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DNA damage response at telomeres contributes to lung aging and chronic obstructive pulmonary disease

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Telomeres are specialized structures at the ends of chromosomes consisting of tandem TTAGGG repeats stabilized by a complex of proteins, known as shelterin (15). Shelterin is thought to arrange telomeric DNA into a loop structure known as the T-loop. It is believed that, during replicative senescence, the progressive loss of telomere repeats destabilizes T-loops, increasing the probability of telomere uncapping, i.e., loss of shelterin (21). Telomere uncapping, whether by inhibition of shelterin or telomere shortening due to extensive replication, has been shown to activate the DNA damage response (DDR) in a manner similar to double-strand breaks (DSBs) (14). Uncapped telomeres become associated with DDR factors, such as phosphorylated forms of the histone protein 2A.X (H2A.X) and ataxia telangiectasia mutated (ATM), which can activate a signaling cascade leading to culmination of senescence (14). More recently, it has been shown that a DDR can induce senescence, irrespective of telomere length, which has been attributed to telomeres being particularly susceptible to oxidation-induced damage and to the inability of telomeres to repair DSBs (20, 25, 30). Moreover, it has been shown in vivo that, with age, telomeres colocalizing with DDR proteins...
increase in the skin of baboons (23) and in the liver, brain, and gut of mice, which can occur irrespectively of length (20, 25).

Telomere shortening has been associated with COPD in circulating leukocytes (45), alveolar epithelial cells (27, 36), and pulmonary vascular endothelial cells (5). However, it is unclear whether telomere shortening in COPD is due to aging, and the role of telomere dysfunction in the aging lung and to COPD-associated accelerated lung aging. In our study, we investigate the role of telomere dysfunction in the aging mouse lung and its potential role in cigarette smoke-induced COPD.

METHODS

Study subjects. Patients undergoing lung resection for localized lung tumors were recruited as controls from the Freeman Hospital, Newcastle upon Tyne, UK (Table 1). Samples from patients with advanced COPD were obtained from an archive of explant lung tissue taken at the time of lung transplantation at the Freeman Hospital. A smaller number of cases were used for immuno-fluorescence in situ hybridization (immuno-FISH) analysis due to limited availability of tissue at time of staining. All samples were parenchymal, and only airways with a diameter of <2 mm and without cartilage were included in the analysis. The clinical characteristics of these subjects are the same as those listed in Table 1. All subjects gave written, informed consent before inclusion in the study. This work was approved by the County Durham and Tees Valley 2 Research Ethics Committee (Res-11/NE/0291).

Animals. Wild-type C57BL/6 male mice were used (n = 3–5 per age group (6.5, 15 and 24 mo)). TERC−/− C57BL/6 male mice were bred to produce successive generations of mice with decreasing telomere length. Lungs from fourth-generation (G4) mice were collected. All work was completed with the guiding principles for the care and use of laboratory animals. The project was approved by the Faculty of Medical Sciences Ethical Review Committee, Newcastle University (project license no. 60/3864).

Lung tissues from mice exposed to either room air or cigarette smoke were a kind gift from Dr. Mark Birrell, Imperial College London, UK. Male C57BL/6 mice (n = 5/group) at 10 wk of age were subjected to a whole body cigarette smoke exposure system or room air, as previously described (18). Briefly, cigarette smoke was generated using 3R4F cigarettes (cigarette filter removed, Tobacco Health Research Institute, University of Kentucky, Lexington, KY) and pumped into a Teague chamber (136 liters) for 1 h, twice daily (500 ml/min), for 14 days. Mice were killed 24 h after the final exposure.

Cell culture and treatments. Human embryonic lung MRC5 fibroblasts were obtained from European Collection of Cell Cultures (Salisbury, UK) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma, Dorset, UK), supplemented with fetal bovine serum (10% vol/vol), 1-glutamine (2 mM), and penicillin/streptomycin and maintained at 37°C, 5% CO2.

