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


Rationale: Senescence of pulmonary artery smooth muscle cells (PA-SMCs) caused by telomere shortening or oxidative stress may contribute to pulmonary hypertension associated with chronic lung diseases.

Objective: To investigate whether cell senescence contributes to pulmonary vessel remodeling and pulmonary hypertension in chronic obstructive pulmonary disease (COPD).

Methods and Results: In 124 patients with COPD investigated by right heart catheterization, we found a negative correlation between leukocyte telomere length and pulmonary hypertension severity. In-depth investigations of lung vessels and derived cultured PA-SMCs showed greater severity of remodeling and increases in senescent p16-positive and p21-positive PA-SMCs and proliferating Ki67-stained cells in 14 patients with COPD compared to 13 age-matched and sex-matched control subjects who smoke. Cultured PA-SMCs from COPD patients displayed accelerated senescence, with fewer cell population doublings, an increased percentage of β-galactosidase–positive cells, shorter telomeres, and higher p16 protein levels at an early cell passage compared to PA-SMCs from controls. Both in situ and in vitro PA-SMC senescence criteria correlated closely with the degree of pulmonary vessel wall hypertrophy. Because senescent PA-SMCs stained for p16 and p21 were virtually confined to the media near the Ki67-positive cells, which predominated in the neointima and hypertrophied media, we evaluated whether senescent cells affected normal PA-SMC functions. We found that senescent PA-SMCs stimulated the growth and migration of normal target PA-SMCs through the production and release of paracrine soluble and insoluble factors.

Conclusion: PA-SMC senescence is an important contributor to the process of pulmonary vascular remodeling that underlies pulmonary hypertension in chronic lung disease. (Circ Res. 2011;109:00-00.)

Key Words: pulmonary hypertension ■ remodeling ■ senescence ■ smooth muscle cells

Pulmonary hypertension (PH) may occur as a complication of various chronic lung diseases. Among these diseases, chronic obstructive pulmonary disease (COPD) is becoming increasingly prevalent and is expected to become the third leading cause of death worldwide by 2020. COPD is characterized by slowly progressive airflow obstruction, resulting in dyspnea and exercise limitation. COPD is also one of the most common causes of PH and cor pulmonale. Extensive pulmonary vessel remodeling with prominent intimal thickening, medial hypertrophy, and muscularization of the small arterioles are cardinal pathological features of PH in COPD. These structural changes are considered the main cause of the increase in pulmonary vascular resistance, but their pathogenesis remains uncertain.

COPD is an age-related disease associated with telomere shortening. One consequence of reduced telomere length is early replicative senescence of somatic cells, characterized by growth arrest, loss of specialized cellular functions, and genomic instability. Premature cell senescence also may occur through nontelomeric signals in response to various types of stress such as oxidative stress. Senescent cells survive in vivo but acquire many changes in the expression of genes encoding various cytokines, proteases, and growth factors. These changes in gene expression may act not only in reinforcing the senescence-related growth arrest but also in a paracrine manner to promote degenerative or hyperproliferative changes in neighboring cells. There is now widespread agreement that senescent cells can be dele-
Subjects and 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 previously13 (Table 1). The second group consisted of 27 patients treated with lung resection surgery for localized lung tumors who were recruited prospectively at the Hotel-Dieu Teaching Hospital (Paris, France); of these 27 patients, 14 had COPD and 13 were defined as controls (Table 2). In this second group, lung tissue samples and derived cell cultures were studied. Inclusion criteria for COPD were at least a 10-pack-year history of tobacco smoking 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 (Créteil, France). All patients and controls signed an informed consent document before study inclusion.

Laboratory Investigations

Pulmonary vascular remodeling was quantified based on histomorphometric analyses and cell senescence was assessed by in situ analysis of lung tissue sections.4 Senescent PA-SMCs within the pulmonary vascular wall of distal pulmonary vessels were identified by p16 and p21 immunostaining and proliferative cells were identified by Ki67 immunostaining described within the expanded Methods section available in the Online Supplement at http://circres.ahajournals.org.

