Accelerated epithelial cell senescence in IPF and the inhibitory role of SIRT6 in TGF-β-induced senescence of human bronchial epithelial cells

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Minagawa S, Araya J, Numata T, Nojiri S, Hara H, Yumino Y, Kawaiishi M, Odaka M, Morikawa T, Nishimura SL, Nakayama K, Kuwano K. Accelerated epithelial cell senescence in IPF and the inhibitory role of SIRT6 in TGF-β-induced senescence of human bronchial epithelial cells. Am J Physiol Lung Cell Mol Physiol 300: L391–L401, 2011. First published December 17, 2010; doi:10.1152/ajplung.00097.2010.—Reepithelialization of remodeled air spaces with bronchial epithelial cells is a prominent pathological finding in idiopathic pulmonary fibrosis (IPF) and is implicated in IPF pathogenesis. Recent studies suggest that epithelial senescence is a risk factor for development of IPF, indicating such reepithelialization may be influenced by the acceleration of cellular senescence. Among the sirtuins (SIRT) family, SIRT6, a class III histone deacetylase, has been demonstrated to antagonize senescence. We evaluated the senescence of bronchiolization in association with SIRT6 expression in IPF lung. Senescence-associated β-galactosidase staining and immunohistochemical detection of p21 were performed to evaluate cellular senescence. As a model for transforming growth factor (TGF)-β-induced senescence of abnormal reepithelialization, we used primary human bronchial epithelial cells (HBEC). The changes of SIRT6, p21, and interleukin (IL)-1β expression levels in HBEC, as well as type I collagen expression levels in fibroblasts, were evaluated. In IPF lung samples, an increase in markers of senescence and SIRT6 expression was found in the bronchial epithelial cells lining cystically remodeled air spaces. We found that TGF-β-induced senescence in primary HBEC by increasing p21 expression, and, whereas TGF-β also induced SIRT6, it was not sufficient to inhibit cellular senescence. However, overexpression of SIRT6 efficiently inhibited TGF-β-induced senescence via proteasomal degradation of p21. TGF-β-induced senescent HBEC secreted increased amounts of IL-1β, which was sufficient to induce myofibroblast differentiation in fibroblasts. These findings suggest that accelerated epithelial senescence plays a role in IPF pathogenesis through perpetuating abnormal epithelial-mesenchymal interactions, which can be antagonized by SIRT6.

idiopathic pulmonary fibrosis; cell senescence; SIRT6

IDIOPATHIC PULMONARY FIBROSIS (IPF) is characterized pathologically by irregular scars composed of dense collagen fibrosis, alternating with areas of fibroblastic proliferation, and cystically remodeled air spaces lined by metaplastic bronchiolar epithelium (2, 3). IPF occurs in older individuals, and increased cellular senescence is a major feature of advanced age, raising the possibility that cellular senescence may contribute to the pathogenesis of IPF. Indeed, mutations in human telomerase reverse transcriptase (hTERT) and human telomerase, which determine replicative cell senescence by regulating telomere length, are present in 8–15% of familial IPF cases (30). Furthermore, recent reports demonstrate that telomere shortening in alveolar epithelium is a risk factor for the development of IPF (1).

Abnormal bronchial epithelial cell proliferation, covering remodeled alveolar space following alveolar epithelial cell apoptosis, is postulated to occur as part of the pathogenic sequence in IPF (11). Reactive oxygen species and transforming growth factor (TGF)-β are major proapoptotic stimuli in the alveolar space of IPF and are also known to induce cellular senescence (8, 14, 17), which is phenotypically characterized by resistance to apoptosis (26, 33), indicating that cell senescence might be a representative adaptive response of lung epithelial cells to proapoptotic microenvironments. Although the pathological nature of phenotypic alteration remains to be determined, enhanced proinflammatory and profibrotic cytokine production, including interleukin (IL)-1β, have been postulated to be negative aspects of cellular senescence (7). Our laboratory has recently reported an important role for squamous metaplasia induced by intrinsic TGF-β activation in small airway remodeling via release of IL-1β as a paracrine regulator in chronic obstructive pulmonary disease (COPD) (4). Furthermore, increased concentration of IL-1β in bronchoalveolar lavage fluids of patients has been implicated in IPF pathogenesis in association with IL-17A (35). These findings suggest that induction of epithelial cell senescence, following reepithelialization with bronchial epithelial cells, may account for a part of abnormal epithelial-mesenchymal interactions with uncontrolled cytokine secretion of IL-1β.