Primary human small airway epithelial cells were isolated from bronchial brushings carried out during research bronchoscopy (normal controls) or from explant lung tissue specimens (COPD) (Table 2). The work was performed under approval of the Newcastle 1 Research Ethics Committee. Primary human bronchial epithelial cells were cultured on 0.5% Purecol-coated (Invitrogen, Carlsbad, CA) dishes in small airway epithelial cell growth medium (L/SABM), supplemented with 2% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin (Lonza, Basel, Switzerland).

MRC5 fibroblasts (population doublings 20–25) were grown until replicative senescence and cultured with DMEM plus 5% cigarette smoke extract (CSE) or DMEM alone. CSE was generated by bubbling smoke from one research-grade cigarette (University of Kentucky; 4A1) into 25 ml DMEM. The solution was filtered (0.2 μm), and the resulting CSE designated 100%. The CSE solution was diluted to 5% in sterile DMEM and used immediately. CSE or DMEM alone was replenished every 48 h. Identical experiments under hypoxic conditions (3% O2) were run in parallel. Human primary small airway epithelial cells (passages 1–3) were treated with two exposures of 5% CSE or media alone (control), 48 h apart.

Chemical inhibitors used were Kur55933 (ATM chemical inhibitor) (10 μM, diluted in DMSO) (R&D, 3544). Inhibitors were replaced every 48 h, along with 5% CSE or fresh DMEM.

Immunofluorescence. Cells grown on coverslips were fixed with 2% paraformaldehyde, permeabilized with PBG-Triton and incubated with the primary antibody at 4°C overnight. The following day, cells were incubated with fluorescein-conjugated secondary antibody (Alexa Fluor 488 or 594; Invitrogen) for 45 min at room temperature. Primary antibodies used were as follows: rabbit polyclonal anti-Ki67 (ab15580; 4 μg/ml Abcam), mouse monoclonal anti-γH2AX (no. 05–636; 0.25 μg/ml Millipore), and mouse monoclonal anti-p16 (SC-81156; 1:500 Santa Cruz).

Immuno-FISH. Immuno-FISH was performed as described (25). Briefly, cells grown on sterile coverslips were fixed with 2% paraformaldehyde and incubated with anti-γH2AX antibody (mouse monoclonal, no. 05–636, Millipore) overnight at 4°C. After application of the secondary antibody, cells were washed with PBS, and FISH was performed. Ten microinjections of C3-T34-polymerase-specific (C3TA2)3 peptide nucleic acid probe (4 ng/μl) (Panagene) was applied to the cells, followed by denaturation at 80°C and hybridization for 2 h at room temperature in the dark. Cells were washed three times for 10 min with wash buffer (70 ml formamide (70%), 30 ml saline-sodium citrate 2%) and three times for 5 min with Tris-buffered saline-Tween 0.5%. Cells were incubated with 4,6-diamidino-2-phenylindole (DAPI), mounted, and imaged using a Leica DMS500B fluorescence microscope. In depth Z-stacking was used (images were captured as stacks separated by 0.247 μm with ×100 objective) followed by Huygens (SVI) deconvolution.

For immuno-FISH in formalin-fixed, paraffin-embedded murine and human lung tissues, sections were dewaxed in 100% Histoclear and hydrated in 100, 90, and 70% ethanol (2 min incubations) and in distilled water (2 × 5 min). For antigen retrieval, the slides were placed in 0.01 M citrate buffer and heated until boiling for 10 min. After cooling down to room temperature, the slides were washed twice with distilled water for 5 min. After blocking in normal goat serum (5%) in BSA/PBS, primary antibody (rabbit polyclonal anti-γH2AX 1:400) (Cell Signaling, 9718) was applied and incubated at 4°C overnight. The next day, slides were washed three times in PBS, incubated with secondary antibody for 30 min, washed three times in PBS, and incubated with Avidin DCS (1:500) for 20 min. Following incubation, slides were washed three times in PBS and dehydrated with 70, 90, and 100% ethanol for 3 min each. Sections were denatured for 5 min at 80°C in hybridization buffer [70% formamide (Sigma), 25 mM MgCl2, 1 M Tris, pH 7.2, 5% blocking reagent.

