Insufficient autophagy in idiopathic pulmonary fibrosis

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Advanced age is one of the most important risk factors for development of IPF (33a). Increased cellular senescence is a major feature of aging and hence is proposed to be a part of the pathogenic sequence of IPF. Recently, we found accelerated senescence of epithelial cells, including metaplastic cells, in active fibrosing lesions of IPF (25). Furthermore, interleukin (IL)-1β secretion by senescent human bronchial epithelial cells (HBEc) was sufficient to induce myofibroblast differentiation in lung fibroblasts, possibly playing a key role in fibrosis development (25). Collectively, it is plausible that accelerated senescence of epithelial cells plays a role in IPF pathogenesis through perpetuating abnormal epithelial-mesenchymal interactions (5, 25). However, the regulatory mechanisms of accelerated cellular senescence in IPF are incompletely understood.

ER stress response is known to induce autophagy, a lysosomal degradation pathway (3). Autophagy is implicated in elimination of damaged proteins and organelles as a central component of the integrated stress response and is a more selective process than originally understood (11, 21). p62 has been shown to be a adaptor protein capable of binding both polyubiquitinated substrates and microtubule-associated protein 1A/1B-light chain 3 (LC3), a component for autophagosome formation (18). Therefore, concomitant accumulation of p62 and ubiquitinated protein is thus now widely recognized as at least partly reflecting insufficient autophagy (18). Autophagy diminishes with aging, and accumulation of damaged proteins and organelles is one of the typical manifestations of cellular senescence (35), indicating the pivotal involvement of autophagic regulation of cellular senescence, especially in those epithelial cells with ER stress responses in IPF. Practically, we have recently reported the involvement of insufficient autophagy in the regulation of cigarette smoke extract (CSE)-induced HBEc senescence in association with chronic obstructive pulmonary disease pathogenesis (9). Defective autophagy has been proposed to contribute to excess production of extracellular matrix in fibroblasts, indicating the further involvement of autophagy in myofibroblast differentiation, a profibrotic phenotype of fibroblasts for fibrosis development (7).

In this context, we explored the regulation of cell senescence and myofibroblast differentiation by autophagy in our in vitro models and autophagy status in IPF lungs by means of immunohistochemical evaluation.

MATERIALS AND METHODS

Cell culture and reagents. Normal airways were collected from first- through fourth-order bronchi from pneumectomy and lobectomy specimens. HBEc were isolated with protease treatment and characterized as previously described (2). Freshly isolated HBEc...
were plated onto rat-tail collagen type I-coated (10 μg/ml) dishes and incubated overnight, and then the medium was changed to bronchial epithelial growth medium (Lanza, Tokyo, Japan). HBEC were serially passaged and used for experiments until passage 4. 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). Lung fibroblasts were serially passaged and used for experiments until passage 6. HBEC showed >95% positive staining with anti-cytokeratin (Lu-5; BioCare Medical, Concord, CA) and <5% positive staining with the anti-vimentin (Sigma-Aldrich, Tokyo, Japan) antibody (data not shown). Lung fibroblasts demonstrated >95% positive staining with anti-vimentin antibodies, and <5% positive staining with the anti-cytokeratin antibody (data not shown). Antibodies used were mouse anti-p21, rabbit anti-binding immunoglobulin protein (BIP), rabbit anti-eukaryotic initiation factor (eIF2), rabbit anti-phospho-eIF2, rabbit anti-phospho-histone H2A.X (Ser139) (Cell signaling Technology, Tokyo, Japan), goat anti-type I collagen (Southern Biotech, Birmingham, AL), mouse anti-α-smooth muscle actin (α-SMA, Sigma-Aldrich), mouse anti-poly and mono ubiquitin (Enzo Life Sciences, Tokyo, Japan), rabbit anti-p62, rabbit anti-ATG5 (MBL, Nagoya, Japan), and mouse anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). Pepstatin A (Peptide Institute, Osaka, Japan), E64d (Peptide Institute), bafilomycin A, recombinant TGF-β1 (R&D Systems, Minneapolis, MN), 3-methyladenine (3-MA) (Affinity BioReagents, Golden, CO), and tunicamycin (Sigma-Aldrich) were purchased. Torin1, a selective and potent small molecule inhibitor of a mammalian target of rapamycin (mTOR) as an inducer of autophagy, was kindly provided by Drs. Gray and Sabatini (Whitehead Institute, Cambridge, MA).