TGF-β, a major multifunctional cytokine, is known as a mediator implicated in IPF pathogenesis (29). TGF-β is known to delay wound healing via inhibition of epithelial proliferation and migration, induce apoptosis, and expand the mesenchymal compartment via enhancement of fibroblast recruitment, fibroblast contractility, and extracellular matrix deposition (28). TGF-β has also been shown to induce both replicative and premature senescence in epithelial cells and to suppress hTERT expression via a smad3-mediated signaling pathway (17, 22).

Growing evidence reveals that sirtuins, NAD-dependent protein deacetylases, play a regulatory role in the process of cellular senescence via deacetylation or ADP-ribosylation of
transcription factors and intracellular proteins (15). Within the seven mammalian sirtuins, SIRT1–7, SIRT1 and SIRT6 are associated with physiological senescence in mammals, demonstrated by the premature aging-like phenotype of mice deficient in SIRT1 and SIRT6 (10, 24). SIRT1 expression levels have been shown to be reduced in macrophages and lung tissues in patients with COPD, and this reduction was associated with increased inflammatory cytokine production mediated by NF-κB (27). SIRT6-deficient mice demonstrate genomic instability caused by a deficiency of base excision repair activity (24), which is responsible for repair of single-strand DNA breaks. Accumulation of DNA damage likely plays a crucial role in cellular senescence (26). SIRT6-mediated deacetylation in NF-κB-driven gene expression has also been implicated in determination of lifespan (18). Taken together, these data suggest that sirtuins may be involved not only in cellular senescence, but also in a senescence-associated secretion phenotype as part of the pathogenic sequence in IPF.

In the present study, we demonstrate increased senescence of lung epithelial cells in IPF lung tissue, despite enhanced SIRT6 expression. In vitro models using cultured primary human bronchial epithelial cells (HBEC), TGF-β induces cellular senescence and increases expression of SIRT6. Endogenous SIRT6, however, is only modestly antagonizing to senescence. Overexpression of SIRT6 efficiently inhibits TGF-β-induced cell senescence, while knock down with short interfering RNA (siRNA) further increases efficiency of TGF-β-induced senescence. Negative regulation of TGF-β-induced cellular senescence by SIRT6 is mainly mediated by posttranslational proteasomal degradation of p21/waf-1. Secretion of the profibrotic mediator IL-1β by senescent epithelial cells induces myofibroblast phenotypic differentiation in lung fibroblasts. Finally, overexpression of wild-type SIRT6 in HBEC suppresses IL-1β production, suggesting a potential role for SIRT6 in inhibition of cellular senescence and fibrosis.

MATERIALS AND METHODS

Cell culture, antibodies, and reagents. Normal airways were collected from first through fourth-order bronchi from pneumonectomy and lobectomy specimens from resections performed for primary lung cancer. Informed consent was obtained from all surgical participants as part of an approved ongoing research protocol by the ethical committee of Jikei University School of Medicine. HBEC were isolated with protease treatment, and freshly isolated HBEC were plated onto rat-tail collagen type I-coated (10 μg/ml) dishes, incubated overnight, and then the medium was changed to bronchial epithelial growth medium (Clonetics, San Diego, CA). Lung fibroblasts were cultured from lung tissues by the explant technique. Briefly, fibroblasts outgrown from lung fragments were cultured in fibroblast growth media (DMEM with 10% FCS and penicillin-streptomycin). Confluent cells were passaged by trypsin treatment (0.025%) and used for the experiments. Cultures were characterized immunohistochemically using anti-prolifer-4-hydroxylase (DAKO, Tokyo, Japan), anti-vein-mitin (Sigma-Aldrich), and anti-cytokeratin antibodies (Lu-5, BioCare Medical, Concord, CA), as previously described (4). HBEC showed >95% positive staining with anti-cytokeratin and <5% positive staining with the anti-vein-mitin antibody. Lung fibroblasts demonstrated >95% positive staining with anti-prolifer-4-hydroxylase and anti-vein-mitin antibodies, and <5% positive staining with the anti-cytokeratin antibody (data not shown).