Cultured PA-SMCs collected from pulmonary arteries of patients with COPD and controls were subjected to repeated cell passages to determine their threshold for replicative senescence and the total number of cell population doublings (PDL). Two main criteria were used for subsequent analyses: the PDL and the percentage of senescent cells found in lungs of patients with COPD compared to control subjects who smoke.5,12 However, the role for cell senescence in the lung alterations characteristic of COPD has not yet been examined.

Here, we reasoned that senescent cells in COPD may contribute to the process of pulmonary vascular remodeling and, therefore, to the pathogenesis of PH. First, to evaluate pulmonary vascular cell senescence by studying lung specimens and derived cultured pulmonary artery smooth muscle cells (PA-SMCs) from 14 patients with COPD and 13 age-matched and sex-matched control subjects who smoke. Finally, we investigated the propensity of senescent cells to release soluble and insoluble factors and to alter the migration and proliferation of normal target PA-SMCs, thereby contributing to the process of pulmonary vascular remodeling.

Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PA-SMC</td>
<td>pulmonary artery smooth muscle cell</td>
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<tr>
<td>α-SMA</td>
<td>alpha-smooth muscle actin</td>
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<tr>
<td>β-gal</td>
<td>beta-galactosidase</td>
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<tr>
<td>Akt</td>
<td>serine/threonine kinase</td>
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<tr>
<td>BMI</td>
<td>body mass index</td>
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<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
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<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
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<tr>
<td>IL-1β</td>
<td>interleukin-1-beta</td>
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<tr>
<td>IL-6</td>
<td>interleukin-6</td>
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<tr>
<td>IL-8</td>
<td>interleukin-8</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemoattractant protein-1</td>
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<tr>
<td>Pap</td>
<td>mean pulmonary artery pressure</td>
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<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<tr>
<td>PDL</td>
<td>population doubling level</td>
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<tr>
<td>PH</td>
<td>pulmonary hypertension</td>
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<tr>
<td>Sap</td>
<td>mean systemic arterial pressure</td>
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<tr>
<td>TGF-β</td>
<td>transforming growth factor-beta</td>
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<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-alpha</td>
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<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
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Table 1. Pulmonary Hemodynamic, Physiological, and Biological Variables in 124 Patients With Chronic Obstructive Pulmonary Disease and Correlations With Telomere Length in Circulating Leukocytes

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean±SEM</th>
<th>r</th>
<th>P</th>
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<tbody>
<tr>
<td>Females/males</td>
<td>29/95</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Telomere length (T/S ratio)</td>
<td>0.56±0.01</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Age, y</td>
<td>64.1±0.7</td>
<td>−0.20</td>
<td>0.02</td>
</tr>
<tr>
<td>Pack-years</td>
<td>50.7±2.4</td>
<td>0.05</td>
<td>0.52</td>
</tr>
<tr>
<td>FEV1, %</td>
<td>41.5±1.6</td>
<td>0.04</td>
<td>0.65</td>
</tr>
<tr>
<td>FEV1, L</td>
<td>1.1±0.0</td>
<td>0.09</td>
<td>0.29</td>
</tr>
<tr>
<td>FVC, L</td>
<td>2.7±0.1</td>
<td>0.06</td>
<td>0.45</td>
</tr>
<tr>
<td>FEV1/FVC, %</td>
<td>47.4±1.2</td>
<td>0.02</td>
<td>0.82</td>
</tr>
<tr>
<td>Pap, mm Hg</td>
<td>4.6±0.6</td>
<td>−0.20</td>
<td>0.04</td>
</tr>
<tr>
<td>Powp, mm Hg</td>
<td>10.4±0.4</td>
<td>0.04</td>
<td>0.64</td>
</tr>
<tr>
<td>Sap, mm Hg</td>
<td>5.7±0.3</td>
<td>0.02</td>
<td>0.82</td>
</tr>
<tr>
<td>Cardiac index, L/min/m²</td>
<td>2.7±0.1</td>
<td>0.02</td>
<td>0.82</td>
</tr>
<tr>
<td>PVR, Wood units</td>
<td>3.1±0.1</td>
<td>−0.29</td>
<td>0.01</td>
</tr>
<tr>
<td>IL-6, pg/mL</td>
<td>3.8±0.4</td>
<td>−0.20</td>
<td>0.04</td>
</tr>
<tr>
<td>IL-8, pg/mL</td>
<td>13.4±0.9</td>
<td>0.05</td>
<td>0.59</td>
</tr>
<tr>
<td>IL-1β, pg/mL</td>
<td>8.1±0.1</td>
<td>0.02</td>
<td>0.85</td>
</tr>
<tr>
<td>TGF-β, pg/mL</td>
<td>28.4±1.4</td>
<td>0.03</td>
<td>0.77</td>
</tr>
<tr>
<td>TNF-α, pg/mL</td>
<td>1.7±0.1</td>
<td>−0.03</td>
<td>0.73</td>
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<tr>
<td>MCP-1, pg/mL</td>
<td>53.07±23.0</td>
<td>−0.18</td>
<td>0.06</td>
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FEV1 indicates forced expiratory volume in 1 second; FVC, forced vital capacity; IL, interleukin; MCP, monocyte chemoattractant protein-1; Pap, mean pulmonary artery pressure; Sap, mean systemic arterial pressure; TGF, transforming growth factor; TNF, tumor necrosis factor.
Table 2. Comparison of Clinical Characteristics and Pathological Variables Between Patients With Chronic Obstructive Pulmonary Disease and Control Smokers