Table 1. Clinical characteristics of patients with COPD and controls (tissue samples)

|                      | Patients with COPD | Controls |
|----------------------|--------------------|----------|
| n                    | 19                 | 11       |
| Sex, male/female     | 11/8               | 3/8      |
| Age, yr              | 52.84 ± 6.9†       | 70.45 ± 7.56 |
| FEV1, liter          | 0.53 ± 0.22†       | 1.79 ± 0.32 |
| FEV1, %              | 17.63 ± 6.99†      | 84.36 ± 9.45 |
| FVC, liter           | 1.89 ± 0.51†       | 2.69 ± 0.59 |
| FVC, %               | 52.05 ± 14.15†     | 95.9 ± 16.8 |
| Smoking history, pack-yr | 39.95 ± 77.1 | 31.3 ± 17.93 |
| GOLD score, I/II/III/IV | 0/0/1/18          | 0/0/0    |

Values are means ± SD; n, no. of subjects. COPD, chronic obstructive pulmonary disease; FEV1, forced expiratory volume in 1 s; FEV1, %, percentage of predicted FEV1; FVC, forced vital capacity; FVC, %, percentage of predicted FVC; GOLD, Global Initiative for Chronic Obstructive Lung Disease. *P < 0.001, †P < 0.0001 compared with controls.
Table 2. Clinical characteristics of patients with COPD and controls (small airway epithelial cells)

| Patients with COPD | Normal Controls |
|--------------------|----------------|
| **n**              | 5              | 5              |
| Sex, male/female   | 4/1            | 4/1            |
| Age, yr            | 56 ± 6.9       | 50.4 ± 6.9     |
| FEV₁, liter        | 0.53 ± 0.2*    | 3.59 ± 1.1     |
| FEV₁, %            | 16 ± 4.5†      | 106.8 ± 17.7   |
| FVC, liter         | 2.57 ± 1.1     | 4.9 ± 1.7      |
| FVC, %             | 61.6 ± 20.8*   | 118.2 ± 23.3   |
| Smoking history, pack-yr | 40.4 ± 23.7* | 0             |
| GOLD score, I/II/III | 0/0/0/5      | 0/0/0/0       |

Values are means ± SD; n, no. of subjects. *P < 0.05, †P < 0.01 compared with controls.

Results

Patients with COPD show increased telomere-associated foci in small airway epithelial cells. To assess telomere dysfunction, we obtained explant lung tissue from patients undergoing transplantation for COPD (n = 10) and from controls (n = 9) undergoing pulmonary resection for localized lung cancer (Table 1). We performed telomere specific quantitative FISH (Q-FISH), together with immunofluorescence staining against DNA damage protein γH2A.X (immuno-FISH). Analysis revealed a significant increase in percentage of small airway epithelial cells containing telomere-associated DNA damage foci (TAF) in patients with COPD (Fig. 1, A and B). No significant differences in telomere FISH intensity were detected (Fig. 1C). Similarly, analysis of individual telomeres in small airway epithelial cells in the COPD lung revealed no differences in telomere length in base pairs.

To determine whether other senescence markers were increased in patients with COPD, we conducted immunohistochemistry against p16, p21, and SIRT1. p16 and p21 are cyclin-dependent kinase inhibitors and tumor suppressors, expressed in most senescent cells (31). Decreased expression of SIRT1 (a NAD-dependent deacetylase) has been associated with cellular senescence (50). Consistent with a senescent-associated phenotype, using a semiquantitative scoring method, we observed increased p16 and decreased SIRT1 expression in small airway epithelial cells from patients with COPD (Fig. 1D). No significant differences in p21 expression were observed (data not shown). Using immuno-FISH (p16 and γH2A.X), we found that p16-positive cells have more TAF than p16-negative cells (Fig. 1E), suggesting that TAF may be involved in senescence induction.