Antibodies used were rabbit anti-SIRT6 (Cell Signaling Technology, Beverly, MA), mouse anti-p21 (Cell Signaling Technology), rabbit anti-p53 (Cell Signaling Technology), goat anti-type I collagen (Southern Biotech), and alexa flour 546 anti-rabbit IgG (Invitrogen). Recombinant active TGF-β1 (R&D Systems, Minneapolis, MN), IL-1β receptor antagonist (Affinity BioReagents, Golden, CO), rat tail type I collagen (Sigma-Aldrich, Tokyo, Japan), and N-carbobenzyloxyl-l-leucinyl-l-leucinyl-l-norleucinal (MG132) (Cosmo Bio, Tokyo, Japan) were purchased.

Senescence-associated β-galactosidase staining. Senescence-associated β-galactosidase (SA-β-gal) staining was performed using HBECS (2 × 10^5) grown on 12-well culture plates and frozen lung sections of normal, IPF, or COPD cases on glass slides, according to the manufacturer’s instructions (β-galactosidase staining kit, BioVision Research Products).

Immunofluorescence staining, immunohistochemistry, and flow cytomtery. Immunofluorescence staining and immunohistochemical staining were performed as previously described (6). The primary antibodies were applied according to the manufacturer’s instructions. Percentages of positive staining of p21 were evaluated by counting of total and positive epithelial cell numbers in five representative lung fields of normal, mild-to-moderate fibrosis, fibroblastic foci, and dense fibrosis, respectively, at a magnification of ×200 on immunohistochemistry slides in each case (n = 5). Cell cycle analysis with flow cytometry was performed as previously described (4). Briefly, treated cells were harvested and immediately immobilized by 70% ice-cold ethanol overnight. Then the cells were incubated with 100 μg/ml of RNase and 50 μg/ml of propidium iodide in PBS-Triton X-100 (0.05%) for 40 min at 37°C. The quantity of cells in each cell cycle phase was measured on a FACScan using the FL-2 channel (Becton Dickinson).

Western blotting. HBEC grown on six-well culture plates were treated with TGF-β for the indicated time points and lysed in RIPA buffer (Thermo Fisher Scientific) with protease inhibitor cocktail (Roche Diagnostics) and 1 mM sodium orthovanadate. Western blotting was performed as previously described with minor modification (5, 6). After transfer to polyvinylidene difluoride membrane (Immobilon-P, Millipore, MA), blotting with specific primary antibodies are performed overnight at 4°C. Proteins were detected by horseradish peroxidase-conjugated secondary antibody (Bethyl Laboratories), followed by chemiluminescence detection (ECL; GE Healthcare) with a LAS-4000 UVmini system (Fujifilm).

Plasmids, siRNA, and transfection. The SIRT6 expression vector was the kind gift of Kartin F. Chua, Stanford University. The SIRT6, p21, and negative control siRNAs were purchased (Applied Biosystems, CA), and transfections of HBEC were performed with the Amaxa Nucleofector (Lonza, Switzerland), using matched optimized transfection kits for airway epithelial cells.

RNA isolation, polymerase chain reaction. RNA isolation, reverse transcription, and polymerase chain reaction were performed as previously described (4, 5). The primers used were SIRT6 sense primer, 5'-CCCCGATCAACGGGTCTTCTAC-3'; SIRT6 antisense primer, 5'-GCC-TTACCCCTTTTGGGGG-3'; p21 sense primer, 5'-CCGTGACTCATGCTTGTGACCT-3'; p21 antisense primer, 5'-GCCGTTTTGAGTGTTAGAATCT-3'; IL-1β sense primer, 5'-CCTGTTGCCTGGGCTCAA-3'; IL-1β antisense primer, 5'-GGGTTGCTGATGACCCCTTGGG-3' (20); type 1 collagen sense primer, 5'-CAGGGGCTTTTCCACCATCAT-3'; type 1 collagen antisense primer, 5'-GCCATTTTCTACGGG-TACTTC-3'; β-actin sense primer, 5'-TGACGCGGTACCAACCCACTGTTGCC-3'; β-actin antisense primer, 5'-CTAGAACGAGTGGCTGGTG-GACGATGGGAGGG-3'. These primer sets yielded PCR products of 136, 130, 204, and 662 bp for SIRT6, p21, IL-1β, and β-actin, respectively. Aliquots of the PCR products were subjected to agarose gel electrophoresis in Tris-acetate-EDTA buffer and visualized by ethidium bromide
staining. Primer sequences for SIRT6, p21, and type I collagen were from Primer Bank (http://pga.mgh.harvard.edu/primerbank).

