Inhibitory effects of carbocisteine on type A seasonal influenza virus infection in human airway epithelial cells

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Yamaya M, Nishimura H, Shinya K, Hatachi Y, Sasaki T, Yasuda H, Yoshida M, Asada M, Fujino N, Suzuki T, Deng X, Kubo H, Nagatomi R. Inhibitory effects of carbocisteine on FluA virus infection in airway epithelial cells. Am J Physiol Lung Cell Mol Physiol 299: L160–L168, 2010. First published June 11, 2010; doi:10.1152/ajplung.00376.2009.—Type A human seasonal influenza (FluA) virus infection causes exacerbations of chronic obstructive pulmonary disease (COPD). 1-carbocisteine, a mucolytic agent, reduces the frequency of common colds and exacerbations in COPD. However, the inhibitory effects of 1-carbocisteine on FluA virus infection are uncertain. We studied the effects of 1-carbocisteine on FluA virus infection in airway epithelial cells. Human tracheal epithelial cells were pretreated with 1-carbocisteine and infected with FluA virus (H1N1). Viral titers in supernatant fluids, RNA of FluA virus in the cells, and concentrations of proinflammatory cytokines in supernatant fluids, including IL-6, increased with time after infection. 1-carbocisteine reduced viral titers in supernatant fluids, RNA of FluA virus in the cells, the susceptibility to FluA virus infection, and concentrations of cytokines induced by virus infection. The epithelial cells expressed sialic acid with an α2,6-linkage (SAα2,6Gal), a receptor for human influenza virus on the cells, and 1-carbocisteine reduced the expression of SAα2,6Gal. 1-carbocisteine reduced the number of acidic endosomes from which FluA viral RNA enters into the cytoplasm and reduced the fluorescence intensity from acidic endosomes. Furthermore, 1-carbocisteine reduced NF-κB proteins including p50 and p65 in the nuclear extracts of the cells. These findings suggest that 1-carbocisteine may inhibit FluA virus infection, partly through the reduced expression of the receptor for human influenza virus in the human airway epithelial cells via the inhibition of NF-κB and through increasing pH in endosomes. 1-carbocisteine may reduce airway inflammation in influenza virus infection.

SAα2,6Gal; bronchial asthma; chronic obstructive pulmonary disease

MUCOLYTIC AGENTS INCLUDING N-acetylcysteine, 1-carbocisteine, and ambroxol have various physiological functions such as antioxidant effects (39), reduction of mucin SAC protein production (27), and improvement of airway mucociliary transport (20). Mucolytic agents are also suggested to have clinical benefits in reducing the frequency of exacerbations and improvement of quality of life in patients with chronic obstructive pulmonary disease (COPD) (40).

Exacerbations of COPD have been reported to be caused by viral and bacterial infection (22) as well as by exposure to oxidants in airways. Of the viruses, type A seasonal influenza (FluA) is one of the pathogens that cause COPD exacerbations especially in the winter season (19). Influenza vaccine reduces the mortality rate in COPD patients in the winter season (14) and is recommended in the management of COPD patients in stable conditions. The clinically used anti-influenza drugs, which include neuraminidase inhibitors such as oseltamivir and zanamivir, and M2 ion channel blockers such as amantadine, are beneficial for human influenza infection (1). On the other hand, a mucolytic agent, 1-carbocisteine, reduces the frequency of common colds as well as the frequency of exacerbations in COPD patients (36, 40). Yang et al. (35) demonstrated that a mucolytic agent, ambroxol, inhibits influenza-virus proliferation in the mouse airway. Likewise, 1-carbocisteine inhibits the infection of rhinovirus (37), a major cause of COPD exacerbations. However, the effects of 1-carbocisteine on influenza virus infection have not been studied.

In an infection of FluA virus, the viruses are attached to sialic acid (SAα2,6Gal), a receptor for human influenza virus, on airway epithelial cells (18). The viruses are then delivered into the cytoplasm, and the RNA of the viruses is released from acidic endosomes into the cytoplasm of the cells (17). As we demonstrated, 1-carbocisteine reduces the number of acidic endosomes in human tracheal epithelial cells (37). However, the effects of 1-carbocisteine on FluA virus infection are still uncertain.