Western blotting. HBEC grown on six-well culture plates were lysed in RIPA buffer (Thermo Fisher Scientific, Waltham, MA) with protease inhibitor cocktail (Roche Diagnostics, Tokyo, Japan) and 1 mM sodium orthovanadate (Sigma Aldrich) or lysed with Laemmli sample buffer. Western blotting was performed as previously described with minor modification (2). After transfer to PVDF membrane (Immobilon-P, Millipore, Billerica, MA), blotting with specific primary antibodies was performed overnight at 4°C. Proteins were detected by horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology Japan, Tokyo, Japan), using matched optimized transfection kits; 0 (less than 10%), 1 (11–49%), and 2 (more than 50%). Patient characteristic features are presented in Table 1, and smoking status of Brinkman index was significantly different between control and IPF groups.

Statistics. 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, respectively. Significance was defined as P < 0.05. Statistical software used was Prism v.5 (GraphPad Software, San Diego, CA).

RESULTS

Autophagy suppresses ER stress responses and ER stress-induced cell senescence in human bronchial epithelial cells. The ER stress response is known to induce autophagy, which removes the expanded and disorganized ER to relieve cell stress (3). Thus autophagy status was determined after induction of ER stress. Abnormal proliferation of metaplastic epithelial cell, covering remedodeled alveolar space, has been demonstrated to be mainly derived from bronchiolar basal cells and Clara cells (12). And our recent finding showed that accelerated cellular senescence was mainly observed in HBEC for in vitro experiments. Tunicamycin (TM), an inducer of ER stress via disruption of protein glycosylation, increased ER stress responses as determined by increases of BIP and phospho-eIF2 expression levels (Fig. 1A). TM

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>Control (n = 5)</th>
<th>IPF (n = 5)</th>
<th>P Value</th>
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<tbody>
<tr>
<td>Age, yr</td>
<td>67.6 ± 6.6</td>
<td>69.2 ± 2.6</td>
</tr>
<tr>
<td>Male, % of group</td>
<td>60</td>
<td>80</td>
</tr>
<tr>
<td>Brinkman index</td>
<td>120 ± 216.8</td>
<td>829 ± 485.5</td>
</tr>
<tr>
<td>%VC</td>
<td>108.5 ± 17.3</td>
<td>87.6 ± 12.5</td>
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<tr>
<td>FEV1/FVC</td>
<td>76.9 ± 4.3</td>
<td>83.4 ± 8.6</td>
</tr>
<tr>
<td>PaO2</td>
<td>87.0 ± 7.9</td>
<td>84.8 ± 10.8</td>
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<tr>
<td>PaCO2</td>
<td>40.4 ± 1.0</td>
<td>38.8 ± 3.3</td>
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Values are mean ± SD. FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; IPF, idiopathic pulmonary fibrosis; NA, not assessed; NS, not statistically significant; PaO2, partial pressure of oxygen in arterial blood; PaCO2, partial pressure of carbon dioxide in arterial blood.
significantly increased the percentage of senescent HBEC as measured by an increase in staining for both SA-β-Gal and phospho-Histone H2A.X (Ser139), a marker of DNA damage, and in p21 by Western blotting (Fig. 1, A–C). Autophagy induction by Torin1, mTOR inhibitor, significantly suppressed TM-induced cell senescence accompanied by autophagy activation and a decrease in ER stress responses as determined by reduced BIP and phospho-eIF2 expression levels (Fig. 1, A–C). TM induced autophagy, and Torin1 further enhanced autophagy induction as determined by

