27-Hydroxycholesterol accelerates cellular senescence in human lung resident cells

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Running head: 27-hydroxycholesterol in COPD
Abstract

Background: Cellular senescence is reportedly involved in the pathogenesis of chronic obstructive pulmonary disease (COPD). We previously showed that 27-hydroxycholesterol (27-OHC) is elevated in the airways of COPD patients compared to those in healthy subjects. The aim of this study was to investigate whether lung fibroblasts of COPD patients are senescent and to determine the effects of 27-OHC on senescence of lung resident cells, including fibroblasts and airway epithelial cells.

Methods: Localization of senescence-associated proteins and sterol 27-hydroxylase was investigated in the lungs of COPD patients by immunohistochemical staining. To evaluate whether 27-OHC accelerates cellular senescence, lung resident cells were exposed to 27-OHC. Senescence markers and fibroblast-mediated tissue repair were investigated in the 27-OHC-treated cells.

Results: Expression of senescence-associated proteins was significantly enhanced in lung fibroblasts of COPD patients. Similarly, expression of sterol 27-hydroxylase was significantly upregulated in lung fibroblasts and alveolar macrophages in these patients. Treatment with the concentration of 27-OHC detected in COPD airways significantly augmented expression of senescence-associated proteins and senescence-associated β-galactosidase activity, and delayed cell growth through the prostaglandin E2-reactive nitrogen species pathway. The 27-OHC-treated fibroblasts impaired tissue repair function. Fibroblasts from lungs of COPD
patients showed accelerated senescence and were more susceptible to 27-OHC-induced cellular senescence compared to those of healthy subjects.

**Conclusions:** 27-OHC accelerates cellular senescence in lung resident cells and may play a pivotal role in cellular senescence in COPD.

**Key words:** COPD, senescence, lung resident cells, oxysterols
Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by a reduction in expiratory airflow due to narrowing of the peripheral airways and pulmonary emphysema (1, 4, 16). Protease/anti-protease imbalance and oxidative stress are involved in the pathogenesis of COPD (1) and lung cellular damage, including apoptosis, autophagy, and senescence, plays a pivotal role in the pathogenesis (2, 20). We and another group have shown impaired fibroblast-mediated tissue repair capacity in COPD patients, with the degree of impairment associated with airflow obstruction (29, 33). These findings suggest that impaired tissue repair could attenuate wound healing in lungs of COPD patients and might cause development of emphysema.

Cellular senescence may cause impairment of the fibroblast-mediated tissue repair capacity in COPD (11, 25). Airway epithelial cells are senescent in the lungs of COPD patients and secrete high levels of inflammatory cytokines and chemokines (3). Cigarette smoke and oxidative stress, which are major causes of COPD, also promote senescence of lung resident cells (10-12, 26, 32, 35), but the mechanisms of lung cellular senescence remain unclear. Because cellular senescence influences tissue repair and inflammation (20), targeting of these mechanisms may be a therapeutic approach in COPD.

Oxysterols are oxygenated derivatives of cholesterol (6). 27-Hydroxycholesterol (27-OHC) is an oxysterol produced by enzymatic oxidation of cholesterol by sterol 27-hydroxylase (6).
Production of 27-OHC is enhanced in atherosclerotic lesions of arteries (7, 17) and in brains of patients with Alzheimer's disease (15). In addition, 27-OHC has pro-inflammatory effects including interleukin (IL)-8 production (14, 22), upregulation of expression of adhesion molecules (14, 24), and apoptosis (14, 28). These findings suggest that 27-OHC may be excessively produced in senescence-related diseases and could have pro-inflammatory effects.

We have shown that production of 27-OHC and sterol 27-hydroxylase is augmented in the airways of COPD patients (21), and these findings led us to explore whether 27-OHC accelerates senescence in lung resident cells, including fibroblasts and airway epithelial cells, which maintain the normal architecture of the lungs and repair damaged tissues.

In this study, we used concentrations of 27-OHC similar to those detected in sputum from COPD patients to examine 1) whether lung fibroblasts of COPD patients show evidence of senescence; 2) which cells express sterol 27-hydroxylase; 3) whether long term exposure to 27-OHC promotes senescence in lung fibroblasts and airway epithelial cells and which signal transduction pathway modulates cellular senescence; 4) whether cellular senescence impairs the tissue repair function; and 5) whether fibroblasts from patients with COPD are more susceptible to the effects of 27-OHC compared to healthy subjects.
Materials and Methods

Materials

Commercially available reagents were obtained as follows: mouse monoclonal anti-p53 antibody, mouse monoclonal anti-p16 antibody, mouse monoclonal anti-cyclooxygenase-1 (COX-1) antibody, mouse monoclonal anti-COX-2 antibody, mouse monoclonal anti-β-actin antibody and mouse monoclonal anti-lamin A/C antibody were from Santa Cruz Biotechnology (Dallas, TX); rabbit polyclonal anti-p21 antibody, rabbit polyclonal anti-p-retinoblastoma (pRB) antibody and rabbit polyclonal anti-p-p53 antibody were from Cell Signaling Technology (Beverly, MA); rabbit polyclonal anti-sterol 27-hydroxylase antibody and 27-hydroxycholesterol (27-OHC) were from Avanti Polar Lipids (Alabaster, AL); mouse anti-CD68 antibody was from BioLegend (San Diego, CA); fibronectin ELISA kit, goat fluorescein isothiocyanate (FITC) polyclonal secondary antibody and rabbit Dylight 650 polyclonal secondary antibody from Abcam (Cambridge, UK); indomethacin, a non-selective cyclooxygenase inhibitor, and celecoxib, a selective cyclooxygenase-2 inhibitor, were from Sigma (St. Louis, MO); MnTBAP, a peroxynitrite scavenger, was from Calbiochem (La Jolla, CA); diaminofluorescein-2 (DAF-2), a fluorescent indicator, was from Sekisui Medical (Tokyo, Japan); PF-04418948, a selective EP<sub>2</sub> receptor antagonist and EIA prostaglandin-kit were from Cayman Chem (San Diego, CA); Dulbecco’s Modified Eagle’s Medium (DMEM), fetal calf serum (FCS) and antibiotic-antimycotic were purchased from
Preparation of human lung tissues and sputum