development using the NovaRed kit (Vector Laboratories, SK-4800). Nuclei were counterstained with Carrazi hematoxylin, and sections were dehydrated through graded ethanol solutions, cleared in xylene, and mounted in di-butyl phthalate in xylene (human) or normal goat serum diluted 1:60 in 0.1% BSA in PBS (mouse). Sections were incubated with the primary antibody overnight at 4°C or for 45 min to 1 h at room temperature. For human tissue, anti-mouse (Dako, K4006) or anti-rabbit (Dako, K4010) secondary antibody conjugated with a horseradish-peroxidase-labeled polymer was added (Envision+ System-HRP, Dako), and slides were treated with 3,3-diaminobenzidine for 5–10 min. For mouse tissue, biotinylated secondary antibody was added and detected using the rabbit peroxidase ABC System-HRP, Dako), and slides were developed using the NovaRed kit (Vector Laboratories, PK-4001), according to the manufacturer’s instructions. Substrate was developed using the NovaRed kit (Vector Laboratories, SK-4800). Nuclei were counterstained with Carrazi hematoxylin, and sections were dehydrated through graded ethanol solutions, cleared in xylene, and mounted in di-butyl phthalate in xylene (Thermo Scientific, LAMB-DPX).

Staining was analyzed with a NIKON ECLIPSE-E800 microscope, butyle phthalate in xylene (Thermo Scientific, LAMB-DPX). Endogenous peroxidase activity was blocked by immersing sections in 0.3% H2O2 (Sigma, H1009) diluted in methanol for 30 min. To retrieve antigens, sections were boiled in 0.01 M citrate (pH 6.0) or 0.01 M EDTA buffer (pH 8.0). Sections were blocked in 5% nonfat milk protein diluted in PBS and incubated at 37°C for 16 h in freshly prepared Senescence-Associated Gamma H2A.X (γH2A.X) antibody (Q-FISH). Analyses revealed a significant increase in percentage of small airway epithelial cells containing telomere-associated DNA damage foci (TAF) in patients with COPD (Fig. 1, A and B). No significant differences in telomere FISH intensity were detected (Fig. 1C). Similarly, analysis of individual telomeres in small airway epithelial cells in the COPD lung revealed no differences in telomere length in base pairs.

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Following ex vivo analysis, we investigated whether TAF were increased in small airway epithelial cells isolated from the COPD lung (Table 2). By immuno-FISH, we found a significant increase in percentage of cells positive for TAF from patients with COPD (Fig. 3A), without significant differences in telomere FISH intensity (Fig. 3B). Because we found no significant differences in telomere length using Q-FISH, we compared telomere length in small airway epithelial cells isolated from COPD patients and age-matched controls (Table 3), using quantitative real-time PCR. Similarly, we detected no statistically significant differences (Fig. 3B). Small airway epithelial cells isolated from the COPD lung had increased positivity of Sen-β-Gal; however, this failed to reach statistical significance, with extensive interpatient variability observed (Fig. 3, C and D).

Telomere-associated foci increase in small airway epithelial cells in mice with age and following cigarette smoke exposure. Following our observation that TAF were increased in small airway epithelial cells of patients with COPD, we investigated whether TAF increased in small airway epithelial cells during physiological aging. Mice have long telomeres and express ubiquitously the enzyme telomerase; hence it was believed that telomere dysfunction did not play a role in cellular senescence in murine tissues (39). However, our group demonstrated that TAF accumulate in liver and intestine with age (25), and TAF have been shown to quantitatively predict mean and maximum lifespan in both short- and long-lived mouse cohorts (29).

We found a significant increase in percentage of cells positive for TAF from 6.5 until 24 mo of age (as well as mean number of TAF per cell, not shown) (Fig. 4, A and B). No significant changes in telomere FISH intensity were found; however, a tendency for decreased FISH intensity in older animals was observed (Fig. 4C).