IL-1β enzyme-linked immunosorbent assay. Airway epithelial cells were cultured in collagen-coated tissue culture plates for 24 h in the presence of recombinant TGF-β1 (2 ng/ml), washed two times with PBS, then incubated in serum-free DMEM for 48 h. IL-1β was measured in conditioned media with an IL-1β Quantikine ELISA kit (R&D Systems).

Statistics. Data are shown as the average (±SE) taken from at least three independent experiments. Student’s t-test was used for comparison of two data sets, analysis of variance for multiple data sets. Tukey’s or Dunn’s test were used for parametric and nonparametric data, respec-

Table 1. Assessment of senescence-associated β-galactosidase staining in lung epithelial cells in normal, IPF, and COPD

<table>
<thead>
<tr>
<th>Normal</th>
<th>Patient, no.</th>
<th>Age, yr/Sex</th>
<th>SA-β-gal</th>
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<td>N 1</td>
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<td>Patient, no.</td>
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<td>76/F</td>
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N, normal; IPF, idiopathic pulmonary fibrosis; COPD, chronic obstructive pulmonary disease; F, female; M, male. Note: senescence-associated β-galactosidase (SA-β-gal) staining of frozen lung sections: −, no staining; +, mild-to-moderate staining; ++, diffuse staining.

Fig. 1. Senescence-associated β-galactosidase (SA-β-gal) staining in lung tissues. A: normal lung (n = 6; case 1). B: chronic obstructive pulmonary disease (COPD; n = 3). Shown is COPD in Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage III (case 1). C: idiopathic pulmonary fibrosis (IPF) lung (n = 6). Shown is IPF (case 1) with arrowhead indicating cuboidal metaplasia. D: SA-β-gal-positive staining of epithelial cells covering fibroblastic focus (FF). Black arrowhead indicates FF, and red arrowhead indicates bronchial epithelial cells. E and F: IPF lung with nuclear staining (case 2). Arrowhead indicates bronchial epithelial cells.
Epithelial cell senescence and increased SIRT6 expression in IPF lung. Cell senescence was measured using SA-β-gal staining in lung epithelial cells from IPF, normal, and COPD patients. SA-β-gal staining was positive in all IPF cases (n = 6, mean age = 64.8 yr), was not present in normal (n = 6, mean age = 62.7 yr) and was barely detected in COPD (n = 6, mean age = 66.3 yr) [Fig. 1; Supplemental Fig. S1 (The online version of this article contains supplemental data); Table 1]. SA-β-gal staining was present in both bronchial epithelial cells (Fig. 1D, red arrowhead, and Fig. 1F, arrowhead) and cuboidal metaplasia lining cystically dilated air spaces (Fig. 1C, arrowhead). SA-β-gal-positive cells were also detected on the surface of fibroblastic foci (Fig. 1D, arrowhead). To further confirm senescence in IPF epithelial cells, we also performed immunohistochemical staining for p21/waf-1, a senescence-associated cyclin-dependent kinase inhibitor. Consistent with SA-β-gal staining, positive staining of p21/waf-1 was not present in normal cases (n = 5, data not shown). However, in IPF cases, semiquantitative assessment showed increased positive staining of p21/waf-1 on epithelial cells covering mild-to-moderate fibrosis and fibroblastic foci (mean = 17.1 and 17.2%) (Fig. 2, B–D), but only slight staining on areas of normal tissue or dense fibrosis (mean = 1.3 and 3.0%) (Fig. 2, A and E), indicating accelerated cellular senescence of epithelial cells lining the alveolar space, especially in the area of ongoing fibrosis in IPF lung (Fig. 2F). Consistent with cell senescence, immunofluorescence staining demonstrated increased expression of SIRT6 in epithelial cells lining the cystically dilated air spaces in IPF (Fig. 3E), but not in normal lung (Fig. 3D). Furthermore, double immunofluorescence staining for SIRT6 and p21 demonstrated...
clear colocalization in epithelial cells in IPF (Fig. 3J), indicating a possible involvement of SIRT6 in the cell senescence process of IPF.