Twenty-two controls who had never smoked, 23 ex-smokers without COPD, and 23 ex-smokers with COPD took part in the lung tissue study after giving written informed consent (Table 1). Seven controls, 8 asthma patients and 12 COPD patients participated in the sputum study (Table 2). COPD was diagnosed according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines. All subjects had undergone surgery for lung cancer after receiving pulmonary function tests. Peripheral lung tissues were obtained from the subpleural parenchyma of the lobe resected at surgery, avoiding areas involving tumors. The tissues were used for immunohistostaining and culture of lung fibroblasts and bronchial epithelial cells. Sputum samples were obtained using a hypertonic saline inhalation method (18). The obtained sputum was treated with Sputasol (Oxoid Ltd., Basingstoke, UK) to dissociate the sulfide bonds of the mucus. After centrifugation, four sets of slides of the cell pellet in each sample were prepared using a Shandon 4 cytocentrifuge (Shandon Southern Instruments, Sewickley, PA). All experiments were approved by the ethics committee of Tohoku University Graduate School of Medicine.

Cell Culture
Human fetal lung fibroblasts (HFL-1) were obtained from the American Type Culture Collection (Rockville, MD). Four strains of adult lung fibroblasts and bronchial epithelial cells were obtained from lung tissues resected surgically from COPD or non-COPD patients with lung cancer at our institution. HFL-1 and adult lung fibroblasts were cultured on tissue culture dishes with DMEM supplemented with 10% FCS, 100 μg/ml penicillin and 250 μg/ml streptomycin. Adult bronchial epithelial cells (passages 3-6) were cultured in serum-free Keratinocyte Basal Medium supplemented with 10 ng/ml recombinant epidermal growth factor and 30 mg/ml bovine pituitary extract. The cells were cultured at 37°C in a humidified atmosphere of 5% CO2 and passaged. HFL-1 cells were used between the 14th and 20th passages. Adult fibroblasts from the patients were used between the 3rd and 7th passages.

**In vitro study protocol**

**Single exposure model**

To evaluate the expression of senescence-associated proteins, including p53, p21, p16, p-pRB and p-p53, the cells were treated with various concentrations of 27-OHC (10^{-9} - 10^{-6} M) for 24 hours and cell lysates were harvested. To evaluate p-p53 translocation into nuclei, the nuclear fraction was obtained by a Nuclear Extraction Kit (Active Motif, Carlsbad, CA).

**Chronic exposure model**
HFL-1 cells were exposed to 27-OHC (10^{-9} - 10^{-6} M) in DMEM with 10% FCS at the 15^{th} passage and were passaged every 3 days to the 20^{th} passage for a total of 2 weeks to examine the senescence-associated β-galactosidase (SA-β-gal) activity and cell growth rate. Adult bronchial epithelial cells were exposed 27-OHC (10^{-9} - 10^{-7} M) in the media for 2 weeks. To evaluate the role of prostaglandins (PGs), including PGE2 and PGD2, on 27-OHC-induced cellular senescence, cells were treated with 27-OHC in the presence or absence of a non-selective COX inhibitor, indomethacin; a selective COX-2 inhibitor, celecoxib; or a selective EP2 receptor antagonist, PF-04418948 for 2 weeks. Similarly, cells were treated with 27-OHC in the presence or absence of a peroxynitrite scavenger, MnTBAP, for 2 weeks. After final treatment with 27-OHC for 48 h, SA-β-gal activity and cell growth were examined.

**Immunohistochemical localization of p53, p16, vimentin, CD68 and sterol 27-hydroxylase**

Lung tissues from healthy controls who had never smoked, healthy ex-smokers and ex-smokers with COPD, and sputum cells were used in immunohistological examination of p53, p16, vimentin, CD68 and sterol 27-hydroxylase. Briefly, after fixation with 4% paraformaldehyde in PBS for 30 min at room temperature, the specimens were blocked with blocking reagent (Dako Japan Ltd., Kyoto, Japan) for 1 h at room temperature and rinsed. Fluorescence immunohistochemistry was performed to detect localization of p53, p16, vimentin, CD68 and sterol 27-hydroxylase. The specimens were incubated with goat
polyclonal anti-vimentin antibody (1:100 dilution, Sigma, St. Louis, MO) plus mouse
monoclonal anti-p53 antibody (1:50 dilution), mouse monoclonal anti-p16 antibody (1:50
dilution), mouse monoclonal anti-CD68 antibody (1:100 dilution), rabbit polyclonal
anti-sterol 27-hydroxylase antibody (1:200 dilution), or non-specific polyclonal rabbit IgG as
a negative control at 4°C overnight. After washing, the samples were incubated with goat
anti-mouse or anti-rabbit IgG conjugated with FITC (1:50 dilution) for 60 min at room
temperature. Then, after washing, they were incubated with rabbit anti-goat or anti-mouse IgG
conjugated with Dylight 650 (1:50 dilution) for 60 min at room temperature. After washing,
the samples were stained with Fluoromount-G containing DAPI (Southern Biotech,
Birmingham, AL) and viewed using a multiphoton confocal LSM 780 NLO microscope
system (Carl Zeiss, Jena, Germany).

