SUPPLEMENTAL MATERIAL

Pulmonary Artery Smooth Muscle Cell Senescence Is a Pathogenic Mechanism for Pulmonary Hypertension in Chronic Lung Disease

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Role of Cell Senescence in Pulmonary Hypertension

DETAILED METHODS

Study population

We evaluated two groups of patients. The first group consisted of 124 patients with COPD who underwent right heart catheterization and telomere length measurement. The data from 91 of these patients in whom inflammatory biomarkers were assayed have been published previously [1] (Table 1). The inclusion criteria for the patients in this cohort were a history of smoking, an FEV_1/FVC ratio <70%, and an arterial partial pressure of oxygen (PaO_2) <80 mm Hg. About 50 of these patients were included in a previously published prospective study comparing telomere length in patients with COPD and controls [2]. Right atrial pressure, pulmonary artery pressures (systolic, diastolic, and mean), and pulmonary wedge pressures were measured. Cardiac output (CO) was determined using thermodilution. Derived hemodynamic variables were calculated using standard formulas: cardiac index (cardiac output/body surface area, CI) as $L \cdot min^{-1} \cdot m^2$ and pulmonary vascular resistance (PVR in Wood Units) as mean pulmonary artery pressure (Pap) – pulmonary wedge pressure /CO.

The second group consisted of 27 patients treated with lung resection surgery and recruited prospectively at the Hotel-Dieu Teaching Hospital (Paris, France), including 14 with COPD and 13 defined as controls (Table 2). Most of these patients underwent lobectomy or pneumonectomy for localized lung tumors. In this group, lung tissue samples and derived cell cultures were studied; pulmonary arteries for the study were taken at a distance from tumor areas. Systolic pulmonary artery pressure was assessed using echocardiography. Inclusion criteria for COPD were an at least 10-pack-year history of tobacco smoking and a forced expiratory volume in 1 second (FEV_1)/forced vital capacity (FVC) ratio <70%. Inclusion criteria for the control smokers were a smoking history greater than 10 pack-years, an FEV_1/FVC ratio greater than 70%, and the absence of chronic cardiovascular, hepatic, and renal disease. None of these patients had received chemotherapy. This study was approved by the institutional review board of the Henri Mondor Teaching Hospital. All patients and controls signed an informed consent document before study inclusion.

Assessment of pulmonary vascular remodeling

The morphologic characteristics of the pulmonary muscular arteries were analyzed in lung tissue sections stained with hematoxylin-phloxin-saffron [3]. Arteries (20 to 30 for each lung) with an external diameter between 100 to 500 μm and complete elastic laminas were evaluated. The areas occupied by the muscular and intimal layers were analyzed using image J software (http://rsbweb.nih.gov/ij/). Masson trichrome staining was also performed to identify the extracellular matrix in the vessel wall and quantified using ImageJ (http://rsbweb.nih.gov) software as the percentage of the wall surface area.

Immunohistochemistry

Paraffin-embedded sections were deparaffinized using xylene and a graded series of ethanol dilutions then incubated in citrate buffer (0.01 M, pH 6) at 90°C for 20 minutes. Endogenous peroxidase activity was blocked with 3% H_2O_2 and 10% methanol in phosphate-buffered saline (PBS) for 10 minutes. Slides were incubated for 60 minutes in 1% bovine serum albumin and 5% goat serum in PBS then incubated overnight with anti-p21 mouse antibody (1:50, Cell signaling, Boston MA, USA), anti-p16 mouse antibody (1:1000, Abcam, Cambridge, MA, USA), and anti-Ki67 rabbit antibody (1:500, Abcam, Cambridge, MA, USA). We used the ABC Vectastain kit (Vector Labs, Burlingame, CA, USA) to mark the primary antibodies according to the user's guide. The staining substrate was DAB (FastDAB, Sigma-Aldrich, St Louis, MO, USA) and the sections were counterstained with hematoxylin [4].