Fig. 1. Autophagy induction suppresses endoplasmic reticulum (ER) stress-induced cell senescence in human bronchial epithelial cells (HBEC). A: Western blotting (WB) using anti-binding immunoglobulin protein (BIP), anti-phospho-eukaryotic initiation factor (eIF), anti-eIF, anti-LC3, anti-p21, and anti-β-actin of cell lysates from control (lanes 1 and 3) and tunicamycin (TM) (0.1 μg/ml) (lanes 2 and 4) treated in the absence or presence of Torin1 (250 nM) (left). For anti-LC3 WB, cells were treated in the presence of protease inhibitors (E64d 10 μg/ml and pepstatin A10 μg/ml). Cell lysates were collected after 24-h treatment. Representative experiment of 3 showing similar results. Middle: average (± SE) of relative increase in BIP normalized to β-actin. Right: average (± SE) of relative increase in P-eIF2 normalized to eIF2 taken from densitometric analysis of WB from 3 independent experiments. Open bar is no treatment, filled bar is TM treatment. *P < 0.05. B: photographs of senescence-associated β-galactosidase (SA-β-Gal) staining of HBEC treated with control or TM (0.1 μg/ml) in the absence or presence of Torin1 (250 nM) (left). Right: percentage (± SE) of SA-β-Gal-positive cells from 3 independent experiments. Original magnification is ×100. C: photographs of immunofluorescent staining of phospho-Histone H2A.X (Ser139) in HBEC treated with control or TM (0.1 μg/ml) in the absence or presence of Torin1 (250 nM) (left) (Bar = 20 μm). Right: percentage (± SE) of phospho-Histone H2A.X (Ser139)-positive cells from 3 independent experiments.
increased conversion of LC3 from LC3-I to LC3-II (Figs. 1A and 2A). HBEC were transfected with siRNA to LC3B and ATG5, which inhibits autophagosome formation. Western blotting of HBEC showed that LC3B and ATG5 siRNA efficiently reduced protein levels of LC3 and ATG5, respectively (Fig. 2, A and C). Knockdown of LC3B resulted in increased accumulation of p62, reflecting insufficient autophagy, and slight enhancement of ER stress responses determined by BIP and phospho-eIF2 expression levels (Fig. 2A). Autophagy inhibition by knockdown of LC3B and ATG5 significantly enhanced TM-induced cell senescence as measured by SA-β-Gal-positive staining HBEC (Fig. 2, B and D). These data indicate that induction of autophagy suppresses ER stress and senescence in HBEC.

Fig. 2. Autophagy inhibition enhances ER stress-induced cell senescence in HBEC. A: WB using anti-BIP, anti-phospho-eIF2, anti-eIF2, anti-LC3, anti-p62, anti-p21, and anti-β-actin of cell lysates from the control siRNA (lanes 1 and 2) and LC3B siRNA (lanes 3 and 4)-transfected HBEC. After 24-h incubation, transfected HBEC were treated with TM (0.1 μg/ml). For anti-LC3 WB, cells were treated in the presence of protease inhibitors (E64d 10 μg/ml and pepstatin A10 μg/ml). Protein samples were collected after 16-h incubation in the presence (lanes 2 and 4) or absence of TM (lanes 1 and 3). Middle: average (± SE) of relative increase in BIP normalized to β-actin; right: average (± SE) of relative increase in P-eIF2 normalized to eIF2, which are taken from densitometric analysis of WB from 3 independent experiments. Open bar is no treatment, filled bar is TM treatment. *P < 0.05. B: photographs of SA-β-Gal staining of control or LC3B siRNA-transfected HBEC. After 24-h incubation, transfected HBEC were treated with TM (0.1 μg/ml) for 24 h. (Bar = 20 μm) Right: percentage (± SE) of SA-β-Gal-positive cells from 3 independent experiments. C: WB using anti-ATG5 and anti-β-actin of cell lysates from the control siRNA (lane 1) and ATG5 siRNA (lane 2)-transfected HBEC. Protein samples were collected after 24-h incubation. D: photographs of SA-β-Gal staining of control or ATG5 siRNA-transfected HBEC. After 24-h incubation, transfected HBEC were treated with TM (0.1 μg/ml) for 24 h. Original magnification is ×100. Right: percentage (± SE) of SA-β-Gal-positive cells from 3 independent experiments.
Autophagy inhibition induces myofibroblast differentiation of lung fibroblasts. We next examined the role of autophagy in lung fibroblast differentiation (7). Lung fibroblasts were transfected with siRNA to LC3B and ATG5, respectively, to elucidate the involvement of autophagy in myofibroblast differentiation. Transforming growth factor (TGF)-β clearly induced autophagy as shown by increased LC3-II and decreased p62 levels (Fig. 3A). Knockdown of LC3B and ATG5 were confirmed by Western blotting, and autophagy inhibition was demonstrated by means of decreased LC3-II conversion and lack of decrease in p62 following TGF-β treatment (Fig. 3A).