Senescence-associated proteins and vimentin double-immunopositive cells were
semi-quantified as follows. Three areas per lung specimen were randomly chosen by two
investigators who were blinded to the background of the subjects. Senescence-associated
proteins, vimentin double-immunopositive cells, and all cells in each randomly chosen area
were counted and semi-quantified using Image J (National Institutes of Health, Frederick,
MD). To measure the immunopositive area for sterol 27-hydroxylase, immunohistochemistry
was performed using the diaminobenzidine (DAB) reaction to visualize immunopositive cells,
followed by counterstaining with hematoxylin. Cells were viewed by microscopy
(BX53-33-SDO, Olympus, Tokyo, Japan) and photographed with a digital camera (DP71-SET, Olympus). Sterol 27-hydroxylase immunopositive cells were semi-quantified using Image J in five areas per lung specimen that were randomly chosen by two investigators blinded to the background of the subjects.

**Western blotting**

Cells were seeded in 60-mm dishes at a density of $1 \times 10^5$/ml. At 90% confluence, the cells were starved with SF-DMEM for 24 h and then treated with various concentrations of 27-OHC for 24 h to examine senescence-associated proteins. To evaluate the expression of inducible nitric oxide synthase (iNOS), 3-nitrotyrosine (3-NT), COX-1, and COX-2, the cells were exposed to 27-OHC for 2 weeks. The cells were washed with ice-cold PBS and homogenized in cell lysis buffer. Equal amounts of protein were loaded and separated by electrophoresis on 10% SDS polyacrylamide gels before transfer to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA). The following antibodies were used for detection of the target proteins: rabbit polyclonal anti-sterol 27-hydroxylase antibody (1:1000 Abcam plc. Cambridge, UK), mouse monoclonal anti-p53 antibody (1:1000 dilution), mouse monoclonal anti-p16 antibody (1:1000 dilution), rabbit polyclonal anti-p21 antibody (1:1000 dilution), rabbit polyclonal anti-pRB antibody (1:1000 dilution), rabbit polyclonal anti-p-p53 antibody (1:1000 dilution), rabbit polyclonal anti-iNOS antibody (1:1000 dilution), rabbit polyclonal
anti-3-NT antibody (1:1000 dilution), rabbit polyclonal anti-COX-1 antibody (1:1000 dilution), goat polyclonal anti-COX-2 antibody (1:1000 dilution), mouse monoclonal anti-laminA/C antibody (1:1000 dilution), or mouse monoclonal anti-β-actin antibody (1:10000 dilution). Bound antibodies were visualized using peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK) with a chemiluminescence imaging system (LAS-4000 mini, Fujifilm, Tokyo, Japan). Band intensity was quantified by densitometry (Quantity One software, Bio-Rad).

Cell proliferation assay

Cells (1 x 10^5 per well) in 12-well plates were treated with 27-OHC at various concentrations for 2 weeks. On day 3, the cells were trypsinized and the cell number was counted using a hemocytometer.

SA-β-gal assay

SA-β-gal enzyme activity was measured using a Senescence Detection kit (BioVision, Mountain View, CA). Development of cytoplasmic blue pigment was photographed using an inverted microscope equipped with a color CCD camera (Nikon Instruments, Inc., Lewisville, TX). The percentage of the blue area was calculated using Image J software.
Nitric oxide (NO) release

HFL-1 cells were plated in a 96-well microplate (1×10^5 cells/well) and cultured for 6 h. The cells were subsequently cultured for 24 h in 200 μl of fresh medium containing various concentrations of 27-OHC. The cells were washed once with PBS and incubated for 1 h at 37 °C in PBS containing 10 mM diaminofluorescein-2 (Sekisui Medical Co., Ltd., Tokyo, Japan). After incubation, supernatants were transferred to 96-well microplates and measured with a fluorescence microplate reader using excitation at 495 nm and emission at 515 nm (Softmax pro, Molecular Devices, Sunnyvale, CA).

Measurement of fibronectin and prostaglandins

Fibronectin, PGD₂ and PGE₂ in media were determined using enzyme-linked immunosorbent assay (ELISA) kits.

Fibroblast-mediated collagen gel contraction assay

Collagen gels were prepared as described previously (31). Briefly, rat tail tendon collagen (RTTC), distilled water and 4x concentrated DMEM were combined to give a final mixture of 0.75 mg/ml collagen, a physiological ionic strength of 1x DMEM, and pH of 7.4. The collagen contraction assay used the following cells at the 20th passage: 1. vehicle-pretreated, vehicle-exposed; 2. indomethacin-pretreated, vehicle-exposed; 3. MnTBAP-pretreated,
vehicle-exposed; 4. vehicle-pretreated, 27-OHC-exposed; 5. indomethacin-pretreated, 27-OHC-exposed; and 6. MnTBAP-pretreated, 27-OHC-exposed cells. Cells were trypsinized and suspended in SF-DMEM and then mixed with the neutralized collagen solution so that the final cell density in the solution was $3 \times 10^5$ cells/ml and the final concentration of collagen was 0.75 mg/ml. Aliquots (0.5 ml/well) of the mixture of cells in collagen were cast into each well of 24-well tissue culture plates (Falcon) and allowed to gel. After gelation was complete, normally within 20 min at room temperature, the gels were gently released from the 24-well tissue culture plates and transferred into 60-mm tissue culture dishes (three gels in each dish) that contained 5 ml of freshly prepared DMEM without FCS. The gels were then incubated at 37°C in a 5% CO$_2$ atmosphere for 3 days. Gel contraction was quantified using an Optomax V image analyzer (Optomax, Burlington, MA) daily. Data are expressed as percentages of the original gel size.