Paraffin-embedded sections were deparaffinized using xylene and a graded series of ethanol dilutions then incubated in citrate buffer (0.01 M, pH 6) at 90°C for 20 minutes. Tissue was permeabilized with 0.1% Triton X-100 in PBS for 10 minutes. Endogenous peroxidase activity was blocked with 3% H_2O_2 and 10%

methanol in PBS for 10 minutes. Slides were incubated for 60 minutes in 1% bovine serum albumin and 5% goat serum in PBS. We performed double-label immunohistochemistry in two steps. In step one, the slides were incubated overnight with anti-p21 mouse antibody (1:50, Cell signaling, Boston MA, USA), anti-p16 mouse antibody (1:1000, Abcam, Cambridge, MA, USA), or anti-Ki67 rabbit antibody (1:500, Abcam, Cambridge, MA, USA). We used the ABC Vectastain kit (Vector Labs, Burlingame, CA, USA) to label the primary antibodies according to the user's guide. The staining substrate was DAB (FastDAB, Sigma-Aldrich, St Louis, MO, USA). In step two, slides were incubated for 1 hour with primary antibodies against smooth muscle actin (SMA) (1:600, Sigma-Aldrich, St Louis, MO, USA) or von Willebrand Factor (1:1000, Abcam Cambridge, MA, USA). Secondary antibodies were anti-rabbit (1:300, Dako, Glostrup, Denmark) or anti-mouse (1:300, Dako) coupled to horseradish peroxidase. The staining substrate was histogreen (Abcys, Paris, France) and the sections were counterstained with hematoxylin [4].

Culture of pulmonary artery smooth muscle cells

PA-SMCs were cultured from explants as previously described [5]. To determine the phenotypic characteristics of cultured PA-SMCs, we assessed the cells from each culture for expression of muscle-specific contractile and cytoskeletal proteins, including smooth muscle cell α-actin and desmin.

Cell replication

After cell outgrowth from the explants, cells were passaged (passage 1), seeded in 25-cm^2 flasks, and cultured to confluence. The cells were then counted and seeded (passage 2) in 75-cm^2 culture flasks. The experiments started at this point and the cells were serially passaged until senescence. The onset of cell replicative senescence was defined based on cessation of cell division, labeling for SA-beta galactosidase (β-Gal), and cell morphology criteria. At each passage, cells were harvested for quantification of DNA (telomere length measurement), RNA (real-time RT-PCR), and protein (Western blotting). Cells were also counted using a hemocytometer, and population-doubling levels (PDL) were calculated as $(\log_{10}Y - \log_{10}X)/\log_{10}2$, where X is the initial number of seeded cells and Y the final number.

Senescence associated β-galactosidase staining

At each passage, cells were washed twice in PBS, fixed for 10 minutes at 37°C in 4% paraformaldehyde, washed twice in PBS, and incubated for 24 h at 37°C in SA-βGal staining solution (1 mg/ml X-Gal, 5 mmol/L potassium ferrocyanide, 5 mmol/L potassium ferricyanide, 150 mmol/L NaCl, 2 mmol/L $MgCl_2$, and 40 mmol/L citrate [titrated to pH 6.0 with NaH_2PO_4]).

Protein extraction and immunoblotting

For protein extraction, isolated PA-SMCs were washed with PBS and lyzed with RIPA lysis buffer. Base ingredients (10 mM sodium phosphate, pH 8; 150 mM NaCl; 0.5% SDS; 1% Na-deoxycholate; and 1% NP40) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM NaF, sodium orthovanadate, and cocktail inhibitors (1/100) were from Sigma Aldrich. For protein analysis using Western blotting, samples were subjected to electrophoresis in 10% or 15 % polyacrylamide gels under reducing conditions. After electrophoresis, proteins from the gel were electroblotted onto polyvinylidene difluoride membranes (Millipore, Molsheim, France) for 2 h. After transfer, the membrane was saturated with PBS/5% milk. The membrane was then incubated overnight at 4 °C with the appropriate antibodies: rabbit polyclonal anti-P-p53 (Ser15) antibody was used at 1:1000 dilution (Cell Signaling Technology, Boston, MA, USA), monoclonal anti-p21Waf1/Cip1 (DCS60) antibody was used at 1:2000 dilution (Cell Signaling Technology), monoclonal anti-p16 (F-12) (sc-1661) antibody was used at 1:500 dilution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and anti–β-actin antibody (Sigma, Saint-Quentin-Fallavier, France) was used at 1:5000 dilution. Protein expression was reported as the protein/ β-actin ratio and expressed as arbitrary units.

Measurements of soluble factors by ELISA

Soluble factors were measured in plasma and cell medium. For cell medium determinations, PA-SMCs from early and late passages were grown to confluence in DMEM containing 15% fetal calf serum (FCS). The medium was then removed and the cells subjected to growth arrest in medium containing no FCS. After 48 hours of incubation, the conditioned medium was used for quantitation of IL-6, IL-8, MCP-1, TNF-α, IL-1β, and TGF-β using Quantikine ELISA kits (R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The cells were washed twice with PBS, trypsinized and counted.