<table>
<thead>
<tr>
<th></th>
<th>Patients (n=14)</th>
<th>Controls (n=13)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females/males</td>
<td>3/11</td>
<td>4/9</td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>64.2±1.8</td>
<td>59.1±3.3</td>
<td>0.15</td>
</tr>
<tr>
<td>Pack-years</td>
<td>49.4±6.1</td>
<td>37.1±4.0</td>
<td>0.10</td>
</tr>
<tr>
<td>FEV₁, %</td>
<td>74.7±4.1</td>
<td>91.9±5.5</td>
<td>0.003</td>
</tr>
<tr>
<td>FEV₁, L</td>
<td>2.05±0.15</td>
<td>2.69±0.25</td>
<td>0.027</td>
</tr>
<tr>
<td>FVC, L</td>
<td>3.2±0.2</td>
<td>3.8±0.4</td>
<td>0.21</td>
</tr>
<tr>
<td>FEV₁/FVC</td>
<td>63.6±1.7</td>
<td>77.0±1.4</td>
<td>0.0001</td>
</tr>
<tr>
<td>Systolic Pap, mm Hg</td>
<td>33.2±2.9</td>
<td>25.0±1.6</td>
<td>0.04</td>
</tr>
<tr>
<td>Wall thickness area, Ø 0–99 μm, 10⁻⁴ mm²</td>
<td>41.0±3.3</td>
<td>27.4±1.7</td>
<td>0.001</td>
</tr>
<tr>
<td>Wall thickness area, Ø 100–199 μm, 10⁻⁴ mm²</td>
<td>123.2±5.2</td>
<td>87.9±5.4</td>
<td>0.00001</td>
</tr>
<tr>
<td>Wall thickness area, Ø 200–399 μm, 10⁻⁴ mm²</td>
<td>292.0±13.0</td>
<td>242.9±23.2</td>
<td>0.05</td>
</tr>
<tr>
<td>Wall thickness area, %</td>
<td>72.0±1.9</td>
<td>55.8±1.9</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

*FEV₁ indicates forced expiratory volume in 1 second; FVC, forced vital capacity; Pap, mean pulmonary artery pressure.*

Data are means±SEM. P values are for the comparisons between patients and those who smoke.

β-galactosidase (β-gal)-positive cells at passage 2 and at senescence. The amounts of p53, p16, and p21 proteins were determined at passage 2 and at senescence by Western blotting. Telomere length was assessed using a real-time quantitative polymerase chain reaction-based assay and expressed as the ratio of the telomere repeat copy number over the single-gene copy number (36B4 gene). Genomic DNA was extracted either from blood samples or from smooth muscle cells at passage 2 and at senescence. Soluble factors (IL-6, IL-8, MCP-1, TNF-α, IL-1β, and TGF-β) were measured in plasma and cell media using an enzyme-linked immunosorbent assay. Functional studies were performed to investigate the impact of paracrine soluble and insoluble factors released by senescent cells on the proliferation and migration of target PA-SMCs as described in the Online Supplement at http://circres.ahajournals.org.

