in epithelia derived from along the entire airway (Fig. 2, B and C). In selected airway ECs, NEU3 protein was detected on immunoblots as a doublet, and, on the basis of tunicamycin inhibition experiments (Fig. 2D), the upper band may constitute an N-linked glycosylation isoform and the lower band a nonglycosylated or O-linked glycosylation isoform. siRNA-induced silencing of NEU3 diminished sialidase activity for the ganglioside substrate by \( \frac{70}{10022} \% \) (Fig. 3F). Finally, NEU3 was detected in human tissues, including trachea, main stem and segmented bronchi, and alveoli (Fig. 6).

The NEU3 immunostaining was localized to the superficial epithelia, including the ciliated brush border, but was reduced in subepithelial tissues.

Although prior reports have documented endogenous sialidase activity in animal and human lung tissues (27, 58), information on which specific sialidase gene products are expressed, in which lung cells, and at the mRNA, protein, or catalytic levels, was unclear. In guinea pig lungs, sialidase activity for the synthetic 4-MU-Neu5Ac substrate was found in lysosomal, microsomal, and cytosolic fractions (58). NEU3 displays very little enzymatic activity for 4-MU-Neu5Ac (63) and is classically associated with the plasma membrane (36, 26, 46).

**DISCUSSION**

We now have demonstrated heat-labile sialidase activity for a ganglioside substrate in human respiratory airway epithelia derived from trachea, bronchus, small airways, and alveoli (Fig. 1). In airway epithelia, NEU3 was expressed at both mRNA (Fig. 2A) and protein (Fig. 2, B and C) levels and could be immunolocalized, as anticipated, to the plasma membrane (Figs. 3C and 5) as well as to the cytosolic and nuclear subcellular compartments (Fig. 4). NEU3 protein was detected in comparable abundance in epithelia derived from along the entire airway (Fig. 2, B and C). In selected airway ECs, NEU3 protein was detected on immunoblots as a doublet, and, on the basis of tunicamycin inhibition experiments (Fig. 2D), the upper band may constitute an N-linked glycosylation isoform and the lower band a nonglycosylated or O-linked glycosylation isoform. siRNA-induced silencing of NEU3 diminished sialidase activity for the ganglioside substrate by \( \frac{70}{10022} \% \) (Fig. 3F). Finally, NEU3 was detected in human tissues, including trachea, main stem and segmented bronchi, and alveoli (Fig. 6). The NEU3 immunostaining was localized to the superficial epithelia, including the ciliated brush border, but as well as to nuclei, but was reduced in subepithelial tissues.

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In humans, sialidase activity was detected in bronchoalveolar lavage fluid (BALF) from 8 of 9 patients with idiopathic pulmonary fibrosis and from 10 of 35 patients with sarcoidosis, whereas BALF from normal subjects was devoid of enzyme activity (27). The cellular source for this sialidase activity was not identified. To define the relative abundance of NEU3 mRNA in human tissues, Northern blots of RNA from human tissues were hybridized with a cDNA for human NEU3 (63). The NEU3 gene was expressed at relatively low levels in lung. In a similar study, Northern analysis of human poly(A) + mRNA from 50 different tissues revealed NEU3 transcripts in adult and fetal lung and adult trachea (40). In a more recent study, qRT-PCR was used to quantify mRNA expression of the four mammalian sialidases in human lung tissue (20). NEU3 was expressed at a lower level than either NEU1 or NEU4 but at a higher level than NEU2. We previously reported that NEU3 is expressed in human lung microvascular and pulmonary arterial endothelia at the mRNA, protein, and catalytic levels (11), as well as in human cells of monocyte-macrophage lineage (57). In the present report, we now have established that NEU3 is expressed at the mRNA and protein levels in human respiratory epithelia derived from along the entire airway (Figs. 2 and 6).