Telomere length assay

Telomere length was assessed using a real-time quantitative polymerase chain reaction (PCR)-based assay [6]. Briefly, the ratio of the telomere repeat copy number over the single-gene copy number (T/S) was determined using an Applied Biosystems 7900HT thermocycler in a 384-well format, using the comparative Ct method ($T/S = 2^{-\Delta\Delta Ct}$). Genomic DNA was extracted from smooth muscle cells using the QIAamp DNA Kit (Qiagen, Courtaboeuf, France) and quantified using a spectrophotometer. Each sample was run in triplicate, using the SYBR Green method (Invitrogen, Cergy-Pontoise, France) and 30 ng of DNA. The sequences and final concentrations of the primers for the telomere and 36B4 (acidic ribosomal phosphoprotein PO, a single-copy gene for normalization) were as follows: Tel F, 5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3', 300 nM; Tel R, 5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3', 300 nM; 36B4F, 5'-CAGCAAGTGGGAAGGTGTAATCC-3',300 nM; and 36B4R, 5'-CCCATTCTATCATCAACGGGTACAA-3', 300 nM. Telomere length is expressed as the ratio of the telomere repeat copy number over the single-gene copy number (36B4 gene).

Extracellular matrix and soluble factors

After identification, presenescent ($4 \cdot 10^4$) and senescent ($6 \cdot 10^4$) PA-SMCs were allowed to attach to 12-well culture dishes overnight and were incubated in serum and growth factor-free medium for 2 days to generate similar cell numbers. The media from presenescent and senescent PA-SMCs incubated in serum and growth factor-free medium for 2 days were used for soluble factor assays and for evaluations of effects on growth or migration of normal target PA-SMCs previously attached to 12-well culture dishes. To determine the contribution of secreted matrices, we allowed presenescent and senescent PA-SMCs in serum-free medium to deposit extracellular matrix onto the culture dishes for 2 days. The cells were then detached using 2 mM EDTA for 30 min at 37°C. Two brief washes with PBS were then used to remove the cells. The target PA-SMCs used to study migration or proliferation were incubated in serum-free medium for 2 days then re-suspended in complete medium and plated on PA-SMC-depleted dishes prepared as described above.

Cell proliferation assay

Cell proliferation was assessed using the tetrazolium salt (MTT) assay (Sigma, Lyon, France). Briefly, PA-SMCs were seeded onto 12-well culture dishes pre-coated with extracellular matrix prepared earlier or in the presence of medium from presenescent or senescent cells or in the presence of 20 ng/mL platelet-derived growth factor (PDGF). After 72 h, the medium was changed to the same medium supplemented with 100 μg/mL of MTT, and the cells were incubated for 4 h. After washing with saline, 1 ml of dimethylsulfoxide (DMSO, Sigma)was added and the mixture was shaken for a few minutes to achieve complete dissolution. Aliquots (200 μL) of the resulting solutions were transferred to 96-well plates and absorbance was recorded at 520 nm using the Microplate Spectrophotometer System.