**TGF-β induces cellular senescence in a p21-dependent manner in HBECs.** To assess the effect of TGF-β on cellular senescence, we used primary HBECs. TGF-β1 increased the percentage of SA-β-gal staining cells and concomitantly induced the expression of p21/waf-1, as assessed by RT-PCR and Western blotting (Fig. 4, A, C, and D). However, p53, an upstream regulator of p21, was not induced, suggesting that increased mRNA and protein expression of p21 by TGF-β1 was p53 independent. Cell cycle analysis also showed an increase of senescent cells, as reflected by an accumulation of cells in the G0/G1 phase (mean ± SD = 44.5 ± 4.8% in control; mean ± SD = 54.6 ± 4.8% in TGF-β1-treated cells). Furthermore, the accumulation of cells in the G0/G1 phase was slightly increased after an additional 48-h incubation without TGF-β (mean ± SD = 55.2 ± 5.7%) (Fig. 4B), suggesting irreversible cell cycle arrest after TGF-β treatment. p21/waf-1 expression was necessary to induce cellular senescence by TGF-β1, since p21/waf-1 knockdown with siRNA efficiently inhibited TGF-β1-induced cell senescence in HBEC (Fig. 4, E and F). Collectively, these results suggest that TGF-β irreversibly induces cellular senescence in HBEC.

**SIRT6 regulates TGF-β-induced cellular senescence in HBEC.** To investigate the mechanism and functional consequence of increased expression of SIRT6 in senescent epithelial cells in IPF, we examined the effect of TGF-β1 on SIRT6 expression. TGF-β1 increased SIRT6 expression at the mRNA and protein level in a dose-dependent manner (Fig. 5, A and B). Despite the upregulation of SIRT6 expression, TGF-β1 still induced cell senescence in HBEC, which was further confirmed by clear colocalization of SIRT6 and p21 (Supplemental Fig. S2), raising questions regarding the regulatory role of SIRT6 in TGF-β-induced cell senescence. To address this, we employed an expression vector and siRNA for SIRT6. Wild-type SIRT6 vector efficiently increased the amount of SIRT6 expression levels, and siRNA clearly reduced SIRT6 expression, as assessed by RT-PCR and Western blotting (Fig. 5C). Overexpression of SIRT6 significantly suppressed the percentage of SA-β-gal staining cells after TGF-β1 treatment. In contrast, SIRT6 siRNA dramatically increased the percentage of SA-β-gal staining cells, indicating that TGF-β1-induced intrinsic SIRT6 upregulation alone is not sufficient to completely inhibit senescence, but SIRT6 has the ability to antagonize TGF-β-induced cellular senescence in HBEC (Fig. 5D).

We analyzed the effect of SIRT6 on p21 expression levels in the presence or absence of TGF-β1, to further investigate the mechanism of SIRT6 mediated antisenescence effect in HBEC. Wild-type SIRT6 overexpression dramatically suppressed p21/waf-1 expression at the protein level (Fig. 6, B and C); however, no significant suppression at the mRNA level was observed (Fig. 6A). The proteasome pathway is known to regulate p21/waf-1 expression levels by posttranslational degradation. MG132, a proteasome inhibitor, efficiently inhibits the SIRT6 overexpression-mediated suppression of p21/waf-1 levels, indicating a mechanism of posttranslational modification and degradation of p21/waf-1 induced by SIRT6 during TGF-β-induced cellular senescence. MG132 demonstrated no apparent cell toxicity by Trypan blue dye exclusion study, with >95% viability (data not shown). These results suggest that SIRT6 antagonizes TGF-β-induced cell senescence by suppressing p21/waf-1 expression via posttranslational modification for proteasomal degradation.