Influenza virus infection induces airway inflammation through the production of proinflammatory cytokines and chemokines such as IL-6, IL-8, and IFN-γ-inducible protein (IP)-10 (7, 30) and through the epithelial cell damage, both of which are associated with exacerbations of COPD (12). 1-
CARBOCISTEINE AND SEASONAL INFLUENZA

Fig. 1. The time course of viral release in supernatant fluids of human tracheal epithelial cells obtained at different times after exposure to 0.5 × 10^3 tissue culture infective dose (TCID50) U/cell type A human seasonal influenza (FluA) virus in the presence of L-carbocisteine (10 μM) or vehicle of L-carbocisteine (0.1% double-distilled water; ○). The rates of change in FluA virus concentration in the supernatant fluids are expressed as TCID50 U/ml/24 h. Results are means ± SE from 5 different tracheae. Significant differences from viral infection alone are indicated by *P < 0.05 and **P < 0.01.

carbocisteine reduces the production of proinflammatory cytokines after rhinovirus infection (37), whereas the inhibitory effects of L-carbocisteine on the production of proinflammatory cytokines by FluA virus infection are uncertain.

In the present study, we studied the effects of L-carbocisteine on FluA virus infection in cultured human tracheal epithelial cells that are targets of the viruses. We also examined the effects of L-carbocisteine on the expression of SAα2,6Gal, a receptor for human influenza virus, and on acidic endosomes to examine the inhibitory mechanisms.

MATERIALS AND METHODS

Media components. Reagents for cell culture media were obtained as follows: DMEM, Ham’s F-12 medium, and FCS were from Gibco BRL Life Technologies (Palo Alto, CA); PBS, MEM, and trypsin were from Sigma (St. Louis, MO), and Ultroser G (USG) was from Pall BioSepa (Cergy-Saint-Christophe, France).

Human tracheal epithelial cell culture. Isolation and culture of the human tracheal surface epithelial cells were performed as described previously (29, 34). The human tracheal surface epithelial cells were plated at 5 × 10^5 viable cells/ml in plastic tubes with round bottoms (16-mm diameter and 125-mm length; Becton Dickinson) coated with human placental collagen because attachment of the cells in plastic tubes was much better than that in glass tubes (data not shown) (34). Cells were cultured in 1 ml of a mixture of DMEM/Ham’s F-12 (DF-12) medium (50:50 vol/vol) containing 2% USG and antibiotics (29, 34). The tubes were laid with a slant of ~5° and kept stationary, and cells were immersed in 1 ml of medium and cultured at 37°C in 5% CO2-95% air in a humid incubator (29, 34). Because of this laid position of the plastic tubes, the cells attached and proliferated on the inner surface of the lateral wall of the tubes and the round shape of the bottom of the tubes. The surface area of culture vessels of the plastic tubes covered by the cells became 11.7 ± 0.2 cm² (n = 3). Cells in the tubes were cultured at 37°C in 5% CO2-95% air. We confirmed the presence of a dome formation when the cells made confluent cell sheets on days 5–7 of culture using an inverted microscope (MIT-2; Olympus, Tokyo, Japan) (29) as described by Widdicombe et al. (32).

Tracheas for cell cultures were obtained after death from 36 patients (age, 71 ± 4 yr; 12 female, 24 male) without complications with bronchial asthma. Five patients were complicated with COPD. The causes of death included malignant tumor other than lung cancer (n = 17), acute myocardial infarction (n = 5), renal failure (n = 3), congestive heart failure (n = 3), cerebral bleeding (n = 2), rupture of an aortic aneurysm (n = 2), cerebral infarction (n = 1), sepsis (n = 1), mitral stenosis (n = 1), and malignant lymphoma (n = 1). Of 36 patients, 15 were exsmokers, and 21 had never smoked. This study was approved by the Tohoku University Ethics Committee.

Culture of Madin-Darby canine kidney cells. Madin-Darby canine kidney (MDCK) cells were cultured in T-25 flasks (Becton Dickinson) in MEM containing 10% FCS supplemented with 5 × 10⁴ U/l penicillin and 50 mg/l streptomycin (15). The cells were then plated in plastic dishes (96-well plate; Becton Dickinson) or in plastic tubes with round bottoms (16-mm diameter and 125-mm length: Becton Dickinson). Cells in the plastic dishes or tubes were cultured at 37°C in 5% CO2-95% air.

Viral stocks. FluA virus (H3N2) was prepared in our laboratory from a patient complaining of fever and rhinorrhea (15). FluA virus was identified by the hemadsorption inhibition (HI) test using an antisera (New York/55/2004) as described previously (15). To generate stocks of FluA virus, MDCK cells in plastic tubes were cultured in the medium (1.1 ml) containing 100 μl of FluA virus stock solution [1.0 × 10⁵ 50% tissue culture infective dose (TCID50) units in 100 μl] and 1 ml of MEM supplemented with 5 × 10⁴ U/l penicillin, 50 mg/l streptomycin, and 3.5 μg/ml trypsin. Cells in the plastic tubes were cultured at 33°C in 5% CO2-95% air (15). To obtain the FluA virus solution, 7 days after infection with FluA virus, cells and medium were frozen by immersing the tubes in ethanol at −80°C, thawed, and sonicated. The virus-containing fluid was frozen in aliquots at −80°C.