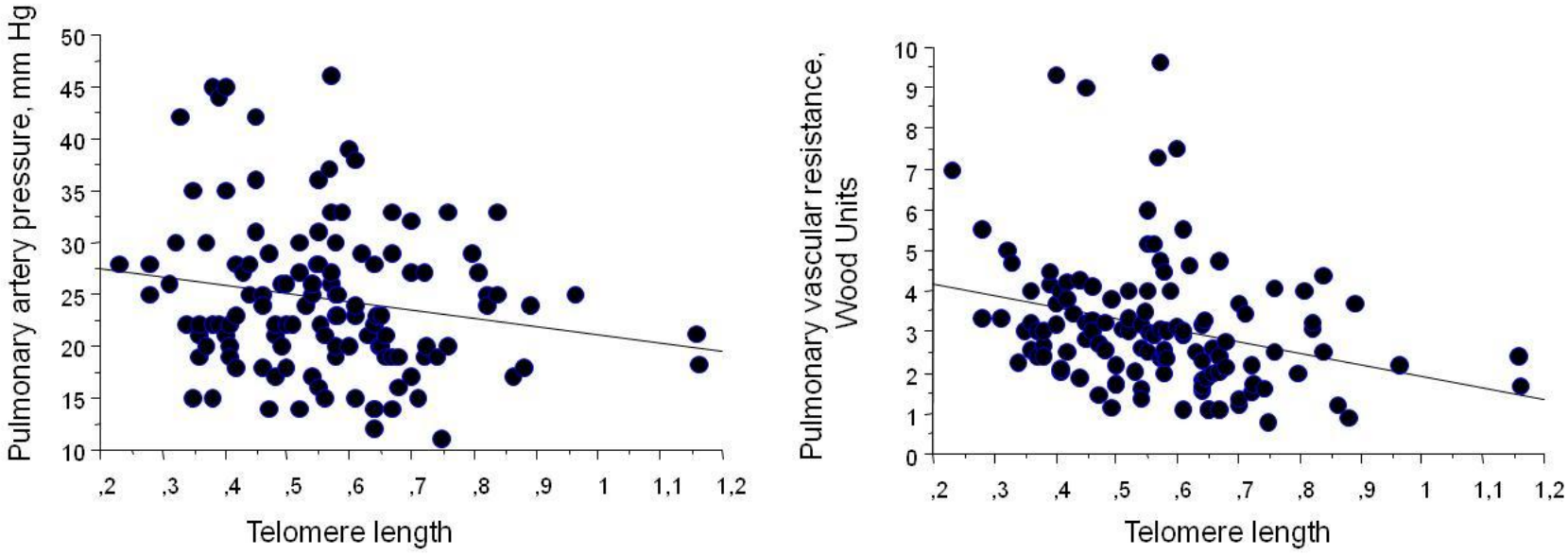
Cell migration assay

The cell migration assay was performed as previously described [7]. PA-SMCs were subjected to growth arrest in medium containing no FCS for 48 or 72 h then resuspended at $30 \cdot 10^6$ cells/mL in culture medium containing 15% FCS and 0.3% agarose. The cells were maintained at 37° C to prevent setting of the agarose. Three-microliter drops of the cellular suspension were plated in the center of each well of a 24-well tissue culture plate. Wells used for the migration assay were precoated with poly-DL-ornithine (0.5 g/ml; Sigma, St. Louis, MO, USA). The preparation was placed at 4°C for 20 min to allow the agarose to gel. Then, 0.9 ml of medium was added to cover the drops. The preparation was incubated at 37°C in 5% CO_2 for 24 h. Samples were fixed and stained with Diff-Quik kit (Siemens Healthcare Diagnostics, Saint Denis, France). Images were imported into ImageJ analysis software for calculation of cell migration under each condition.