Autophagy inhibition by knockdown of LC3B and ATG5 further enhanced their expression (Fig. 3B). On the other hand, knockdown of LC3B and ATG5 demonstrated no significant increase in senescent cells as measured by SA-β-Gal staining (Fig. 3C). Regardless of siRNA transfections, TGF-β significantly decreased the percentage of senescent fibroblasts (Fig. 3C). Conversely, Torin1 treatment suppressed α-SMA and type I collagen expression levels without significant changes in percentage of senescent cells, supporting the notion that autophagy negatively regulates myofibroblast differentiation but is not apparently involved in modulation of cellular senescence in fibroblasts (Fig. 3, D and E).

Autophagy-related protein expression in IPF lung. To evaluate the autophagy status in IPF lungs, we performed immunohistochemical staining for autophagy-related proteins. The conversion of LC3 from LC3-I (free form) to LC3-II (phosphatidylethanolamine-conjugated form) represents a key step in autophagosome formation (14), and membrane-bound LC3-II indicates autophagic vacuoles. Interestingly, we observed clear dot-like staining of LC3, which resembles autophagosomes, only in AECII cytoplasm in relatively normal areas of IPF lung without structural distortion (Fig. 4, D, E, and J), but not in AECI in normal lung (Fig. 4, A and B). The cell-type-specific LC3 staining in AECII in IPF lung was further confirmed by concomitant immunofluorescence detection of SP-C (Fig. 4K). In contrast, no obvious staining of LC3 was observed in any other regions, regardless of whether the fibrosing process was mild or severe. LC3 expression was not detected in cuboidal metaplastic cells (Fig. 4H). On the other hand, immunohistochemical staining of Beclin1, which is also an important component for autophagy, showed high cytoplasmic staining of a diffuse nature in all areas of normal lung (Fig. 5, A–C) and IPF lung (Fig. 5, D–I). However, the cytoplasm of AECII in normal regions in IPF lung tended to have relatively higher expression of Beclin1 than epithelial cells in other lesions (Fig. 5, E and J). Interestingly, AECII in normal lung also demonstrated relatively higher expression of Beclin1 (Fig. 5B).

p62 expression in normal and IPF lung tissues. In normal lung, only AECII expressed p62. No expression of p62 was observed in type I alveolar epithelial cells (AECI) or fibroblasts and was faintly detected in airway epithelial cells (Fig. 6, A–C). Consistent with normal lung, p62 was expressed only in AECII in relatively normal areas without structural distortion of IFP (Fig. 6, D, E, and M). Metaplastic epithelial cells, including cuboidal, squamous, and bronchiolar-type cells covering the airspace with mild to moderate structural distortion accompanied by subepithelial fibroblast proliferation, strongly expressed p62 (Fig. 6, F–J). In dense fibrotic lesions with severe structural remodeling, metaplastic epithelial cells also expressed p62 (Fig. 6K), whereas less prominent expression was observed in honeycomb change areas within a few subepithelial fibroblasts (Fig. 6L). p62 expression was also observed in subepithelial fibroblasts, which is obvious in PF, the known leading edge of fibrosis development (Fig. 6F).