Detection and titration of influenza viruses. Detection and titration of influenza viruses in supernatant fluids was performed with the

Fig. 2. Concentration-response effects of L-carbocisteine on the viral release in supernatant fluids collected during 3 days (72 h) to 5 days (120 h) after infection. The cells were treated with L-carbocisteine or vehicle (Control; 0.1% double-distilled water) from 3 days before FluA virus infection until the end of the experiments after FluA virus infection. The rates of change in FluA virus concentration in the supernatant fluids are expressed as TCID50 U/ml/24 h. Results are means ± SE from 7 different tracheae. Significant differences from vehicle alone (Control) are indicated by *P < 0.05 and **P < 0.01.
described with some modification. A stock solution of tracheal epithelial cells was performed with methods previously described (5, 15, 34). Furthermore, the rates were obtained by epithelial cells were cultured in 1 ml of DF-12 medium containing 2% USG supplemented with 5 × 10^4 U/ml penicillin, 50 mg/l streptomycin, and 3.5 μg/ml trypsin (15) and added into the replicate MDCK cells in the wells (200 μl/well) of 96-well dishes. MDCK cells in the wells were then cultured at 33°C in 5% CO₂-95% air for 7 days, and the presence of the typical cytopathic effects (CPE) of influenza virus was examined in all replicate cells as described previously (5, 15). The number of wells that showed CPE of influenza was counted in each dilution of supernatant fluids. Then, the dilution of virus-containing supernatant fluids, which showed CPE in >50% of replicate wells, and the dilution of the fluids, which showed CPE in <50% of replicate wells, were estimated. Based on these data, TCID₅₀ was calculated with the method as previously described (5). Because the human tracheal epithelial cells were cultured in 1 ml of DF-12 medium containing 2% USG, viral titers and cytokine concentrations were estimated. In brief, to measure the release of FluA virus and proinflammatory cytokines during the first hour after virus infection, we collected the supernatant fluids at 1 h after FluA virus infection. After collecting supernatant fluids at 1 h after infection, the cells were rinsed with PBS, and 1 ml of DF-12 medium containing 2% USG was replaced. Furthermore, to measure the release of FluA virus and proinflammatory cytokines from 1 h to 1 day after FluA virus infection, we collected supernatant fluids at 1 day (24 h) after virus infection. To measure the release of FluA virus and proinflammatory cytokines during 1–3 days after virus infection, supernatant fluids were collected at 1 day after infection, the cells were rinsed with PBS, and 1 ml of DF-12 medium containing 2% USG was replaced. Supernatant fluids were also collected at 3 days after infection. Likewise, to measure the release of FluA virus and proinflammatory cytokines during 3–5 days after FluA virus infection, after collecting supernatant fluids at 3 days after infection, the cells were rinsed with removed, and the cells were rinsed one time with 1 ml of PBS. The cells were then fed with 1 ml of fresh DF-12 medium containing 2% USG supplemented with antibiotics. The tubes were laid with a slant of ~5° and kept stationary in a humid incubator, and cells were cultured at 33°C in 5% CO₂-95% air as described previously (29, 34). The supernatant fluids were stored at ~80°C for the determination of viral titers and cytokine concentrations.

**Treatment with l-carbocisteine.** The cultured human tracheal epithelial cells were treated with l-carbocisteine (10 μM) from 3 days before FluA virus infection until the end of the experiments after FluA virus infection (34, 37) unless we describe other concentrations or treatment periods. The concentrations of l-carbocisteine (10 μM) were chosen because the maximum concentrations of l-carbocisteine in the serum became >10 μM after oral ingestion of 1,500 mg of l-carbocisteine (9).

To examine the concentration-dependent effects of l-carbocisteine on FluA virus infection, cells were treated with l-carbocisteine at concentrations ranging from 10 nM to 100 μM.