Fibroblast chemotaxis assay

Cell migration was assessed using a Boyden blindwell chamber (Neuroprobe Inc., Gaithersburg, MD), as previously described (31). The same cells used in the collagen gel contraction assay were examined in the chemotaxis assay. Briefly, 26 μl of SF-DMEM containing fibronectin (20 μg/ml) were placed into the bottom wells. Eight-micrometer pore polycarbonate membranes (Neuroprobe Inc.), which were pre-coated with 5 μg/ml gelatin in
0.1% acetic acid, were used. The cells were trypsinized and suspended in SF-DMEM at a density of 1 x 10^6/ml. Fifty microliters of cell suspension were then added into each top well. Cells were allowed to migrate at 37°C in a 5% CO_2 atmosphere for 6 h. Cells that had not migrated were scraped off the upper surface of the membrane, and the membranes were air-dried. Cells were then stained with PROTOCOL (Fisher Scientific, Swedesboro, NJ) and mounted on a glass microscope slide. Chemotaxis was assessed by counting the number of cells in five high-power fields (5 HPF). Wells with SF-DMEM were used as negative controls and those with chemoattractant alone were used as positive controls.

Collagen Assay

The amounts of collagen was measured in the supernatants using a Sircol Collagen Assay kit (Biocolor Ltd., Belfast, Northern Ireland).

Statistical analysis

Data were evaluated by one way analysis of variance (ANOVA) followed by a Scheffé test to adjust for multiple comparisons, with P<0.05 considered significant.
Results

Detection of p53 and p16 in lung fibroblasts

Investigation of acceleration of cellular senescence of fibroblasts in lungs of COPD patients was performed by immunohistochemistry. Results for immunohistostaining of p53 and p16, which are senescence markers, are shown in Figures 1 and 2. More p53- and p16-immunopositive cells were observed in the COPD group than in the healthy groups (Figs. 1A,B, 2A,B). Among the immunopositive cells, immunoreactivity in COPD lung fibroblasts was significantly enhanced compared to those in the healthy groups (p < 0.01, Figs. 1A,B, 2A, B). To determine if p53 is phosphorylated in lung fibroblasts, translocation of phosphorylated p53 (p-p53) into nuclei was examined in primary lung fibroblasts. The level of p-p53 in the nuclear fraction was significantly higher in the COPD group (p < 0.01, Fig 1C), suggesting that p53 was more activated in fibroblasts of COPD patients than in those of healthy subjects.

Analysis of sterol 27-hydroxylase in lung fibroblasts and macrophages

Immunoreactivity against sterol 27-hydroxylase was investigated in lung specimens. More immunopositive cells were observed around the peripheral airway wall and lung parenchyma in the COPD group than in the healthy groups (both p < 0.01, Fig. 3A,B). These immunopositive cells were probably lung mesenchymal cells and alveolar macrophages (Fig 3A). Double immunostaining was performed to clarify whether the mesenchymal cells
expressed sterol 27-hydroxylase. The sterol 27-hydroxylase immunopositive cells also expressed vimentin, which is a marker of mesenchymal cells (Fig. 4A). Many other immune cells also expressed sterol 27-hydroxylase, and these immunopositive cells were thought to be macrophages (Fig. 3A). To confirm this finding, double immunostaining was performed with CD68, a marker of macrophages (Fig. 4B). The sterol 27-hydroxylase immunopositive cells expressed CD68, indicating that the cells were macrophages. To examine whether lung fibroblasts in COPD expressed more sterol 27-hydroxylase, primary lung fibroblasts were obtained (Table 3). Expression of sterol 27-hydroxylase in lung fibroblasts from COPD patients was significantly higher than that in those from healthy subjects (p < 0.01, Fig. 3C).

Sterol 27-hydroxylase immunopositive cells in sputum of COPD patients were also significantly increased compared with sputum from healthy subjects and asthma patients (p < 0.01, Fig 3D). Regarding the mechanism of expression in vitro, exposure to H2O2 significantly enhanced production of sterol 27-hydroxylase (p < 0.01, Fig 3E).

**Effects of 27-OHC on cellular senescence of lung fibroblasts and airway epithelial cells.**

Previously, we found that 27-OHC was present in sputum from patients with COPD at ~3 x 10^{-7} M. We hypothesized that exposure to 27-OHC might accelerate senescence of lung fibroblasts and airway epithelial cells. Patients with COPD are continuously exposed to such concentrations of 27-OHC, and thus long-term exposure may be physiologically more
relevant. Lung fibroblasts were cultured with various concentrations of 27-OHC for 72 h followed by passage and continuously exposed to 27-OHC for 2 weeks (20th passage cells). The effects of 27-OHC on expression of senescence-associated proteins were then investigated. Treatment with 27-OHC significantly augmented expression of p53 (p < 0.01, Fig. 5A), p21 (p < 0.01, Fig. 5B), and p16 (p < 0.05, Fig. 5C), and significantly reduced phosphorylation of retinoblastoma (p-pRB) (p < 0.01, Fig. 5D). Continuous exposure to 27-OHC also increased SA-β-gal activity in a concentration-dependent manner (p < 0.01, Fig. 5E,F) and reduced cell proliferation significantly compared to that of the control group (p < 0.01, Fig 5G). These results suggest that chronic exposure to a pathophysiological concentration of 27-OHC induces senescence in lung fibroblasts. Similarly, treatment with 27-OHC significantly accelerated senescence in adult bronchial epithelial cells, as assessed by expression of senescence-associated proteins (p < 0.01, Fig 6A,B), SA-β-gal activity (p < 0.01, Fig 6C), and cell proliferation (p < 0.01, Fig 6D).

**Role of reactive nitrogen species (RNS) in 27-OHC-induced senescence of fibroblasts**

RNS are overproduced in COPD lungs and may be involved in the pathogenesis of COPD through oxidative/nitrosative stress (18, 19, 30, 31). Treatment with 27-OHC significantly enhanced iNOS expression and NO release in a concentration-dependent manner (p < 0.01, Fig.7A,B). Production of 3-NT, a footprint of RNS production, was significantly augmented
by treatment with 27-OHC (p < 0.01, Fig. 7C). Pretreatment with MnTBAP, a potent RNS scavenger, suppressed 27-OHC-augmented 3-NT production to control levels (p < 0.01, Fig. 7D) and restored 27-OHC-induced SA-β-gal accumulation (p < 0.01, Fig. 7E), suggesting that RNS can modulate senescence of fibroblasts induced by 27-OHC.