Statistical analysis

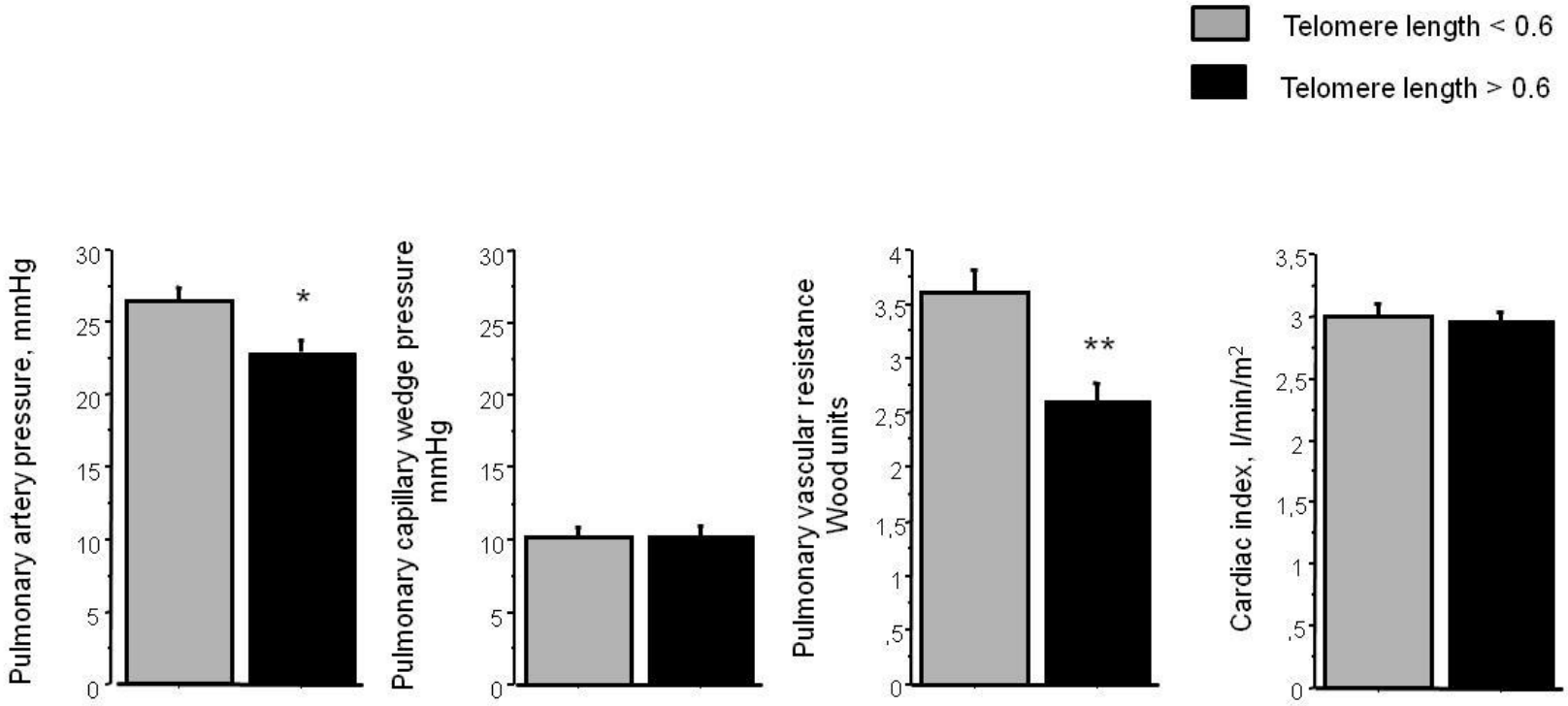
Data are expressed as mean±SEM. Patients with COPD and controls were compared using the unpaired t-test for quantitative variables and the chi-square test for categorical variables. Correlations between variables were evaluated using least-square linear regression techniques. The effects of senescence in cells from patients with COPD and controls were assessed by using a paired t-test. *P* values less than 0.05 were considered significant. Data were analyzed using Stata statistical software (release 8.0; StataCorp, College Station, TX, USA).

Online Figure I



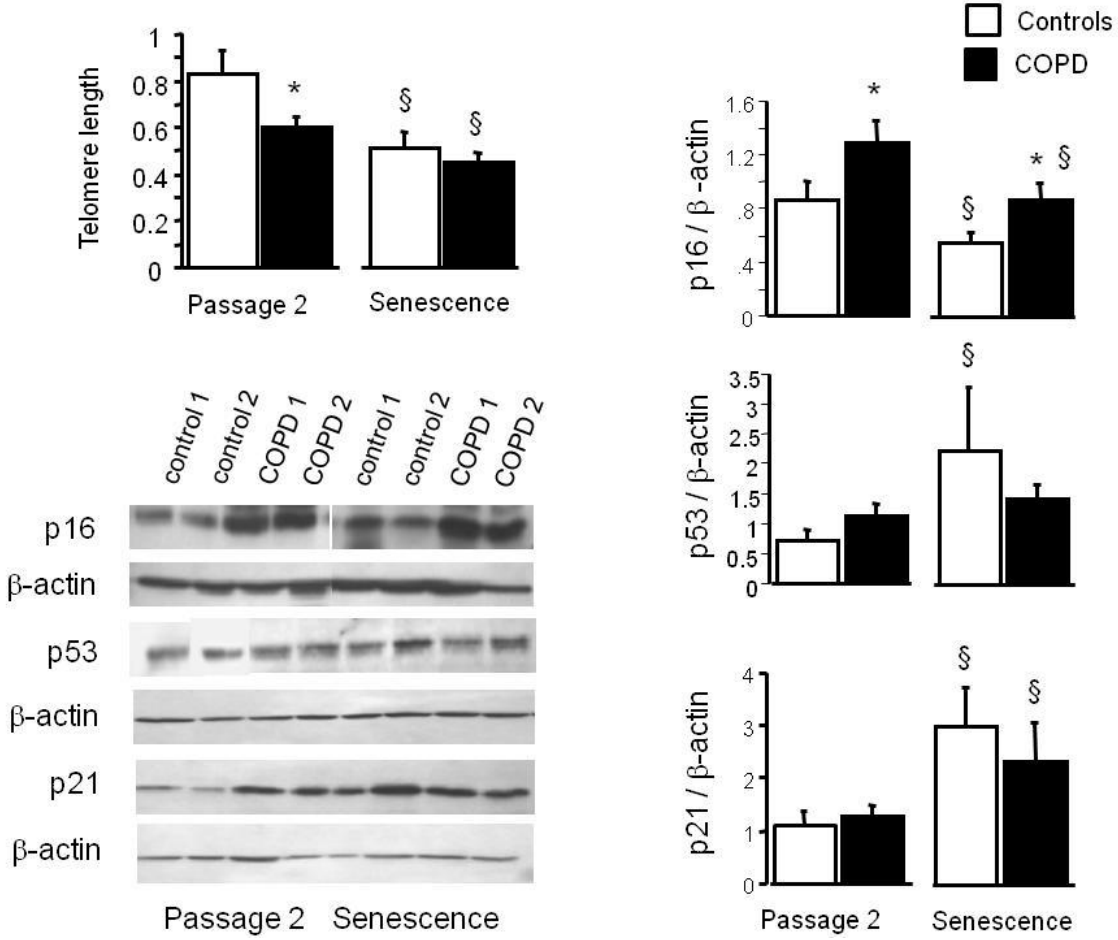
Online Figure I: Correlations between telomere length, pulmonary artery pressure (r= -0.20, *P*<0.04) and pulmonary vascular resistance (r= -0.29, *P*<0.01) in patients with COPD. Telomere length is expressed as the ratio of the telomere repeat copy number over the single-gene copy number (36B4 gene).