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 χ² 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 using a paired t test; P<0.05 was considered significant. Data were analyzed using Stata statistical software (release 8.0; StataCorp).

Results

Relationship Between Telomere Length and Pulmonary Hemodynamics in Patients With COPD Investigated by Right Heart Catheterization

The characteristics of the 124 patients with COPD are reported in Table 1. Telomere length was associated with age but not with the degree of airflow limitation or with Sap or creatinine levels. Telomere length correlated negatively with Pap (range, 11–47 mm Hg) and pulmonary vascular resistance (range, 1–9.5 Wood units; Table 1 and Online Figure I). Telomere length also correlated negatively with circulating IL-6 levels, which correlated positively with Pap (r=0.31; P<0.01) and pulmonary vascular resistance (r=0.42; P<0.001). Patients with telomere lengths less than or equal to the median value (0.6, T/S ratio) had higher Pap and pulmonary vascular resistance values than did patients with telomere lengths >0.6 (Online Figure II).

Characteristics of Patients With COPD and Controls Included in the Study of Lung Tissue and Derived Cultured Cells

Table 2 reports the clinical features of the surgical patients with and without COPD, as well as the histological findings from their lung specimens. The group with COPD did not differ significantly from the group of control subjects who smoke regarding age, sex ratio, smoking history, body mass index, or mean Sap. Systolic Pap was higher in the patients with COPD than in the controls and correlated positively with the wall thickness area (r=0.38; P<0.05). Pulmonary vascular remodeling (wall thickness area) was more severe in patients with COPD than in controls, whether remodeling was assessed based on selected vessel size categories or based on all vessel size categories in a given individual.

In Situ Analysis of p16-Stained, p21-Stained, and Ki67-Stained PA-SMCs, of p16-Stained and p21-Stained Endothelial Cells, and of Collagen in Distal Pulmonary Vessels From Patients With COPD and Controls

Senescent PA-SMCs were identified as p21-stained and p16-stained cells. Preliminary experiments were performed in proximal pulmonary arteries to verify that α-SMA–positive cells, when positive for β-gal activity, were also positive for p16 and p21, indicating that they were senescent PA-SMCs. In distal pulmonary vessels, the number of senescent cells expressed as the percentage of α-SMA–positive PA-SMCs also positive for p16 and p21 was considerably higher in pulmonary vessels from patients with COPD than in those from controls (Figure 1A). Similar differences in Ki67-positive proliferating cells were observed (Figure 1A). Of note, senescent p16-positive or p21-positive PA-SMCs and Ki67-stained cells predominated in remodeled pulmonary arteries, as shown by the tight relationship between the percentage of cells stained for p16, p21, or Ki67 and the wall thickness area ratio (Figure 1A). In addition, the percentage of Ki67⁺ cells correlated positively with the percentage of p16-stained (r=0.81; P<0.001) and p21-stained (r=0.84; P<0.001) PA-SMCs. At sites of vascular hypertrophy, proliferating cells were found chiefly in the neointima or hypertrophied media, whereas senescent cells were virtually confined to the media, with only a few senescent cells in the neointima (Figure 2).

The percentage of senescent endothelial cells expressed as the percentage of vWF-stained cells also positive for p21 or p16 was higher in pulmonary vessels from patients with COPD than in those from controls and correlated positively with the wall thickness area ratio (Figure 1B). Similarly,
more fibrosis was found in vessels from patients with COPD than in controls (Figure 1C).

Replicative Senescence of Cultured PA-SMCs From Patients With Chronic Obstructive Lung Disease and Controls

Cultured PA-SMCs from pulmonary arteries of patients with COPD and controls were subjected to repeated cell passages to determine their threshold for replicative senescence and the total number of cell population doublings (PDL). As shown in Figure 3A, PA-SMCs from patients with COPD began senescing after passage 2 to 3, whereas those from controls began senescing after passage 5 to 6. Consequently, the PDL was twice as high in controls as in patients with COPD (Figure 3B). The percentage of β-gal–positive cells was higher in patients with COPD than in controls at passage 2,
and then increased with subsequent passages and reached similar values in COPD patients and controls at the stage of cellular senescence (Figure 3C, D). In the overall population of surgical patients with and without COPD, PDL was tightly and inversely related to the wall thickness area ratio ($r= -0.61; P<0.001$), indicating a close relationship between in vitro criteria for senescence and the severity of pulmonary vascular remodeling (Figure 3E). A less significant relationship was found between the percentage of $\beta$-gal–positive cells and the wall thickness area ratio ($r= 0.38; P<0.05$).