In initial studies of mammalian sialidases, each enzyme was named on the basis of its subcellular localization (1, 35, 42, 55). NEU3 was designated the plasma membrane-associated sialidase (36, 40). When a cDNA for human NEU3 was transfected into COS-7 cells, the protein was immunolocalized to the plasma membrane (40, 71). The murine and bovine enzymes similarly associate with the plasma membrane (36, 45, 70). Biochemical characterization of human NEU3 has revealed a protein that enriches to plasma membrane fractions (40) and is hydrophilic (1, 35, 42, 55), and whose activity increases in the presence of the detergent, Triton X-100 (40). However, on the basis of a Kyte-Doolittle hydrophobicity plot, a typical transmembrane domain is not evident (42). To better understand the mechanism(s) through which NEU3 associates with the plasma membrane, studies have revealed that NEU3 cofractionates with markers of lipid rafts (25) and closely associates with caveolin-1 in distinct membrane microdomains (67). Following EGF stimulation of A431 epidermoid carcinoma cells, NEU3 redistributes from a uniform plasma membrane staining pattern to membrane ruffles (68). In our studies, we utilized flow cytometry of unstimulated, nonpermeabilized airway epithelia to establish surface expression of endogenous NEU3 (Fig. 5) and surface biotinylation to detect HA-tagged NEU3 in the cadherin-containing plasma membrane fractions (Fig. 3C). Furthermore, NEU3 immunostaining of intact human lung tissue localized NEU3 to superficial epithelia, including the brush border (Fig. 6, A–C). However, strong NEU3 signal also could be detected in the intracellular compartment (Fig. 4A). Compatible with this finding, murine NEU3 has been found to be internalized and localized in recycling endosomal structures (70). NEU3 was clearly immunolocalized to nuclei (Fig. 4). Confocal fluorescence microscopy with removal of DAPI signal revealed NEU3 in the nucleus (Fig. 4Aii), as did NEU3 immunoblotting of isolated nuclear subcellular fractions (Fig. 4B). Finally, much (~60%) of the sialidase activity that was detected in intact airway ECs by using the ganglioside substrate, the preferred substrate for NEU3 (40, 42, 63), was found in isolated nuclei (Fig. 4C). NEU3 immunostaining of human lung alveoli, although not definitive, also was suggestive of NEU3 within the nuclei of airway epithelia (Fig. 6D). NEU3 has been demonstrated within the inner membranes of the nuclear envelope of both rodent and human neuroblastoma cells (28, 64). Since gangliosides are expressed in these same nuclear membranes, it is possible that NEU3 regulates their local catabolism. Whether NEU3 in the nucleus might also participate in transcriptional gene regulation is unknown.
Of the four known mammalian sialidases, it is NEU3 that has been most closely associated with the plasma membrane (25, 67, 70). The preferred substrates for NEU3, gangliosides (40, 42, 63), are also localized to the plasma membrane (16). Not only is NEU3 found on the cell surface (Fig. 3C, 5) (25, 67, 70), but it also exerts catalytic activity for sialylated molecules expressed on the surface of neighboring cells (45). These combined data indicate that NEU3 is catalytically active on the cell surface and possibly within the paracellular space. The optimal pH for NEU3 catalytic activity in various buffers for various substrates has ranged from 3.8 to 6.0 (3, 40, 66). Transfection of human NEU3 cDNA into COS-7 cells generated sialidase activity for ganglioside GD1a substrate in sodium acetate buffer with an optimal pH of \( \approx 3.8 \), and in sodium phosphate buffer, with an optimal pH of \( \approx 6.0 \) (66). The pH of airway surface liquid in nasal secretions obtained from normal human subjects was \( \approx 7.2 \), and in cultures of human bronchial epithelial cells, \( \approx 6.7 \) (13). Although these reported airway surface liquid pH values lie outside the reported optimal pH range for NEU3 catalytic activity, it is unclear how accurately the pH of infinitesimally small areas of the microenvironment surrounding a specific molecule, possibly embedded within the glycocalyx, can be measured. In any case, in our in vitro studies, human airway EC sialidase activity for a ganglioside mixture could be detected (Fig. 1, A and B) and NEU3 explained \( >70\% \) of this total sialidase activity (Fig. 3F).

NEU3, through its ability to desialylate gangliosides, is poised to regulate airway EC responsiveness to multiple pathogens and

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**Fig. 6. NEU3 immunostaining of airway epithelial tissues.** Sections (5.0 μm) of human trachea (A), main stem bronchus (B), segmental bronchus (C), and expanded alveoli (D) were processed for NEU3 immunohistochemistry. As a negative control, sections of alveoli were probed with nonimmune rabbit IgG (E). Each section was photographed at \( \times400 \) or \( \times1,000 \) (insets). Scale bar, 50 μm. Arrows indicate immunostaining for NEU3. Each photograph is representative of 2 independent sections.
their toxins. GM1 is a binding site for *Vibrio cholerae* toxin and *Escherichia coli* heat-labile enterotoxin (21) whereas asialoGM1 recognizes *Pseudomonas aeruginosa*-derived pilin (8, 48, 51) and flagellin (2). Another ganglioside, GD1a, binds polyoma viruses, including the human pathogens, JC and BK viruses (14, 15), and flagellin (2). Another ganglioside, GD1a, binds polyoma viruses, recognizing *Escherichia coli* (Fig. 6, A–C). Here, NEU3 is strategically positioned to influence airway EC-pathogen interactions.

NEU3 contains a caveolin-1-binding domain that targets it to distinct membrane microdomains where the siaidase is in close proximity to multiple signaling elements (67). NEU3 physically and/or functionally interacts with caveolin-1 (67), Rac1 (68), integrins β1 (59) and β4 (26), Grb-2 (52), Akt (59), focal adhesion kinase (59), and Ras (62). NEU3 strongly regulates insulin (52, 69) and EGFR (34, 38, 59, 62, 68) signaling pathways and alters expression of multiple metalloproteinases (MMPs), including MMP-2, MMP-7, and MMP-9 (24, 29, 43, 59). In conclusion, this report demonstrates that human airway ECs express catalytically active NEU3 siaidase and implicate NEU3 in multiple biological processes within human airway epithelia.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