### Collection of supernatant fluids for measurements

We measured the time course of the release of FluA virus and proinflammatory cytokines with methods previously described (29, 34) with some modifications. In brief, to measure the release of FluA virus and proinflammatory cytokines during the first hour after virus infection, we collected the supernatant fluids at 1 h after FluA virus infection. After collecting supernatant fluids at 1 h after infection, the cells were rinsed with PBS, and 1 ml of DF-12 medium containing 2% USG was replaced. Furthermore, to measure the release of FluA virus and proinflammatory cytokines from 1 h to 1 day after FluA virus infection, we collected supernatant fluids at 1 day (24 h) after virus infection. To measure the release of FluA virus and proinflammatory cytokines during 1–3 days after virus infection, supernatant fluids were collected at 1 day after infection, the cells were rinsed with PBS, and 1 ml of DF-12 medium containing 2% USG was replaced. Supernatant fluids were also collected at 3 days after infection. Likewise, to measure the release of FluA virus and proinflammatory cytokines during 3–5 days after FluA virus infection, after collecting supernatant fluids at 3 days after infection, the cells were rinsed with
PBS, and 1 ml of fresh medium was replaced. Supernatant fluids were also collected at 5 days after virus infection. The cells were then rinsed with PBS, and 1 ml of fresh medium was replaced. Supernatant fluids were also collected at 7 days after infection to measure the release of FluA virus and proinflammatory cytokines during 5–7 days after FluA virus infection.

To measure the concentrations of proinflammatory cytokines before FluA infection, supernatant fluids were also collected just before FluA infection.

**Effects of L-carbocisteine on susceptibility to influenza virus infection.** The effects of L-carbocisteine on the susceptibility to influenza virus infection were evaluated as previously described (26, 29, 34) using epithelial cells pretreated with L-carbocisteine (10 µM) or vehicle (0.1% double-distilled water) from 3 days before infection with FluA virus until just finishing the virus infection. The epithelial cells were then exposed to serial 10-fold dilutions of FluA virus (H3N2) or vehicle of influenza virus (MEM containing 3.5 µg/ml trypsin) for 1 h at 33°C in 5% CO2-95% air. Because we found in the preliminary experiments that the maximum virus titers were observed in the supernatant fluids collected for 3–5 days, the presence of FluA virus was determined in the supernatant fluids collected for 3–5 days after infection with methods described above to assess whether infection occurred at each dose of influenza virus used.

**Quantification of influenza virus RNA.** To quantify FluA (H3N2) virus RNA and ribosomal RNA (rRNA) expression in the human tracheal epithelial cells after virus infection, real-time quantitative RT-PCR using the TaqMan technique (Roche Molecular Diagnostics) was performed as previously described (34) with some modification.

Each RNA sample (100 ng/10 µl water) was mixed in 40 µl of buffer containing 100 nM forward primer (5'-AGATGAGTCTTCTAAACCGAGGTCTG-3'), 100 nM reverse primer (5'-TGCAAAACATCTTCAAGTCTCG-3'), and other reagents as previously described (25). The TaqMan probe influenza virus [5'-FAM TCAGGC-CCCCTCAAAGCCGA (TAMRA)-3'] was designed for FluA virus (25). The fragment of RNA extracted from the human tracheal epithelial cells at 3 days (72 h) or at 5 days (120 h) after infection by FluA virus was reverse-transcribed into cDNA (30 min at 48°C) and amplified by PCR for 40 cycles (15 s at 95°C and 1 min at 60°C). The standard curve was obtained between the fluorescence emission signals and cycle threshold (Ct) by means of 10-fold dilutions of the total RNA, extracted from 1.0 × 10^6 TCID$_{50}$ U/ml FluA virus in the supernatant fluids of the MDCK cells 7 days after infection with FluA virus (0.5 × 10^-3 TCID$_{50}$ U/cell). Real-time quantitative RT-PCR for rRNA was also performed using the same PCR products. The standard curve was obtained between the fluorescence emission signals and Ct by means of 10-fold dilutions of the RNA extracted from the cells. The expression of FluA virus RNA was normalized to the constitutive expression of rRNA.

**Detection of Sα2,6Gal in human tracheal epithelial cells.** Sα2,6Gal in human tracheal epithelial cells was detected using lectins as previously described (23). In brief, human tracheal epithelial cells were cultured for 7 days on a filter membrane (0.45-µm pore size and 0.6-cm² area; Millicell-CM inserts; Millipore Products Division) coated with collagen gel (PureCol; Inamed) as described previously (33). Because cells were cultured with air-interface methods (33), culture medium was supplied from the basolateral side of the cell monolayer.