**Telomere Length, Telomerase Activity, and Levels of p53, p21, and p16 Protein During Replicative Senescence of PA-SMCs From Patients With COPD and Controls**

The expression of senescent regulatory proteins and telomere length in cultured PA-SMCs was assessed at passage 2 and at replicative senescence. With repeated PA-SMC passages, p53 and p21 increased, p16 decreased, and telomeres shortened until senescence was reached (Online Figure III). At passage 2, patients with COPD and controls differed regarding telomere length and p16 protein, but not regarding p53 or p21. At senescence, telomere length was no longer significantly different between patients and controls, whereas the difference in p16 persisted. No telomerase activity was detected in PA-SMCs at any passage. Of note, PDL correlated strongly with the p16 level measured at passage 2 ($r= -0.61; P<0.001$) and less strongly with telomere length ($r= 0.37; P<0.05$).

**Factors Secreted by PA-SMCs From Patients With COPD and Controls During Replicative Senescence**

Because the cell senescent phenotype is not limited to an arrest of cell proliferation but includes widespread changes in protein expression and secretion, we measured the amounts of several cytokines and growth factors released by PA-SMCs from patients with COPD and controls at passage 2 and at senescence. As shown in Figure 4, soluble factors that increased from passage 2 to senescence included IL-6, IL-8, TNF-α, MCP-1, and TGF-β measured in the culture medium of PA-SMCs deprived of serum for 48 hours (IL1-β was not detectable in any of the samples). Among these factors, IL-6, IL-8, and TNF-α were found in higher concentrations in culture media of passage 2 cells from patients with COPD than from controls. At senescence, the differences were no longer significant, except for the difference in TNF-α. Of note, the amount of IL-6 released by passage 2 cells correlated with the percentage of $\beta$-gal–positive cells ($r=0.40; P<0.05$).

**Contribution of Secreted Soluble and Insoluble Factors to PA-SMC Proliferation and Migration**

To investigate whether secretion of soluble factors by senescent PA-SMCs affected the function of the target PA-SMCs, we evaluated the proliferation and migration of nonsenescent PA-SMCs treated with media from presenescent and senescent PA-SMCs. Growth stimulation of targetPA-SMCs was more marked with medium of senescent PA-SMCs than with medium of presenescent cells (Figure 5A, C). Similarly, the medium of senescent PA-SMCs was more potent in stimu-
lating PA-SMC migration than was the medium of presenescent cells (Figure 6A, B). Neutralizing antibodies to IL-6 and MCP-1, but not to IL-8 and TNF-α, markedly reduced PA-SMC proliferation induced by PA-SMC culture media (Figure 5D). The stimulatory effects of PA-SMC media from senescent and presenescent cells were no longer significantly different in the presence of anti-MCP-1 antibodies or IL-6 antibodies (Figure 5D). In contrast, neutralizing antibodies to IL-8, TNF-α, IL-6, or MCP-1 did not affect PA-SMC migration in response to culture media from nonsenescent cells, whereas they inhibited PA-SMC migration induced by culture media from senescent cells by ~25% (Figure 6D). Thus, neutralizing antibodies to IL-8, TNF-α, IL-6, and -MCP-1 abolished the differences in PA-SMC migration in response to culture media from senescent vs presenescent cells.

To determine the contribution of secreted matrices or insoluble factors, we allowed senescent or nonsenescent PA-SMCs to deposit extracellular matrix onto culture dishes for 3 days, after which we removed the cells without altering the matrix and introduced new healthy PA-SMCs, which were assessed for growth or migration. We found that target PA-SMCs exhibited faster growth (Figure 5B, C) and greater migration (Figure 6C) in dishes coated with matrices secreted by senescent cells compared to nonsenescent cells.