Role of PGs in 27-OHC-induced senescence of fibroblasts

A recent study showed that PGE2 accelerates cellular senescence (11), and thus we next examined the role of PGs in 27-OHC-induced senescence. Treatment with 27-OHC significantly enhanced COX-2 expression (p < 0.01, Fig. 8B), but not that of COX-1 (Fig. 8A). 27-OHC significantly stimulated release of PGE2 in media through COX-2 (p < 0.05, Fig. 8C), but not that of PGD2 (Fig. 8D). Indomethacin and celecoxib both reduced 27-OHC-induced SA-β-gal activity (p < 0.01, Fig. 8F) and 27-OHC-augmented production of RNS (p < 0.01, Fig. 8E) to control levels. Treatment with PF-04418948, a selective EP2 receptor antagonist, also significantly reduced 27-OHC-induced SA-β-gal activity (p < 0.01, Fig. 8G). These results suggest that PGE2, and especially activation of the EP2 receptor, modulates cellular senescence through overproduction of RNS.

Effects of 27-OHC on fibroblast-mediated tissue repair

Next, we examined the tissue repair function in chronically 27-OHC-exposed cells using
collagen gel contraction and chemotaxis assays. Collagen gel contraction mediated by 27-OHC-exposed fibroblasts was significantly inhibited compared with vehicle-exposed cells (p < 0.01, Fig. 9A). Pretreatment with both indomethacin and MnTBAP completely restored the 27-OHC-inhibited gel contraction (both p < 0.01, Fig. 9A). The chemotactic activity of the 27-OHC-exposed cells was significantly inhibited compared with vehicle-exposed cells (p < 0.01, Fig. 9B). Both indomethacin and MnTBAP also restored 27-OHC-inhibited chemotaxis (both p < 0.01, Fig. 9B). Furthermore, chronic exposure to 27-OHC significantly inhibited production of ECM proteins (both p < 0.01, Fig. 9C,D). These findings suggest that 27-OHC not only accelerates cellular senescence but also impairs fibroblast-mediated tissue repair.

Effects of 27-OHC on cellular senescence in primary lung fibroblasts

The effects of 27-OHC on cellular senescence were investigated in primary cells (Table 3). Even in the unstimulated condition, COPD lung fibroblasts showed significantly increased SA-β-gal activity compared with those from control subjects (p < 0.01, Fig. 10A). At a relatively low concentration of 27-OHC (10⁻⁸ M), changes in SA-β-gal activity were significantly greater in COPD cells than in those from controls (p < 0.01, Fig. 10B). As expected, proliferation of COPD cells was significantly slower compared to those from healthy subjects (p < 0.01, Fig. 10C). A low concentration of 27-OHC (10⁻⁸ M) significantly potentiated the delayed growth rate in COPD cells compared to controls (p < 0.01, Fig. 10D).
These results suggest that lung fibroblasts of COPD patients underwent an acceleration of senescence and were more susceptible to 27-OHC-induced cellular senescence.
Discussion

This study shows that senescence of lung resident cells such as fibroblasts is accelerated in patients with COPD compared with healthy subjects. Expression of sterol 27-hydroxylase was upregulated in lung fibroblasts and alveolar macrophages of COPD patients. Chronic exposure to 27-OHC accelerated senescence of lung resident cells through the PGE$_2$-RNS pathway and the 27-OHC-treated cells showed impaired tissue repair. Senescence of COPD fibroblasts was markedly more accelerated and chronic exposure to 27-OHC further potentiated senescence in these fibroblasts. These results suggest that 27-OHC may modulate cellular senescence of lung resident cells in COPD patients.

Recent studies have shown increased senescence in lung cells of patients with COPD (2, 3, 11, 34). Senescence induces irreversible growth arrest, which affects tissue renewal and plays a role in the aging process. Among the cells, lung fibroblasts and airway epithelial cells are believed to be key players in repair of damaged lung tissues. We found that senescence-associated proteins were highly expressed in lung resident cells from COPD patients. Moreover, the activity of SA-β-gal was increased and cell growth was reduced in lung fibroblasts from these patients. Taken together, lung fibroblasts in COPD patients showed accelerated senescence that may have a role in impairment of tissue repair.

We also found that senescence-associated proteins, including p53 and p16, were expressed at higher levels in lung fibroblasts of COPD patients compared to healthy subjects. Many
other cells also expressed senescence-associated proteins, suggesting that lung resident cells accelerate cellular senescence in COPD lungs. However, lung fibroblasts were a relatively small fraction of the positively stained cells. Therefore, the role of fibroblast-mediated cellular senescence in the pathogenesis of COPD remains uncertain and further studies of the effects of senescence in lung fibroblasts are needed.

Cellular senescence is induced by telomere shortening and by telomere-independent signals, such as DNA damage and oxidative stress (8, 9). Tsuji and coworkers showed that both patterns of senescence were accelerated in COPD lungs (34). Little is known about the cellular mechanisms of senescence in these lungs, but the levels of 27-OHC in the airways of COPD patients are 30 times higher than those in healthy subjects (21) and 27-OHC has pro-inflammatory effects (22, 24). Other bioactivities of 27-OHC in COPD are unknown. In this study, we found that chronic exposure to 27-OHC at concentrations found in COPD airways accelerated cellular senescence, which suggests that 27-OHC may affect the pathogenesis of COPD through promotion of cellular senescence.