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**Fig. 5.** A and B: the expression of Sα2,6Gal (arrows), a receptor for human influenza virus, on the cultured human tracheal epithelial cells treated with L-carbocisteine (10 µM, 72 h; Carbocisteine) or vehicle of L-carbocisteine (0.1% double-distilled water, 72 h; Control). Data are representative of 8 different experiments. Bar = 100 µm. C: the fluorescence intensity of Sα2,6Gal on the mucosal surface of the human tracheal epithelial cells treated with L-carbocisteine (10 µM, 72 h; Carbocisteine) or vehicle of L-carbocisteine (0.1% double-distilled water, 72 h; Control). Results are expressed as relative fluorescence intensity (%) compared with that treated with vehicle and reported as means ± SE from 8 samples. Significant differences from control values are indicated by ***P < 0.001. D: concentrations of Sα2,6Gal measured with enzyme-linked lectin assay (ELLA) in the cells treated with L-carbocisteine (10 µM, 72 h; Carbocisteine) or vehicle of L-carbocisteine (0.1% double-distilled water, 72 h; Control). Results are expressed as optical density (OD) and are means ± SE from 5 different tracheae. Significant differences from vehicle alone (Control) are indicated by *P < 0.05.
sheets through the filter membrane. Then, the cells were treated with L-carbocisteine (10 μM) for 3 days and fixed in 10% paraformaldehyde.

To study the expression of SAα2,6Gal in human tracheal epithelial cells, paraffin-embedded cell sheets were cut into 5-μm thick sections with a microtome and mounted on 3-aminopropyltriethoxysilane-coated slides (Matsunami Glass Industries, Tokyo, Japan). Sections were incubated with 250 μl of FITC-labeled *Sambucus nigra* (SNA) lectin (1:100; Vector Laboratories, Burlingame, CA) overnight at 4°C. Sections were incubated with Alexa Fluor 594-conjugated streptavidin (1:250; Molecular Probes, Eugene, OR) for 2 h at room temperature and counterstained with 4',6-diamidino-2-phenylindole (DAPI; Dojindo Molecular Technologies, Kumamoto, Japan). The cover glasses were mounted on the sections and observed with a fluorescence microscope (BZ-8000; Keyence, Osaka, Japan). The excitation wavelengths were 470 nm (FITC), 560 nm (Alexa Fluor 594), and 360 nm (DAPI), and the emitted light from the cells was detected through 495-, 595-, and 400-nm filters, respectively. The fluorescence intensity was calculated using a fluorescence image analyzer system (Lumina Vision; Mitani, Fukui, Japan) equipped with a fluorescence microscope.

We also measured the amounts of SAα2,6Gal in the cells with enzyme-linked lectin assay (ELLA) (11, 21) with some modification. SAα2,6Gal in the cells was extracted using Glycoprotein Isolation Kits (Thermo Scientific). The extracted samples were diluted to concentrations of 15 μg/ml with Coating Solution Concentrate (KPL), and diluted samples were coated on Costar ELISA plates in triplicate overnight at 4°C. The plate was washed four times with TBS and then blocked for 1 h with freshly made TBS-Tween 20. Plates were then incubated for 2 h with biotinylated SNA (diluted 1:200), washed four times with TBS-Tween 20, and then incubated at room temperature with alkaline phosphatase-conjugated streptavidin at 1:1,000 dilution for 1 h. Plates were developed with alkaline phosphatase substrate for 15 min. Absorption readings were taken at 405 nm, and results are expressed as optical density (OD).

Measurement of changes in acidic endosomes. The distribution and the fluorescence intensity of acidic endosomes in the cells were measured as previously described with a dye, LysoSensor DND-189 (Molecular Probes) (29, 34). Because the cells were pretreated with L-carbocisteine for 3 days before FluA virus infection, we studied the effects of a 3-day treatment of L-carbocisteine (10 μM) on the distribution and the fluorescence intensity of acidic endosomes. Because each cell contains many acidic endosomes, we measured the average fluorescence intensity from many acidic endosomes in each cell. We measured the average fluorescence intensity for each cell in 100 human tracheal epithelial cells and calculated the mean value of fluorescence intensity of the cell sheets observed. The mean value of fluorescence intensity of the cell sheets treated with L-carbocisteine (10 μM) was expressed as percentage of control value compared with the fluorescence intensity of the cell sheets treated with vehicle of L-carbocisteine (0.1% double-distilled water).

Measurement of cytokine production. We measured IL-1β, IL-6, and IL-8 of supernatant fluids by specific ELISAs (29, 34). To demonstrate the time course of cytokines release, we expressed the rates of change in cytokine concentration in the supernatant fluids. The rates were obtained by dividing the value of cytokine concentration in supernatant fluids by incubation time and are expressed as pg/ml/24 h.