**Discussion**

We show here that PA-SMC senescence is involved in the process of pulmonary vessel remodeling that underlies PH in patients with COPD. After finding a positive correlation between telomere shortening and PH severity in a large population of patients with COPD, we investigated the pulmonary vessels and derived cultured PA-SMCs from patients with or without COPD. We found that remodeled vessels were characterized by PA-SMC senescence and that cultured PA-SMCs from patients with COPD displayed accelerated senescence. Our finding that in situ and in vitro criteria for PA-SMC senescence correlated closely with the severity of pulmonary vascular remodeling, together with the presence of senescent cells near actively dividing cells at sites of vessel wall hypertrophy, strongly suggests a role for senescent cells in the remodeling process. Moreover, we found that accelerated PA-SMC senescence in COPD was associated with increased expression of soluble and insoluble factors that affected PA-SMC migration and proliferation.
Taken together, these results support a role for PA-SMC senescence in the process of pulmonary vascular remodeling in COPD.

The role for telomere shortening as a pathogenic mechanism was recently highlighted in patients with familial idiopathic fibrosis who harbored a mutation in the telomerase gene. Both COPD and pulmonary fibrosis are age-related diseases associated with telomere shortening. Because short telomeres are associated with increased susceptibility to replicative cellular senescence, one current hypothesis is that cellular senescence represents one mechanism underlying the pathological alterations seen in these chronic lung diseases. Here, we focused on the process of pulmonary vascular remodeling that underlies PH in patients with COPD. In a population of patients with COPD investigated by right heart catheterization, we found that telomere shortening was associated with PH severity independently from the severity of airflow obstruction, age, and smoking history. To evaluate whether cellular senescence was present in pulmonary vessels from patients with COPD and reflected a process related to pulmonary vascular remodeling, we compared pulmonary vessels and derived cultured PA-SMCs from patients with COPD and from sex-matched and age-matched control subjects who smoke. Pulmonary vessels from patients with COPD were characterized by increased wall hypertrophy compared to controls, in keeping with the higher PAP in the patients with COPD than in the controls. Immunohistochemical examination of pulmonary vessels revealed an increased percentage of pulmonary vascular cells stained for p21 and p16, including endothelial and smooth muscle cells, in patients with COPD compared to controls. These cells were identified as senescent cells by experiments performed in the proximal pulmonary arteries, in which β-gal–positive cells were also positive for p16 and p21. We then evaluated whether PA-SMCs derived from pulmonary vessels also exhibited characteristic features of accelerated senescence when studied in vitro. Cultured PA-SMCs from patients with COPD exhibited premature senescence when compared to those of controls, with a marked decrease in cumulative PDL and a higher percentage of β-gal–positive cells measured at an early passage.

In studies of pulmonary vessels and derived cultured PA-SMCs from patients with COPD and from sex-matched and age-matched control subjects who smoke, we found that in situ and in vitro criteria for cell senescence correlated with the severity of pulmonary vascular wall hypertrophy, suggesting a close relationship between cell senescence and the pulmonary vascular remodeling process. The large proportion of senescent cells within the walls of remodeled pulmonary vessels may seem paradoxical, because PH is primarily a proliferative disorder and cell senescence is associated with impaired regenerative capacity in a given tissue. We investigated PA-SMC proliferation in vessels from our patients and found that remodeled vessels from patients with COPD contained more proliferating Ki67-stained PA-SMCs and more accumulated extracellular matrix than those from controls. Thus, remodeled vessels from patients with COPD were paradoxically characterized by a combination of elevated senescent cell counts with an increased proportion of proliferating cells and increased extracellular matrix deposition. Of note, studies of remodeled vessels at sites of vascular hypertrophy revealed senescent cells to be virtually confined to the media, with only a few senescent cells in the neointima, whereas proliferating cells predominated in the neointima and hypertrophied media. Our results therefore support the concept that several PA-SMC subsets are present in the pulmonary vascular wall of remodeled vessels in COPD and that these subsets work in combination to participate in the remodeling process. Similar results have been reported in atherosclerotic lesions characterized by senescent cells and showing presence in the neointima of actively dividing cells, possibly of monoclonal origin. Similarly, endothelial cells with senescence-
associated phenotypes are present in human atherosclerotic lesions.16