Online Figure II



Online Fig II: Comparison of pulmonary artery pressure, pulmonary capillary wedge pressure, pulmonary vascular resistance, and right atrial pressure between patients dichotomized based on the median telomere length. Values are means±SEM. **P*<0.05, ***P*<0.01 compared with values from subjects with telomere lengths less than or equal to 0.6. Telomere length is expressed as the T/S ratio of the telomere repeat copy number over the single-gene copy number (36B4 gene)..

Online Figure III



Online Figure III. Telomere length and Western blotting analysis of p16, p53, and p21 protein levels in PA-SMCs from the 14 patients with COPD and 13 controls determined at passage 2 and at senescence. Each bar is the mean±SEM. **P*<0.01 compared with values for PA-SMCs from controls. § *P*<0.05 compared with corresponding values for PA-SMCs at passage 2.

Supplemental references

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NOVELTY AND SIGNIFICANCE

What is known?

- Chronic obstructive pulmonary disease (COPD) is an age-related disease that is among the most common causes of pulmonary hypertension (PH).
- COPD is associated with telomere shortening, which causes cell senescence.
- The role for telomere shortening as a pathogenic mechanism in COPD is unknown.

What new information does this article contribute?

- PH severity is related to telomere shortening in COPD, and remodeled pulmonary vessels are characterized by an increased number of senescent pulmonary artery smooth muscle cells (PA-SMCs).
- Senescent PA-SMCs produce soluble and insoluble paracrine factors, which stimulate the growth and migration of normal target PA-SMCs, thereby contributing to pulmonary vessel remodeling.

Summary of Novelty and Significance

Chronic obstructive pulmonary disease is increasingly prevalent in industrialized countries and is among the most common causes of PH, a condition that carries a poor prognosis. COPD is also an age-related disease associated with telomere shortening, which causes cell senescence. In 124 patients investigated by right heart catheterization, we found that telomere shortening was associated with PH severity. Investigations of lung vessels and derived cultured PA-SMCs from patients with COPD and age- and sex-matched control smokers showed increased senescent cell counts in remodeled COPD vessels and accelerated senescence in proportion to remodeling severity in derived cultured PA-SMCs. Senescent PA-SMCs, whose in situ location was near actively dividing cells at sites of vessel wall hypertrophy, were shown in vitro to overexpress soluble and insoluble factors that affected PA-SMC migration and proliferation. These results support the concept that PA-SMC senescence is a pathogenic mechanism of pulmonary vascular remodeling and PH. Knowledge of this new pathophysiological pathway might help to identify new biomarkers of disease severity and prognosis. It may open up new therapeutic possibilities targeting cell senescence and, potentially, its effects, including PH, cardiovascular disease, and cancer, which are the main causes of morbidity and mortality in COPD.