Ubiquitin expression in normal and IPF lung tissues. Ubiquitinated proteins are destined for selective degradation not only by the proteasome but also by autophagy, and accumulation of ubiquitinated proteins is an age-related feature of senescent cells, especially in pathological conditions (6, 24). In an immunohistochemical evaluation of ubiquitin, no staining was observed in epithelial cells or fibroblasts in normal lungs (Fig. 7, A–C), whereas AECII in histologically normal areas without structural distortion in IPF lungs demonstrated obvious ubiquitin staining (Fig. 7, D and E), which is consistent with recent findings of increased ER stress responses in AECII in areas of normal architecture in IPF (20). In areas with mild to moderate fibrosis, there was a correlation of staining distribution between p62 and ubiquitin in both epithelial cells and fibroblasts (Figs. 6M and 7, F–K, and M). Both epithelial cells and fibroblasts slightly expressed ubiquitinated proteins in

Fig. 3. Myofibroblast differentiation by autophagy inhibition in lung fibroblasts. A: WB using anti-p62, anti-ATG5, anti-LC3, and anti-β-actin in nonsilencing control siRNA-transfected (lanes 1 and 2), ATG5 siRNA-transfected (lanes 3 and 4), and LC3B siRNA-transfected (lanes 5 and 6) fibroblasts. TGF-β treatment (2 ng/ml) was started 24 h post-siRNA transfection, and protein samples were collected after 24-h treatment. For anti-LC3 WB, cells were treated in the presence of protease inhibitors (50 μg/ml pepstatin A10 μg/ml). Representative experiment of 5 showing similar results. Middle: average (± SE) of relative increase in p62 normalized to β-actin; right: average (± SE) of relative increase in LC3-II normalized to β-actin, which are taken from densitometric analysis of WB from 5 independent experiments. Open bar is no treatment, filled bar is TGF-β treatment. *P < 0.05. B: WB using anti-type I collagen, anti-α smooth muscle actin (α-SMA), and anti-β-actin in nonsilencing control siRNA-transfected (lanes 1 and 2), ATG5 siRNA-transfected (lanes 3 and 4), and LC3B siRNA-transfected (lanes 5 and 6) fibroblasts. TGF-β treatment (2 ng/ml) was started 24 h post-siRNA transfection, and protein samples were collected after 48-h treatment. Cell lysates were for α-SMA, and conditioned medium was for type I collagen (left). Representative experiment of 3 showing similar results. Middle: average (± SE) of relative increase in α-SMA normalized to β-actin; right: average (± SE) of relative increase in type I collagen normalized to β-actin, which are taken from densitometric analysis of WB from 3 independent experiments. C: photographs of SA-β-Gal staining of nonsilencing control, ATG5, and LC3B siRNA-transfected fibroblasts. TGF-β treatment (2 ng/ml) was started 24 h post-siRNA transfection and treated for 48 h. Bar = 100 μm. Bottom: percentage (± SE) of SA-β-Gal-positive cells from 6 independent experiments. D: WB using anti-type I collagen, anti-α-SMA, and anti-β-actin in control-treated (lanes 1 and 2) and Torin1 (250 nM)-treated (lanes 3 and 4) fibroblasts in the presence or absence of TGF-β (2 ng/ml). Protein samples were collected after 48-h treatment. Cell lysates were for α-SMA, and conditioned medium was for type I collagen. Representative experiment of 4 showing similar results. Bottom: average (± SE) of relative increase in α-SMA normalized to β-actin in the presence or absence of Torin1 (250 nM) compared with control HBEC without Torin1 and TGF-β (2 ng/ml) treatment, which are taken from densitometric analysis of WB from 4 independent experiments. E: photographs of SA-β-Gal staining of control and Torin1 (250 nM)-treated fibroblasts for 8 h. Bar = 100 μm. Bottom: percentage (± SE) of SA-β-Gal-positive cells from 3 independent experiments. Open bar is no treatment, filled bar is Torin1 treatment.
AUTOPHAGY IN IPF

A

B

C

D

E

percentage of SA-β-gal positive cells

(%)
areas with honeycomb change (Fig. 7L). These data suggest that increased expression of p62 and ubiquitinated proteins in both epithelial cells and fibroblasts in fibrosing lesions reflects insufficient autophagy activity compared with AECII in relatively normal areas with increased cytoplasmic expression of LC3 and Beclin1 in IPF.