The mechanisms underlying premature PA-SMC senescence in COPD can only be speculated from the present study. We found increased p53 and p21 expression during replicative PA-SMC senescence, in parallel with a decrease in telomere length. Cells from patients with COPD studied at passage 2 had shorter telomeres than those from controls, in keeping with their increased susceptibility to replicative senescence. Although telomere loss is known to activate p53, with subsequent transcription of p21, we found similar levels of p53 and p21 in cells from patients with COPD and controls. In contrast, p16 expression was higher in cells from patients with COPD than in those from controls, suggesting a major role for p16 in driving premature senescence in COPD. Indeed, p16 activation by nontelomeric signals such as oxidative stress leads to premature senescence,17 but it also may occur during replicative senescence as a second barrier to cell proliferation.8 In accordance with this possibility, we found a strong inverse relationship between PDL and the amount of p16 measured in passage 2 cells, as well as a relationship with telomere length. Accelerated PA-SMC senescence in COPD therefore may be attributable to a combination of both telomere shortening and oxidative stress responsible for p16 activation. Other mechanisms also may interact with the senescence process, including reduced expression of HDAC2 and sirtuin, decreased proteasome
activity, and decreased Akt signaling, which have been reported in patients with COPD.

It may be argued that cellular senescence was a consequence of pulmonary vascular remodeling rather than an active contributor, ie, was attributable to the cell divisions involved in lesion development. This possibility is unlikely. In patients with COPD, telomere shortening and accelerated cell senescence exist as a general process that is not restricted to the blood vessels. Cells stained for p16 were present not only in pulmonary vascular cells but also in nondividing alveolar epithelial cells, in keeping with previous studies of patients with emphysema. Second, replicative senescence secondary to increased PA-SMC turnover would have been associated with prominent p21 expression. In contrast, we found a prominent difference in p16 expression, which appeared to be the main mechanism driving accelerated senescence of cultured PA-SMCs in patients with COPD. Moreover, the presence of senescent cells and actively dividing cells at different sites of the vascular lesions is not in favor of cell senescence occurring at exhaustion of their replicative potential. Finally, cells studied in culture were collected from proximal pulmonary arteries, which are not subjected to the same remodeling process as PA-SMCs from distal vessels. Thus, PA-SMC senescence in COPD does not seem to be a consequence of increased cell turnover at sites of vascular hypertrophy. Rather, PA-SMC senescence may be part of the pathogenic mechanisms associated with COPD that lead to pulmonary vessel remodeling and subsequent development of PH. Although we could not examine the relationship between telomere length in circulating leukocytes and in cultured PA-SMCs, such as positive relationship between telomere length in circulating leukocytes and in lung tissues already have been reported in patients with lung fibrosis.

The identification of the exact mechanisms by which PA-SMC senescence contributes to pulmonary vascular remodeling is challenging. Proliferation of neighboring cells may occur via a direct mitogenic effect or via indirect effects mediated by tissue damage or the recruitment of inflammatory cells. In the present study, we found that PA-SMCs undergoing replicative senescence released excessive amounts of several cytokines and mediators. The amount of these secreted factors differed markedly between patients with COPD and controls at an early cell passage but not at senescence, indicating that this difference was mainly attributable to the higher proportion of senescent cells in patients with COPD. Several factors such as IL-6, IL-8, MCP-1, and IL-1β have been demonstrated to make a strong but indirect contribution to pulmonary vascular remodeling. In particular, IL-6 is a
major contributor to hypoxic PH and is closely linked to PH severity in patients with COPD\textsuperscript{13} or idiopathic PH.\textsuperscript{20}