**TGF-β-induced senescent HBEC induces myofibroblast differentiation via IL-1β expression.** As a first step in determining whether accelerated epithelial senescence is involved in pulmonary fibrosis, we investigated the secretion of IL-1β, a...
known profibrotic mediator secreted by airway epithelial cells. IL-1β is a paracrine factor that leads to myofibroblast differentiation of fibroblasts adjacent to airway epithelial cells (4).

RT-PCR showed increased production of IL-1β/H9252 expression in TGF-β/H9252-induced senescent HBEC, whereas overexpression of SIRT6 suppressed not only TGF-β/H9252-induced cell senescence, but also IL-1β/H9252 mRNA expression (Fig. 7A). Consistent with the data from mRNA, IL-1β secretion was suppressed by SIRT6 overexpression, as confirmed by ELISA (Fig. 7B). Conditioned medium from TGF-β/H9252-induced senescent HBEC efficiently induced myofibroblast differentiation, as determined by the increased expression levels of type I collagen (Fig. 7, C and D). Senescent HBEC-derived IL-1β was responsible for myofibroblast differentiation, because addition of IL-1 receptor antagonist to conditioned medium efficiently inhibited type I collagen expression in fibroblasts (Fig. 7, C and D). Taken together, these data indicate that SIRT6-mediated inhibition of IL-1β expression by senescent HBECs might contribute to myofibroblast differentiation and abnormal epithelial-mesenchymal interactions as a paracrine regulator.

Fig. 4. Transforming growth factor (TGF)-β induces cellular senescence via p21 expression in human bronchial epithelial cells (HBEC). A, top: photograph of SA-β-gal staining of control (left) or TGF-β-treated (2 ng/ml for 48 h; right) HBECs (original magnification, ×100). Bottom: percentage (±SE) of SA-β-gal-positive cells from 3 independent experiments. Open bar is no treatment, solid bar is TGF-β (2 ng/ml for 48 h), and striped bar is TGF-β (6 ng/ml for 48 h). *P < 0.05. B: percentage of G1/G0 phase cells by FACS analysis. Open bar is no treatment, solid bar is TGF-β (2 ng/ml for 48 h), and striped bar is TGF-β for 96 h (2 ng/ml for 48 h and additional 48-h incubation without TGF-β). C: p21 expression after TGF-β treatment. Top: RT-PCR using primers to p21 (top blot) and β-actin (bottom blot) was performed from total RNA harvested from control (lane 1) and TGF-β-treated HBEC (2 and 6 ng/ml for 24 h; lanes 2 and 3). Bottom: average (±SE) taken from 3 independent experiments shown as relative expression of p21 compared with β-actin. D: Western blot (WB) using anti-p21, p53, or β-actin for indicated time points of TGF-β treatment. Positive control for p53 is cell lysate of Beas2B cells. Shown is a representative experiment of 3 showing similar results. E: effect of p21 knockdown on TGF-β-induced senescence. Top blot: RT-PCR using primers to p21 and β-actin performed from total RNA harvested from nonsilencing control short interfering RNA (siRNA; lane 1), and p21 siRNA-transfected (lane 2) HBEC in the presence of TGF-β (2 ng/ml) for 24 h. Bottom blot: WB using anti-p21 and β-actin from similarly treated HBEC. Shown are representative data from 3 different experiments with the same results. F: percentage (±SE) of SA-β-gal-positive cells from 3 independent experiments. Open bar is nonsilencing control siRNA transfected without TGF-β, solid bar is nonsilencing control siRNA transfected with TGF-β (2 ng/ml for 48 h), and striped bar is p21 siRNA transfected with TGF-β (2 ng/ml for 48 h). *P < 0.05.