**p21 expression and SA-β-Gal staining in IPF lung.** In our recent study, expression of p21, a senescence-associated cyclin-dependent kinase inhibitor, and SA-β-Gal staining were prominent in fibrosing lesions in IPF lungs (25). Here we have performed further evaluation of p21 expression in IPF lung. p21 expression was not detected in normal lung or in relatively normal areas of IPF lung (Fig. 8, A and B). Nuclear staining of p21 was clearly demonstrated in only epithelial cells covering actively fibrosing lesions, including FF (Fig. 8, C–E). In contrast, p21 expression was barely detected in epithelial cells of honeycomb change areas (Fig. 8F). p21 has been shown to be a key regulator during the

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**Figure 4.** LC3 expressions in normal and idiopathic pulmonary fibrosis (IPF) lung tissues. Immunohistochemical staining of LC3 in normal and IPF lung tissues. Photomicrographs of lung parenchyma (A and B) and airway in normal lung (C), relatively normal area without structural remodeling (D and E), normal airway (NA) (F), fibroblastic focus (FF) (G), mild to moderate fibrosis (MMF) (H), and honeycomb (HC) change areas (I) in IPF. J: average of semiquantitative score (± SE) of LC3-positive cells in total cells from 5 cases. Bar = 200 μm, 100 μm, 50 μm, and 10 μm, respectively. DF, dense fibrosis; TII, type II cell. K: immunofluorescence staining of surfactant protein (SP)-C in IPF lung tissues. Immunofluorescence staining (bottom) was performed after immunohistochemical detection of LC3 (top). Bar = 10 μm.
progression of cellular senescence (17). To elucidate the involvement of p21 in insufficient autophagy-induced cellular senescence in epithelial cells, we employed p21 knock-down using siRNA. Knockdown of p21 using siRNA was efficient because reductions in expression were demonstrated using RT-PCR and Western blotting (Fig. 8H).

3-MA, an autophagy inhibitor, significantly increased the percentage of senescent cells as measured by SA-β-Gal-positive staining in HBEC. p21 siRNA-transfected HBEC demonstrated clear resistance to 3-MA-induced cellular senescence (Fig. 8H). Accelerated cellular senescence in metaplastic (cuboidal and squamous) epithelial cells in IPF was also demonstrated by increased SA-β-Gal staining (Fig. 8, I–K). Consistent with the results, in vitro inhibition of autophagy did not increase the percentage of senescent cell in lung fibroblasts, no obvious p21 and SA-β-Gal staining was observed in fibroblasts regardless of whether the fibrosis was mild or severe. Taken together, these data suggest a linkage between insufficient autophagy and accelerated cell senescence in metaplastic epithelial cells covering mild to moderate fibrosing lesions; conversely, in line with our in vitro data (Fig. 3), myofibroblast differentiation may be also attributed to insufficient autophagy in a cell-type-specific manner.

**DISCUSSION**

In this study, we explored the potential involvement of insufficient autophagy in driving accelerated epithelial cell senescence and myofibroblast differentiation in IPF. We find...
that insufficient autophagy may promote accelerated senescence and that p21 plays a role in the progression to cellular senescence in response to insufficient autophagy in airway epithelial cells (Figs. 1, 2, and 8). In contrast, autophagy inhibition induces myofibroblast differentiation and not senescence of lung fibroblasts (Fig. 3). Using human biospecimens, we demonstrate expression of markers of insufficient autophagy in metaplastic epithelial cells and lung fibroblasts in areas with developing fibrosis. Accelerated cellular senescence was demonstrated in metaplastic epithelial cells but not in fibroblasts within FF, indicating a cell-type-specific role of autophagy in controlling cellular phenotype and differentiation as a part of pathogenic sequence in IPF.