To better-investigate the potential interplay between nonsenescent and senescent cells, we evaluated whether senescent cells affected the migration and proliferation of nonsenescent cells in a paracrine manner. We found that soluble and insoluble factors released by senescent cells stimulated the growth and migration of target PA-SMCs. A similar finding was obtained previously using senescent fibroblasts and cultured epithelial cells and was taken as evidence that senescent cells promoted cell proliferation and tumor growth.\textsuperscript{18} This possibility is consistent with our observation that actively dividing cells in the neointimal lesions of remodeled vessels were surrounded by senescent cells, suggesting cross-talk between the two cell subsets. In our study, cell proliferation in response to culture media from senescent cells were markedly reduced in the presence of neutralizing antibodies to IL-6 and MCP-1, and cell migration was reduced in the presence of neutralizing antibodies to IL-8, TNF-\(\alpha\), IL-6, and MCP-1. The fact that the increased PA-SMC proliferation in response to culture media from senescent compared to presenescent cells was no longer observed in the presence of anti-MCP-1 and anti-IL-6 antibodies suggests an important role for these cytokines in this process.\textsuperscript{13,19,20} The fact that anti-IL-6 and anti-MCP-1 antibodies inhibited proliferation under both basal and stimulated conditions is also consistent with an autocrine effect of these mediators. Moreover, these results suggest that the global action of these soluble factors may be stimulation of growth and migration and not stimulation of senescence. Thus, senescent cells may create a microenvironment that facilitates the migration and growth of nonsenescent cells, thereby inducing neointima formation and vessel remodeling. Whether nonsenescent cells involved in neointima formation exhibit a normal or an abnormal phenotype remains to be elucidated.

There are several important limitations to this study. First, cell senescence in COPD is probably a process applying to the whole body, and it remains unclear whether the observations described here are specific to the pulmonary circulation or apply also to other vascular beds. Second, because we could not study a third group with severe PH or with other forms of PH, we do not know whether our data are relevant to various types of PH or specific of diseases associated with telomere dysfunction. Telomere shortening in circulating leukocytes is found in patients with COPD and lung fibrosis but not in patients with idiopathic PH.\textsuperscript{23} Whether telomere shortening may represent a biomarker of disease severity in various types of PH also remains an open question. In patients with COPD, telomere shortening may constitute a biomarker of overall accelerated aging and, potentially, of its effects, including PH, cardiovascular disease, and cancer, which are the main causes of morbidity and mortality in patients with COPD.

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Disclosures
None.

References


### 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.

COPD is increasingly prevalent in industrialized countries and is among the most common causes of PH, a condition that has a poor prognosis. COPD is also an age-related disease associated with telomere shortening in circulating leukocytes and alveolar cells, which causes cell senescence. In 124 patients investigated by right heart catheterization, we found that telomere shortening in PA-SMCs was associated with PH severity. Investigations of lung vessels and derived cultured PA-SMCs from patients with COPD and age-matched and sex-matched control subjects who smoke showed increased senescent cell counts in remodeled COPD vessels. Accelerated senescence was in proportion to remodeling severity in derived cultured PA-SMCs. Senescent PA-SMCs were located near actively dividing cells at sites of vessel wall hypertrophy. The senescent cells 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 pathophysiologic pathway might help to identify new biomarkers of disease severity and prognosis. It may open new therapeutic possibilities targeting cell senescence and, potentially, its effects, including PH, which is the main causes of morbidity and mortality in COPD.
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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·min\(^{-1}\)·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 \(\mu\)m 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\(_2\)O\(_2\) 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\(_2\)O\(_2\) 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.

**Culture of pulmonary artery smooth muscle cells**

PA-SMCs were cultured from explants as previously described. 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-cm² flasks, and cultured to confluence. The cells were then counted and seeded (passage 2) in 75-cm² 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 MgCl₂, and 40 mmol/L citrate [titrated to pH 6.0 with NaH₂PO₄]).

**Protein extraction and immunoblotting**

For protein extraction, isolated PA-SMCs were washed with PBS and lysed 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 phenylmethylsulfonfyl 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. 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^{-ΔΔCt}). 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'-CGTGGGTTTGAGTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGG TTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGG TTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGT

Extracellular matrix and soluble factors
After identification, presenescent (4·10^4) and senescent (6·10^4) 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. PA-SMCs were subjected to growth arrest in medium containing no FCS for 48 or 72 h then resuspended at 30·10⁶ 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% CO₂ 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: 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: 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. 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