Expression of sterol 27-hydroxylase is upregulated in brains of patients with Alzheimer’s disease (15) and in atherosclerotic lesions of blood vessels (7, 17). These diseases are closely related to senescence and much more 27-OHC is produced in these organs and tissues. We have reported that sterol 27-hydroxylase is strongly expressed in macrophages in patients with COPD (21), and in the current study we confirmed this finding using double immunostaining.
The exact source of increased 27-OHC in COPD lungs remains unknown, but macrophages and fibroblasts could be involved in overproduction of 27-OHC.

27-OHC has various bioactivities, including induction of pro-inflammatory cytokines and apoptosis (14), and may cause oxidative stress, which is a major driving force in aging (14). Oxysterols upregulate the activity of NOX isoenzymes, including constitutive NOX1, NOX4 and induced NOX2, which are multi-subunit NADPH oxidases (14). In the current study, we showed that 27-OHC accelerates lung cellular senescence through RNS, and thus the effects of 27-OHC on cellular senescence could be associated with oxidative stress. RNS are powerful oxidants that activate pro-inflammatory transcriptional factors such as NF-κB and AP-1, and inactivate HDAC2 (13, 19, 27). Since we first found excessive production of RNS in COPD airways (18), RNS have come to be thought of as largely responsible for many of the adverse effects of oxidative stress in COPD (1). In this study, we showed that 27-OHC stimulates cellular production of RNS and that RNS cause acceleration of senescence. Therefore, some RNS production in COPD airways may be mediated by 27-OHC.

PGE\textsubscript{2} appears to have an important role in COPD because the levels are elevated in COPD airways and correlated with the severity of airflow obstruction (23, 29, 33). Dagouassat et al. showed that PGE\textsubscript{2} is overproduced in COPD fibroblasts and could be involved in cellular senescence through unknown ROS (11). In this study, we provided de novo evidence that 27-OHC stimulates production of PGE\textsubscript{2} and that the ROS induced by PGE\textsubscript{2} were RNS.
Because PGE$_2$ plays a pivotal role in cellular senescence (11), inhibition of PGE$_2$ may constitute a therapeutic approach to COPD.

We previously showed that COPD lung fibroblasts have impaired tissue repair function due to multiple mechanisms, including secreting more PGE$_2$ and suppressing Smad signaling (33). The 27-OHC-treated cells showed a similar phenotype to COPD lung fibroblasts in terms of PGE$_2$ production and impairment of tissue repair function. Both a COX inhibitor and a RNS scavenger restored 27-OHC-impaired tissue repair, and these drugs may improve tissue repair function in aged cells.

Expression of sterol 27-hydroxylase is upregulated in lung fibroblasts and alveolar macrophages of COPD patients, with 30-100 times greater 27-OHC levels in COPD airways than in healthy subjects (21). 27-OHC is produced by sterol 27-hydroxylase and through a ROS-related pathway (6). Excessive oxidative stress is observed in COPD airways, and thus ROS may be involved in overproduction of 27-OHC in these airways. Further, we found that oxidative stress stimulated expression of sterol 27-hydroxylase in lung fibroblasts in the current study. Thus, although little is known about the mechanisms of sterol 27-hydroxylase expression, oxidative stress could stimulate expression of sterol 27-hydroxylase.

Interestingly, even a relatively low concentration of 27-OHC ($10^{-8}$ M) also accelerated senescence in COPD cells compared with those from healthy controls. These results suggest that lung architectural cells such as fibroblasts may be susceptible to pro-inflammatory stimuli.
We did not investigate the mechanisms, but it may be useful to clarify the mechanisms of susceptibility to inflammatory stimuli in order to elucidate the pathogenesis of COPD.

In general, colon cancer cells and uterus cancer cells are known to secrete higher levels of 27-OHC. Therefore, we cannot exclude the influence of lung cancer on the findings in the current study. To avoid this influence, we obtained peripheral lung tissues remote from the tumor margin. Further, to minimize the effects of tumors on the results, all lung tissues in the control groups were obtained from non-COPD subjects with lung cancer. The relationship between lung cancer and production of 27-OHC needs to be addressed in further study.

In conclusion, we showed that cellular senescence of lung resident cells was accelerated in COPD and that treatment with 27-OHC induced senescence in lung resident cells via the PGE2-RNS pathway. The 27-OHC-treated cells also showed impaired tissue repair function. COPD fibroblasts were also more susceptible to 27-OHC-induced senescence. The mechanisms of senescence in lung cells in COPD are unclear, but 27-OHC signaling may be an important pathway in acceleration of cellular senescence in the lungs of patients with COPD.
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Author Contributions:

Y Hashimoto: cell culture, biochemical studies, immunohistochemical analysis, interpretation of results.

H Sugiura: design of the study, interpretation of results, technical advice, writing of the manuscript.

S Togo: gel contraction and chemotaxis studies, technical advice, interpretation of results.

A Koarai: technical advice, interpretation of results.

K Abe: recruitment of patients, informed consent of patients, technical advice, interpretation of results.

M Yamada: technical advice, interpretation of results.

T Ichikawa: technical advice, interpretation of results.

T Kikuchi: technical advice, interpretation of results.

T Numakura: recruitment of patients, informed consent of patients.

K Onodera: recruitment of patients, informed consent of patients.

R Tanaka: recruitment of patients, informed consent of patients.
K Sato: recruitment of patients, informed consent of patients.

S Yanagisawa: technical advice, interpretation of results.

T Okazaki: technical advice, interpretation of results.

T Tamada: technical advice, interpretation of results.

T Kikuchi: technical advice, interpretation of results.

Y Hoshikawa: recruitment of patients, informed consent of patients.

Y Okada: recruitment of patients, informed consent of patients.

M Ichinose: design of the study, interpretation of results, writing of the manuscript.

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Conflict of interest

None of the authors has any conflicts of interest related to this manuscript.