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DISCUSSION

Cell senescence has been implicated in several human diseases through deregulation of proinflammatory cytokines and growth factors and is characterized as senescence-associated secretion phenotype (7, 36, 38). To our knowledge, this is the first report that clearly shows extensive senescence in epithelial cells covering remodeling and remodeled alveolar surface of IPF lungs by means of SA-β-gal staining. Our in vitro data also suggest the possible involvement of SIRT6 in HBEC senescence, specifically that induced by TGF-β. It is very intriguing that, compared with COPD lung (where cellular senescence has also been demonstrated and implicated in its pathogenesis in terms of impaired cell repopulation), IPF lung shows dramatically increased cellular senescence, suggesting that the degree of epithelial senescence is involved in the progression of fibrosis (31, 32). Despite wide use as a representative biomarker for cell senescence, there is a limitation for interpreting SA-β-gal staining, which reflects the lysosomal β-D-galactosidase activity and is not required for senescence progression (21). Indeed, distinguishing senescent cells from quiescent or terminally differentiated cells by SA-β-gal staining remains controversial. However, we think concomitantly increased p21 expression in tissue staining, and p21 expression and an irreversible accumulation of cells in the G0/G1 phase in an in vitro model using TGF-β stimulation, further support the cell senescence in our system. Furthermore, the distribution of positive immunostaining of p21, mainly in epithelial cells covering progressive fibrotic lesions, suggests the active involvement of epithelial cell senescence in the pathological sequence in IPF. To better understand the pathological nature of cellular senescence in IPF, we noted that, following alveolar epithelial apoptosis, there is aberrant reepithelialization by bronchial epithelial cells with a tendency of resistance to proapoptotic stimulus, giving rise to pathological activation of epithelial-mesenchymal interactions. Indeed, compared with distal lung epithelial cells, proximal cells have been shown to be relatively resistant to Fas-induced apoptosis, which is important in the pathogenesis of Fas ligand-mediated lung injury (25). Furthermore, it has been reported that the antiapoptotic protein c-FLIP was expressed by alveolar epithelial cells only in areas of injury and fibrosis in lung tissues from IPF cases, but not from control cases (9). Taken together, it is reasonable.

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to postulate that epithelial cells covering the alveolar space of IPF lungs with progressive fibrosis are abnormally resistant to apoptosis. This resistance to apoptosis may change the response of epithelial cells to proapoptotic stimuli, such as reactive oxygen species and TGF-β, such that metaplasia and senescence could result. Supporting this notion, TGF-β clearly induced cellular senescence in our primary HBEC, and p21 upregulation was responsible for TGF-β-dependent HBEC senescence, as determined by p21 specific knockdown with siRNA (Fig. 4F). It is not entirely unexpected that TGF-β induced both p21 expression and cellular senescence in a p53-independent manner (which is a representative upstream transcriptional regulator for p21), because it has been reported that the proximal promoter region of p21 contains a TGF-β response element (12).