NF-κB assay. Nuclear extracts from human tracheal epithelial cells were prepared by using a TransAM NF-κB p50, p52, p65, and Family Kit (Activ Motif) according to the manufacturer’s instructions. After

Fig. 6. A and B: changes in the distribution of acidic endosomes with green fluorescence in the human tracheal epithelial cells 3 days (72 h) after treatment with L-carbocisteine (10 μM; B) or vehicle of L-carbocisteine (0.1% double-distilled water; A). Data are representative of 5 different experiments. C: the fluorescence intensity of acidic endosomes 3 days (72 h) after treatment with L-carbocisteine (Carbocisteine; 10 μM) or vehicle of L-carbocisteine (Control; 0.1% double-distilled water). Results are means ± SE from 5 different tracheae. Significant differences from control values are indicated by **P < 0.01.
centrifugation at 20,000 g for 5 min at 4°C, nuclear extracts were assayed for p50, p65, c-Rel, p52, and RelB content. An equal amount of nuclear lysates was added to incubation wells precoated with the DNA-binding consensus sequence. The presence of subunits including translocated p50, p65, c-Rel, p52, and RelB was assayed by using a TransFactor Family Colorimetric Kit-NF-xB (BD Biosciences/Clontech) according to the manufacturer’s instructions (10). Plates were read at 655 nm, and results are expressed as OD.

Statistical analysis. Results are expressed as means ± SE. Statistical analysis was performed using one-way measures of ANOVA. Subsequent post hoc analysis was made using Bonferroni method. For all analyses, values of P < 0.05 were assumed to be significant. In the experiments using culture of human tracheal epithelial cells, n refers to the number of donors (tracheae) from which cultured epithelial cells were used.

RESULTS

Effects of L-carbocisteine on influenza virus infection. Exposing confluent human tracheal epithelial cell monolayers to FluA virus (H3N2, 0.5 × 10⁻³ TCID₅₀ U/cell) consistently led to infection. No detectable virus was revealed at 1 h after infection, whereas FluA virus was detected in culture medium at 24 h, and the viral content progressively increased between 1 and 24 h after infection (Fig. 1). Evidence of continuous viral production was obtained by demonstrating that each of the supernatant fluids collected during 1–3, 3–5, and 5–7 days after infection contained significant levels of influenza virus (Fig. 1). The viral titer levels in supernatant fluids increased significantly with time for the first 5 days (P < 0.05 by ANOVA). Treatment of the cells with L-carbocisteine (10 μM) significantly decreased the viral titers of FluA virus in supernatant fluids from 24 h after infection (Fig. 1). FluA virus titer levels in supernatant fluids of the cells from 15 exsmokers did not differ from those from 21 patients who had never smoked (data not shown). Likewise, FluA virus titer levels in supernatant fluids of the cells from 5 patients complicated with COPD (5.5 ± 1.1 log TCID₅₀ U/ml/24 h, means ± SE; P > 0.20) did not differ from those from 31 patients without COPD (5.2 ± 0.9 log TCID₅₀ U/ml/24 h). No virus was detected in supernatant fluids after infection of UV-inactivated influenza virus (data not shown).

L-carbocisteine inhibited influenza virus infection concentration-dependently, and the maximum effect was obtained at 100 μM (Fig. 2).

Effects of L-carbocisteine on viral RNA by PCR. Further evidence of the inhibitory effects of L-carbocisteine on FluA viral RNA replication in human tracheal epithelial cells was provided by real-time quantitative RT-PCR analysis. The RNA extraction was performed at 3 days (72 h) and 5 days (120 h) after virus infection. FluA viral RNA replication in the cells was consistently observed at 3 days after infection and increased between 3 and 5 days after infection (Fig. 3). L-carbocisteine (10 μM) decreased the FluA viral RNA at 3 and 5 days after infection (Fig. 3).

Effects of L-carbocisteine on susceptibility to influenza virus infection. Treatment of the cells with L-carbocisteine (10 μM) decreased the susceptibility of the cells to infection by FluA
virus (H3N2). The minimum dose of influenza virus necessary to cause infection in the cells treated with l-carbocisteine (10 μM, 3 days; 3.2 ± 0.3 log TCID₅₀ U/ml, n = 5; P < 0.05) was significantly higher than that in the cells treated with vehicle of l-carbocisteine (0.1% double-distilled water; 1.4 ± 0.2 log TCID₅₀ U/ml, n = 5; Fig. 4).

Effects of l-carbocisteine on Sα2,6Gal expression. Sα2,6Gal, a receptor for human influenza virus, was observed as green lines or spots on the mucosal surface of cultured human tracheal epithelial cell sheets (Fig. 5A). l-carbocisteine (10 μM, 72 h) reduced the number of green lines or spots on the cell sheets (Fig. 5B) and the fluorescence intensity from the receptor (by 54 ± 8% compared with that in vehicle of l-carbocisteine; P < 0.001, n = 8, Student’s t-test; Fig. 5C).