Autophagy has been proposed to control the quality of cellular components to prevent cell senescence (35). Our in vitro experiments clearly demonstrate the inhibition of airway epithelial senescence as a property of autophagy. Historically, inhibition of senescence has been difficult to demonstrate due to the lack of a widely accepted methodology to evaluate autophagic status in fixed tissue samples. Because of the dynamic nature of autophagy, it is difficult to distinguish between increased autophagy flux and impaired subsequent clearance of autophagosomes using the standard comparison of ATG expression levels (17, 26). Indeed, immunohistochemical evaluations of Beclin1 (Fig. 5) and ATG5 (data not shown) demonstrated diffuse cytoplasmic staining that did not allow for clear quantification of autophagic activity.
definitive determination of the autophagy status. Here, we have utilized the recent finding that insufficient autophagy is characterized by an increase of ubiquitinated proteins and p62 (19). Concomitantly increased expression of ubiquitinated proteins, p62, and p21 in our immunohistochemical evaluation indicate that autophagic elimination is not sufficient to prevent phenotypic alteration in metaplastic epithelial cells, resulting in accelerated senescence. Despite the slight increase of ubiquitinated proteins with concomitant expression of p62, no cellular senescence was detected in AECII in uninvolved areas of IPF lungs. The activated form of LC3 (LC3-II: phosphatidylethanolamine-conjugated form) is capable of binding to the

Fig. 7. Ubiquitin expression in normal and IPF lung tissues. Immunohistochemical staining of ubiquitin in normal and IPF lung tissues. Photomicrographs of lung parenchyma (A and B) and airway in normal lung (C), relatively normal area without structural remodeling (D and E), FF (F), mild to moderate fibrosis (G–J), dense fibrosis areas (K), and honeycomb change areas (L) in IPF. M: average of semiquantitative score (± SE) of p62-positive cells in total cells from 5 cases. Bar = 200 μm, 100 μm, 50 μm, and 10 μm, respectively. *P < 0.05.
This property can be exploited to detect autophagy activation experimentally by monitoring enhanced green fluorescent protein-LC3 dot formation (17, 26). Here, we found dot-like staining of LC3 in only AECII in uninvolved areas of IPF lungs, which reflects increased autophagosome formation (Fig. 4). We hypothesize that the consequence of this increased autophagosome formation is the elimination of damaged cellular components resulting in inhi-
tion of cellular senescence. Furthermore, in normal lungs in AECII, increased p62 expression without ubiquitinated protein accumulation may play diverse roles in the cellular pathways attributed to p62 such as nuclear factor (erythroid-derived 2)-like 2 and NF-κB (27).

Fibroblasts in FF also highly express both ubiquitinated proteins and p62 without evidence of cellular senescence, raising two different possibilities. First, autophagy status may not be involved in cellular senescence of fibroblasts. Indeed, we showed that autophagy inhibition by knockdown experiments did not induce cellular senescence but promoted myofibroblast differentiation, which was further supported by opposing effects of Torin1 (Fig. 3). It has been demonstrated that defective autophagy may contribute to excessive production of extracellular matrix by altering the turnover of proteins such as collagen in fibroblasts (7). Because autophagy is essential for cell survival, we speculate that incomplete knockdown of LC3B and ATG5 expression by our siRNA experiment may be important for maintenance of minimal baseline autophagy and also for myofibroblast differentiation. ATG5 knockdown clearly suppressed autophagy and enhanced myofibroblast differentiation of α-SMA and type I collagen expression induced by TGF-β treatment (Fig. 3), indicating that TGF-β-induced autophagy may negatively contribute to myofibroblast differentiation by this pathway, which is at least partly consistent with recent report (16). This suggests that inhibition of autophagy is a novel pathway of myofibroblast differentiation that interacts negatively with the TGF-β pathway. Therefore, autophagy induction is an interesting approach to suppress myofibroblast differentiation. Second, myofibroblasts in IPF are inherently resistant to stress-induced cellular senescence (28, 29), which may be explained by different gene expression profiles (38). For instance, the matricellular protein CCN1 induces cellular senescence in myofibroblasts, resulting in reducing excessive fibrogenesis through upregulation of matrix metalloproteinases accompanied by downregulation of collagen and TGF-β (13). Hence, elucidating the mechanisms for cell-type-specific induction of cellular senescence in myofibroblasts, other than autophagy regulation, may also offer clues for the development of novel approaches for IPF treatment.