The elucidation of the molecular mechanisms of cell senescence may provide clues for understanding the pathogenesis of lung diseases associated with aging. Among the SIRT family, SIRT6 comparable with SIRT1 is one of the most plausible candidates as the regulator in both stress-induced premature and replicative cellular senescence, because of its ability to enhance base excision repair and functional regulation of telomeres by its catalytic activity (23, 24). Although SIRT1 has been implicated in the pathogenesis of lung diseases, including COPD, and is also an important candidate for the investigation in IPF pathogenesis, our preliminary experiments demonstrated that TGF-β-induced SIRT6 but not influenced SIRT1 expression in HBEC. In our findings, IPF lung showed increased expression of SIRT6 in cystically remodeled air space surface-covering cells, consistent with senescent epithelial cells shown by colocalization with p21 (Fig. 3J). Although the precise functional properties of SIRT6 expressed in epithelial cells in IPF lung in terms of anti-senescent activity need to be determined in future studies, we speculate that increased expression of SIRT6 might be an insufficient compensatory mechanism against stress-induced cellular senescence by a proapoptotic stimulus, such as TGF-β, resulting in accelerated cellular senescence in IPF. Indeed, in our in vitro model, TGF-β-induced SIRT6 expression, concomitant with cellular senescence in HBEC and overexpression of SIRT6, efficiently inhibited TGF-β-induced senescence of HBEC, which was mediated by the enhanced degradation of p21 via the proteasomal pathway. p21 has been shown to be a key regulator of cellular senescence and was also recently demonstrated in the regulation of inflammatory pathways in a cigarette-smoking model for COPD (37), suggesting a clinical relevance in manipulations of p21 levels by SIRT6 expression in terms of regulation of both cellular senescence and the inflammatory process. The ubiquitin-proteasome pathway is major intracellular proteolytic machinery and is responsible for diverse cell function and fate, including senescence (16). Although the detailed cellular mechanisms of association between SIRT6 and the ubiquitin-proteasome pathway need to be determined in future studies, it is interesting that SIRT6 regulates p21 expression levels by enhancing proteasomal degradation, suggesting the involvement of the ubiquitin-proteasome pathway in SIRT6-mediated inhibition of TGF-β-induced HBEC senescence in our in vitro model. However, it is not known whether SIRT6 prevent the cellular senescence specifically induced by TGF-β or p21 expression without specificity, which is under investigation. Although recent papers showed the clinical significance of shortening of telomere length in the pathogenesis of IPF, at least in our short-term in vitro experiment, telomerase expression levels were not involved in TGF-β-induced HBEC senescence, as hTERT overexpression had no inhibitory effect on senescence (data not shown).
TGF-β and IL-1β are major profibrotic and proinflammatory cytokines, respectively, and are strongly implicated in IPF pathogenesis. The concentrations of both TGF-β and IL-1β in the bronchoalveolar lavage fluid from IPF cases were significantly higher than those from control cases (14, 35), indicating a possible role for TGF-β and IL-1β in the regulation of lung epithelial cell fate and phenotypic modulation of IPF pathogenesis. TGF-β mediates diverse profibrotic events, including alveolar epithelial cell apoptosis, squamous metaplasia, and epithelial-mesenchymal transition as a source of myofibroblasts during fibrosis (4, 14, 19, 34). IL-1β has also been demonstrated to be an important profibrotic cytokine in a model of airway and lung fibrosis using intratracheal injection of human IL-1β adenovirus (20). Pathologically activated epithelial-mesenchymal interactions after repeated epithelial cell injury have been recently proposed to account for the ineffectiveness of the anti-inflammatory modality of treatment for IPF (13). We showed SA-β-gal and p21 positive cells covering the surface of fibroblastic foci, widely accepted as the leading edge of fibrosis, indicating the possible association between epithelial cells with senescence-associated secretion phenotype and fibroblast phenotypic modulation from the beginning of fibrosis development. In terms of the coordination between TGF-β and IL-1β, IL-1β might be one of the upstream regulators of TGF-β activation in fibroblasts. As our recent model elucidated, the reciprocal interactions of IL-1β secreted from epithelial cells and TGF-β induced from fibroblasts (4). Although physiological relevance in vivo is under investigation, in our in vitro model, we clearly show differentiation of fibroblasts into myofibroblasts via secretion of IL-1β from TGF-β-induced senescent HBEC. We believe that IL-1β secretion was mainly involved in senescent phenotypic change, but is not a direct effect of TGF-β signaling, because TGF-β slightly suppressed IL-1β mRNA after 24-h treatment (data not shown), and IL-1β expression was increased following 24-h incubation without TGF-β, concomitant with positive SA-β-
gal staining. Interestingly, SIRT6 overexpression suppressed both cellular senescence and IL-1β expression, indicating that SIRT6 suppressed not only the senescence process, but also the senescence-associated secretion phenotype of IL-1β. A recent study demonstrated that SIRT6-mediated deacetylation in NF-κB resulted in decreased gene expression important for determination of lifespan (18), raising the possibility that both senescence and IL-1β suppression by SIRT6 in our system might also be mediated by regulation of NF-κB activity.

In summary, we have elucidated the increased senescence and SIRT6 expression in epithelial cells of IPF lungs, including aberrant reepithelialization with bronchial epithelial cell proliferation, probably following normal alveolar epithelial cell apoptosis. Our in vitro study indicates the novel pathway of paracrine secretion of IL-1β leading to pulmonary fibrosis via TGF-β-mediated senescent epithelial cells. SIRT6, a regulator of TGF-β-induced cellular senescence of HBECs, seems to be an ambitious target molecule for understanding the pathogenesis of IPF.

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DISCLOSURES

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

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