ELLA on extracted samples from human tracheal epithelial cells showed the significant amounts of SNA binding (Fig. 5D). Treatment with l-carbocisteine (10 μM, 72 h) reduced the levels of binding of this lectin (Fig. 5D).

Effects of l-carbocisteine on pH in the acidic endosomes. Acidic endosomes in human tracheal epithelial cells were stained green with LysoSensor DND-189 (Fig. 6, A and B). Treatment of the cells with l-carbocisteine (10 μM, 3 days) reduced the number of acidic endosomes with green fluorescence in the cells (Fig. 6B) and the fluorescence intensity from acidic endosomes in the epithelial cells (Fig. 6C) compared with that in cells treated with vehicle of l-carbocisteine (0.1% double-distilled water).

Effects of l-carbocisteine on cytokine production. The secretions of IL-1β, IL-6, and IL-8 all increased after FluA virus infection (Fig. 7), and maximum secretion was observed at 5 days after the infection (data at 1, 3, and 7 days not shown). Treatment with l-carbocisteine (10 μM) reduced the concentration of IL-1β, IL-6, and IL-8 5 days after FluA virus infection as well as baseline concentrations of these cytokines before FluA virus infection (Fig. 7). In contrast, UV-irradiated influenza virus did not increase IL-1β, IL-6, and IL-8 (Fig. 7). Likewise, the secretion of IL-1β, IL-6, and IL-8 in supernatant fluids of the cells from 5 patients complicated with COPD did not differ from those from 31 patients without COPD (data not shown). The magnitude of inhibitory effects of l-carbocisteine in the cells from COPD patients was also similar to that in the cells from patients without COPD (data not shown).

Effects of l-carbocisteine on NF-κB. In the human tracheal epithelial cells before FluA virus infection, a significant amount of p50, p65, c-Rel, p52, and RelB of NF-κB was detected in the nuclear extracts. l-carbocisteine (10 μM, 3 days) significantly reduced the amount of p50, p65, c-Rel, p52, and RelB of NF-κB in the nuclear extracts in the cells (Fig. 8).

DISCUSSION

In the present study, we have shown that viral titers in supernatant fluids and RNA of FluA virus in the human tracheal epithelial cells increased with time, and l-carbocisteine reduced viral titers of FluA virus in supernatant fluids concentration-dependently, RNA of FluA virus replication in the cells, and the susceptibility to FluA virus infection. Immunocytochemical staining with FITC-labeled SNA lectin (23) and an ELLA (11, 21) showed that the cultured human tracheal epithelial cells expressed Sα2,6Gal, a receptor for human influenza virus (18). Treatment of human tracheal epithelial cells with l-carbocisteine reduced the expression of the receptor on the epithelial cells. These findings suggest that l-carbocisteine might inhibit FluA virus infection, partly through the reduced expression of the receptor for human influenza virus in the human tracheal epithelial cells. l-carbocisteine also reduced the number of acidic endosomes from which RNA of FluA enters into the cytoplasm and reduced the fluorescence intensity from acidic endosomes in the cells. These findings suggest that the reduction of acidic endosomes might also relate to the inhibition of FluA virus infection by l-carbocisteine. Furthermore, l-carbocisteine reduced concentrations of proinflammatory cytokines and a monokine, including IL-1β, IL-6, and IL-8 in supernatant fluids. l-carbocisteine may also modulate airway inflammation induced by FluA virus infection.

MDCK cells did not show any morphological change that shows the presence of FluA virus when supernatant fluids collected 1 h after FluA virus infection were added to the MDCK cells. In contrast, supernatant fluids 24 h after infection produced a morphological change on the cells showing the presence of FluA virus (15). These findings suggest that supernatant fluids 24 h after infection contained significant amounts of FluA virions, which were newly produced after infection.
Human seasonal influenza viruses and classical H1N1 swine influenza viruses bind to SAα2,6Gal, and most avian and equine viruses bind to SAα2,3Gal (18). An expression of SAα2,6Gal was observed in epithelial cells in the nasal mucosa, pharynx, tracheae, and bronchi (6, 23). In contrast, SAα2,3Gal was reported not to express on the tracheal epithelial cells (6), whereas Matrosovich et al. (13) demonstrated its expression on ciliated cells in the human trachea. SAα2,3Gal is also expressed in nonciliated cuboidal bronchiolar cells and type II cells lining the alveolar wall (23). In this study, human tracheal epithelial cells expressed SAα2,6Gal but not SAα2,3Gal (data not shown). These findings are consistent with those in previous reports (6, 13, 23). In the present study, immunocytochemistry and ELLA demonstrated the reduced expression of SAα2,6Gal in human tracheal epithelial cells after treatment with L-carbocisteine. Furthermore, the minimum dose of FluA virus necessary to cause infection in the cells treated with L-carbocisteine was significantly higher than that in the cells treated with vehicle of L-carbocisteine, showing the reduced susceptibility to FluA virus infection. These findings suggest that L-carbocisteine might reduce the amount of FluA virus virions attached on the epithelial cells through the reduced expression of SAα2,6Gal in the cells.