Interestingly, a recent paper also demonstrated the impairment of autophagy in IPF lung, which might be caused by TGF-β-mediated mechanisms (32). Conversely, our results showed autophagy induction by TGF-β in fibroblasts, which can be attributed to the differences in origin of fibroblasts and experimental conditions. Actually, instead of MRC-5, a fetal lung fibroblast cell line, we used primary lung fibroblasts derived from adult lung and used for experiments until passage 6. As a part of the underlying mechanism for impairment of autophagy in IPF, we speculate the potential involvement of chronic virus infections. Indeed chronic and latent infection of several viruses, including cytomegalovirus, adenovirus, and Epstein-Barr virus, have been widely implicated in IPF pathogenesis via chronic inflammation and increased ER stress responses in association with apoptosis induction (30). In general, virus infections have been demonstrated to inhibit autophagy not only to prevent xenophagic degradation but also to modulate immune responses (23). Therefore, chronic virus infections may be associated with autophagy insufficiency in IPF and need to be addressed in future studies. IPF is generally assumed to be a disease of aging, and autophagy diminishes with aging. Although it is difficult to sort out cause from consequence of disease progression, accelerated cellular senescence itself may also be involved in the mechanism for autophagy insufficiency in IPF lung.

The biological activities of senescent cells in regenerative processes have been widely attributed to SASP of excessive cytokines and growth factor secretions (8). The p21 and SA-β-Gal-positive senescent metaplastic epithelial cells were distributed mainly in actively fibrosing areas (FF) (25). Therefore, it is reasonable to postulate that SASP may explain the phenotypic modulations via reciprocal interactions between epithelial cells and subepithelial fibroblasts for fibrosis development. Although we have reported that IL-1β, a potential SASP factor, was capable of inducing myofibroblast differentiation in lung fibroblasts (25), many SASP factors, including chemokines for fibrocyte recruitment, are potent candidates for future studies (4). Although abnormal metaplastic epithelial cells have been assumed to be mainly derived from bronchiolar epithelial cells (12), we understand the potential limitations of using HBEC as an experimental model to elucidate the pathogenesis of IPF, where AECII play a pivotal role. Therefore, cell culturing models using alveolar epithelial cells are needed in future studies to further confirm the physiological relevance of our results.

In summary, we have demonstrated concomitant accumulations of ubiquitinated proteins and p62 accompanied by an increase of cellular senescence as measured by p21 expression and SA-β-Gal staining in metaplastic epithelial cells, resulting from insufficient autophagic clearance. On the other hand, insufficient autophagy may also be responsible for phenotypic alteration of fibroblasts to fibrogenic myofibroblasts, indicating that insufficient autophagy is a potential underlying mechanism of accelerated epithelial cell senescence and myofibroblast differentiation in the pathogenic sequence of IPF (Fig. 9). Although cell-type-specific regulatory mechanisms of autophagy for cell senescence and myofibroblast differentiation

![Hypothetical model of involvement of insufficient autophagy in idiopathic pulmonary fibrosis pathogenesis](http://ajplung.physiology.org/)
remain to be determined, sufficient levels of autophagy induction may be a novel medical intervention to prevent accumulation of senescent epithelial cells and myofibroblasts in fibrotic areas in IPF.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