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of 27-hydroxycholesterol in the airways of patients with COPD: Possible role of


27. Pryor WA, Squadrito GL. The chemistry of peroxynitrite: A product from the reaction of


Figure Legends

Figure 1. Detection of p53 in lung fibroblasts.
Lung tissues were obtained from healthy controls who had never smoked, healthy ex-smokers and ex-smokers with COPD. Localization of p53, a senescence-associated protein, was investigated in the lung tissues by immunostaining. Representative photographs of p53 immunoreactivity are shown (A). The boxed area indicates a region of higher magnification. Arrows indicate p53-immunopositive lung fibroblasts. Red: p53; green: vimentin (marker of fibroblasts). The p53 and vimentin double-immunopositive cells were semi-quantified using Image J (B). Four strains of primary lung fibroblasts were obtained from the patients. Cells were treated with 27-OHC and the nuclear fraction was obtained. Translocation of phosphorylated p53 (p-p53) into the nucleus was evaluated by western blotting (C). Relative intensity was calculated by dividing the p-p53 band intensity by the lamin A/C band intensity. Values are the mean ± SEM. **p < 0.01 vs. HNS. ††p < 0.01 vs. HES. HNS = healthy control who had never smoked; HES = healthy ex-smoker; COPD = ex-smoker with COPD.

Figure 2. Detection of p16 in lung fibroblasts.
Lung tissues were obtained from patients. Localization of p16, a senescence-associated proteins, was investigated in these tissues by immunostaining. Representative photographs of p16 immunoreactivity are shown. The boxed area indicates a region of higher magnification.
Arrows indicate p16-immunopositive lung fibroblasts. Red: p16; green: vimentin. p16 and vimentin double-immunopositive cells were semi-quantified using Image J (B). **p < 0.01 vs. HNS. ††p < 0.01 vs. HES. HNS = healthy control who had never smoked; HES = healthy ex-smoker; COPD = ex-smoker with COPD.

Figure 3. Expression of sterol 27-hydroxylase in lungs and lung fibroblasts.

Lung tissues were obtained from patients. Localization of sterol 27-hydroxylase in the lungs was examined by immunohistochemistry. Representative photographs of sterol 27-hydroxylase immunoreactivity are shown (A). Arrows indicate sterol 27-hydroxylase immunopositive cells. The area of sterol 27-hydroxylase immunopositive cells around the peripheral airway wall was semi-quantified using Image J (B). Lung fibroblasts from each group were purified. Four strains were obtained from patients and production of sterol 27-hydroxylase was quantified by western blotting (C). Representative photographs of three strains in each group are shown (C). Band intensity was assessed by densitometry and is expressed relative to the β-actin band intensity. Values are the mean ± SEM (n = 4). Detection of sterol 27-hydroxylase was examined by immunocytochemistry in sputum cells from healthy subjects, asthma patients, and COPD patients. Representative photographs of sterol 27-hydroxylase immunoreactivity are shown and immunopositive cells in sputum were counted (D). Arrows indicate sterol 27-hydroxylase immunopositive cells. Effects of
hydrogen peroxide on production of sterol 27-hydroxylase were examined in lung fibroblasts using western blotting (E). The band intensity for sterol 27-hydroxylase is expressed relative to that of the β-actin band intensity. *p < 0.05, **p < 0.01 vs. HNS. ††p < 0.01 vs. HES. ‡p < 0.05, ‡‡p < 0.01 vs. each control group. HNS = healthy control who had never smoked; HES = healthy ex-smoker; COPD = ex-smoker with COPD.

**Figure 4. Detection of sterol 27-hydroxylase in lung fibroblasts and macrophages.**

Lung tissues were obtained from patients. Localization of sterol 27-hydroxylase was investigated in lung tissues by immunostaining. Representative photographs of the sterol 27-hydroxylase immunoreactivity are shown (A). The boxed area indicates a region of higher magnification. Arrows indicate sterol 27-hydroxylase immunopositive lung fibroblasts. Green: sterol 27-hydroxylase; red: vimentin. To identify whether macrophages expressed sterol 27-hydroxylase, double immunostaining for CD68 and sterol 27-hydroxylase was carried out (B). Arrowheads indicate CD68 and sterol 27-hydroxylase double-immunopositive macrophages. Green: sterol 27-hydroxylase; red: CD68.

**Figure 5. Effects of 27-hydroxycholesterol (27-OHC) on senescence-associated protein production, SA-β-gal activity and cell growth in human lung fibroblasts.**

Human fetal lung fibroblasts (HFL-1) were exposed to various concentrations of 27-OHC for
24 h and cell lysates were harvested. Expression of senescence-associated protein was evaluated by western blotting. Band intensity for each senescence-associated protein, including p53 (A), p21 (B), p16 (C), and phosphorylated-retinoblastoma (p-pRB) (D), was assessed by densitometry and is expressed relative to the β-actin band intensity. SA-β-gal activity (E and F) and cell growth (G) were determined in 20th passage cells after chronic exposure to 27-OHC for 2 weeks. Arrows indicate SA-β-gal-positive cells. Values are the mean ± SEM (n = 4). *p < 0.05, **p < 0.01 vs. control group. n.s. = not significant.

**Figure 6. Effects of 27-OHC on senescence-associated protein production, SA-β-gal activity, and cell growth in adult bronchial epithelial cells.**

Adult bronchial epithelial cells were obtained from healthy subjects and the cells were exposed to various concentrations of 27-OHC for 24 h. Expression of senescence-associated proteins was evaluated by western blotting. Band intensity for each senescence-associated protein, including p53 (A) and p16 (B), was assessed by densitometry and is expressed relative to the β-actin band intensity. SA-β-gal activity (C) and cell growth (D) were determined in 6th passage cells after chronic exposure to 27-OHC for 2 weeks. Values are the mean ± SEM (n = 4). *p < 0.05, **p < 0.01 vs. control group.