The mechanisms for the reduction of SAα2,6Gal expression by L-carbocisteine are uncertain. However, TNF-α, one of the inflammatory mediators in airways, increases the expression glycosyltransferase and sulfotransferase responsible for biosynthesis of sialylated epitopes in the bronchial mucosa (8) through the activation of NF-κB (4). SAα2,6Gal glycotopes on serum glycoproteins also increase in inflamed mice in response to turpentine oil (38). On the other hand, as we (37) previously demonstrated, L-carbocisteine reduces the production of proinflammatory cytokines such as IL-1 through the inhibition of NF-κB activation. We also demonstrated the inhibitory effects of L-carbocisteine on NF-κB activation in this study. These findings suggest that reduced NF-κB might be partly associated with the reduced expression of SAα2,6Gal by L-carbocisteine in the human tracheal epithelial cells.

After attachment of influenza virus to the receptor, viruses enter the airway epithelial cells and are internalized by endocytic compartments via four internalization mechanisms, including: clathrin-coated pits; caveolae; nonclathrin, noncaveole pathway; and macropinocytosis (17). Of these mechanisms, a nonclathrin, noncaveole-mediated internalization pathway depends on low pH (24). Furthermore, after binding on the cell surface, the virus is internalized by receptor-mediated endocytosis, and the low pH in the endosome triggers fusion of the viral and endosomal membranes. The viruses then release their ribonucleoproteins (RNPs), including viral RNA, into the cytoplasm, resulting in the next processes of viral replication (17, 31). As shown previously (37) and in this study, L-carbocisteine reduced the number of acidic endosomes and the fluorescence intensity from acidic endosomes in the cells. Furthermore, L-carbocisteine reduced FluA viral RNA in human tracheal epithelial cells and viral titers of influenza in supernatant fluids. These findings are consistent with the reports that bafilomycin A1 reduces the number of acidic endosomes (16, 28) in the epithelial cells and reduces the growth of influenza virus in MDCK cells (16). Increased pH in acidic endosomes might relate to the inhibition of virus entry and releasing the viral RNPs into the cytoplasm and might inhibit the next processes of viral replication in this study.

In this study, we demonstrated that L-carbocisteine reduces the expression of the receptor for FluA virus on the human tracheal epithelial cells and reduces the number of acidic endosomes from which influenza virus RNA enters into the cytoplasm. However, Yang et al. (35) demonstrated that a mucolytic agent, ambroxol, inhibits influenza virus multiplication and improves the survival rate of mice. They suggested that the inhibitory effects of ambroxol on influenza virus infection may be associated with upregulation on the production of pulmonary surfactant (2), mucus protease inhibitor (3), and immunoglobulin. Further studies are needed to examine the effects of L-carbocisteine on these mechanisms.

The inhibitory effects of L-carbocisteine on the production of proinflammatory cytokines and a monokine observed in this study are consistent with those of a previous report that L-carbocisteine reduces them in airway epithelial cells after rhinovirus infection (37). These findings are also similar to that of an in vivo study by Yang et al. (35) that ambroxol reduces these proinflammatory factors including TNF-α in mice after influenza virus infection. Influenza virus infection induces airway inflammation through the production of proinflammatory cytokines and chemokines such as IL-6, IL-8, and IP-10 (7, 30) and through epithelial cell damage, both of which are associated with exacerbations of COPD (12). Therefore, the inhibitory effects of L-carbocisteine on the production of the cytokines and chemokines may also relate to reduced frequency of exacerbations in COPD patients (36, 40).

In summary, we demonstrated that L-carbocisteine, a mucolytic agent, inhibits FluA virus infection in human tracheal epithelial cells at least by the reduced expression of the receptor on the cells and the reduction of acidic endosomes where the viral RNPs are released into the cytoplasm. L-carbocisteine may also modulate airway inflammation after influenza virus infection.

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DISCLOSURES

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