**Figure 7. Effects of 27-OHC on reactive nitrogen species (RNS) production and effect of
an RNS scavenger on 27-OHC-enhanced cellular senescence.

HFL-1 cells were exposed to various concentrations of 27-OHC for 2 weeks and cell lysates were harvested. Expression of inducible type nitric oxide (NO) synthase (iNOS) was evaluated by western blotting (A). NO release was quantified using chemiluminescence with diaminofluorescein-2 (DAF-2) (B). 3-Nitrotyrosine (3-NT) formation, a footprint of RNS production, was evaluated by western blotting (C). HFL-1 cells were treated with 27-OHC in the presence or absence of MnTBAP, an RNS scavenger. Cells were harvested and assayed for 3-NT formation (D) and SA-β-gal activity (E). Band intensity is expressed relative to the β-actin band intensity. Values are the mean ± SEM (n = 4-6). *p < 0.05, **p < 0.01 vs. control group. ††p < 0.01 vs. 27-OHC treated group.

Figure 8. Effects of 27-OHC on prostaglandin (PG) synthesis and effects of cyclooxygenase (COX) inhibitors on 27-OHC-enhanced cellular senescence.

HFL-1 cells were exposed to various concentrations of 27-OHC for 2 weeks. COX-1 and COX-2 expression were evaluated by western blotting (A and B). PGE₂ and PGD₂ release was quantified by enzyme-linked immunoassay (ELISA) (C and D). Cells were treated with 27-OHC in the presence or absence of indomethacin, a non-selective COX inhibitor; celecoxib, a selective COX-2 inhibitor; and PF-04418948, a selective EP₂ receptor antagonist. Cells were harvested and assayed for 3-NT formation (E) and SA-β-gal activity (F and G).
Relative intensity was calculated by dividing each protein band intensity by the β-actin band intensity. Values are the mean ± SEM (n = 4). *p < 0.05, **p < 0.01 vs. control group. †p < 0.05, ††p < 0.01 vs. 27-OHC treated group.

Figure 9. Effect of 27-OHC on fibroblast-mediated tissue repair.

Cells were treated with 27-OHC in the presence or absence of indomethacin or MnTBAP. The treated cells were cast into three-dimensional collagen gels and floated in medium. Gel size was measured on day 3 (A). The 27-OHC-treated cells were assayed for chemotaxis toward fibronectin using a Boyden chamber. The migrated cell number was counted per five high-power fields (5HPF) (B). Collagen I and fibronectin release in the media was quantified using an ELISA kit (C and D). Values are the mean ± SEM (n = 4). **p < 0.01 vs. control group. ††p < 0.01 vs. 27-OHC treated group.

Figure 10. Effects of 27-OHC on SA-β-gal activity and cell growth in primary lung fibroblasts.

Four strains of lung fibroblasts were obtained from patients. SA-β-gal activity (A and B) and cell growth (C and D) were investigated in these cells after chronic exposure to 27-OHC for 1 week. Values are the mean ± SEM. **p < 0.01 vs. HNS. ††p < 0.01 vs. HES. ‡‡p < 0.01 vs. each control group. HNS = healthy control who had never smoked; HES = healthy ex-smoker;
COPD = ex-smoker with COPD.
Figure 4

(A)

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<tr>
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<th>COPD</th>
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<tbody>
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(B)

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<th>COPD</th>
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Scale bars: 50 µm
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<tr>
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<td>96.6 ± 11.3 **</td>
<td>74.8 ± 14.1 **††</td>
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<td>FVC %predicted</td>
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<td>FEV₁/FVC (%)</td>
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<td>62.0 ± 8.9 **††</td>
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<td>DLCO/VA %predicted</td>
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<td>3 / 20</td>
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Data are presented as mean ± SD. FEV₁ = forced expiratory volume in 1 second; FVC = forced vital capacity; DLCO = diffusing capacity of carbon monoxide; VA = alveolar volume. **p < 0.01 compared with healthy never-smokers; ††p < 0.01 compared with healthy ex-smokers.
Table 2. Characteristics of the sputum study

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<td>Male sex (%)</td>
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<tr>
<td>Age (y)</td>
<td>60 ± 14</td>
<td>56 ± 23</td>
<td>72 ± 6</td>
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<td>FEV₁ (L)</td>
<td>3.02 ± 1.24</td>
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<tr>
<td>FEV₁ %predicted</td>
<td>98.1 ± 12.4</td>
<td>84.4 ± 28.3</td>
<td>66.6 ± 34.1** ††</td>
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<td>FEV₁/FVC (%)</td>
<td>84.6 ± 4.9</td>
<td>74.1 ± 10.9</td>
<td>51.4± 15.8**†</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD. FEV₁= forced expiratory volume in 1 second; FVC = forced vital capacity; **p < 0.01 compared with Healthy subjects; †p < 0.05, ††p < 0.01 compared with Asthma
### Table 3. Characteristics of the subjects in the culture study of fibroblasts

<table>
<thead>
<tr>
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<th>Healthy never-smokers</th>
<th>Healthy ex-smokers</th>
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<td>6</td>
<td>6</td>
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<tr>
<td>Age (y)</td>
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<td>FEV₁ (L)</td>
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<td>FEV₁ (%predicted)</td>
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<td>104 ± 20.1</td>
<td>75.5 ± 8.8†</td>
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<td>FVC (L)</td>
<td>3.16 ± 0.63</td>
<td>3.5 ± 0.62</td>
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<tr>
<td>FEV₁/FVC (%)</td>
<td>79.4 ± 1.7</td>
<td>76.4 ± 4.2</td>
<td>62.8 ± 7.3** ††</td>
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</table>

Data are presented as mean ± SD. FEV₁ = forced expiratory volume in 1 second; FVC = forced vital capacity; *p < 0.05, **p < 0.01 compared with healthy never-smokers; †p < 0.05, **††p < 0.01 compared with healthy ex-smokers.