Telomere dysfunction has been associated with increased expression of p21 (12). Consistently, we found with increasing age that a greater percentage of small airway epithelial cells stained positive for p21 (Fig. 4D). The aging lung is associated with structural changes similar to those that occur in emphysema, including distal air space enlargement (28). Consistent with this, we found increased air space size in mice with age, indicated by a decreasing number of air spaces per visual field (Fig. 4E). Interestingly, telomere FISH intensity did not correlate with air space number; however, there was an inverse correlation between percentage of cells positive for TAF and number of air spaces (P = 0.02) (Fig. 4E). These results suggest that telomere dysfunction may play a role in age-related lung tissue decline.

Cigarette smoke has been associated with early onset-senescence and induction of H2A.X phosphorylation in human pulmonary endothelial cells in vitro (3), and telomere length is reduced in small airway epithelial cells isolated from healthy smokers (49). However, the role of cigarette smoke in activation of a DDR specifically at telomeres has not been fully elucidated. We found that 3-mo-old mice exposed to cigarette smoke, twice daily for 2 wk, had an increased percentage of small airway epithelial cells positive for TAF, similar to levels observed in mice at 15 mo of age. While this increase was not significant (P = 0.06), we found that mean number of TAF increased significantly (P = 0.03) (Fig. 4F). No significant differences in telomere FISH intensity were observed (Fig. 4F). Altogether, these results suggest that small airway epithelial cells accumulate TAF with age, which can be accelerated by cigarette smoke exposure.

Late-generation TERC−/− mice show increased telomere-associated foci (TAF) in small airway epithelial cells and early-onset emphysema. At late generations, mice deficient in the RNA component of telomerase (mTERC−/−) exhibit a number of phenotypes indicative of premature aging, thought to be due to early onset of senescence (8). Late-generation mTERC−/− mice show critically short telomeres in most tissues and premature incidence of TAF. We found that G4 mTERC−/− mice have an increase in percentage of small airway epithelial cells containing TAF and decreased telomere FISH intensity (Fig. 5, A–C). Consistent with the hypothesis that telomere dysfunction contributes to loss of alveolar integrity, we found a significant reduction in number of air spaces in G4 mTERC−/− mice (Fig. 5, D and E). The correlation between TAF and number of air spaces we report is strengthened when G4 mTERC−/− are added (Fig. 5F); however, telomere FISH intensity still does not correlate (not shown).

Cigarette smoke extract induces TAF and senescence markers in primary human airway epithelial cells and MRC5 fibroblasts. In vitro exposure to cigarette smoke has been shown by several groups to result in expression of senescence-associated markers (3, 38). Nevertheless, the role of telomere dysfunction in cigarette smoke-induced senescence is less clear. Recent data from our group and others have revealed that stress-induced activation of a DDR at telomeres is persistent compared with nontelomeric damage, mostly because of inhibition of DNA repair mechanisms at telomere regions (20, 25). This suggests that TAF, given their persistence, may be excellent markers for age-related accumulated damage.

To determine whether CSE induced TAF in isolated small airway epithelial cells, we cultured cells isolated from healthy nonsmoking controls (n = 5) and exposed them to 5% CSE for 48 h. Small airway epithelial cells had increased TAF following CSE exposure (Fig. 6, A and B); however, analysis of Sen-β-Gal expression revealed no significant increases (not shown). Our data shows that TAF are induced as a consequence of CSE and may precede the induction of other senescence markers.

Epithelial cells cannot be cultured for prolonged periods of time without induction of epithelial-to-mesenchymal transition, a process whereby epithelial cells lose their epithelial features and acquire mesenchymal characteristics. This limits our ability to determine the effects of chronic cigarette smoke exposure on telomere dysfunction and other senescence-associated phenotypes. Therefore, we used normal human fetal lung fibroblasts (MRC5), which can be cultured for longer periods of time and are not overly sensitive to the effects of cigarette smoke exposure. MRC5 cells were cultured for 60 days in the presence or absence of 5% CSE to determine the effects of long-term cigarette smoke exposure. Consistent with previous observations, we show that long-term exposure to CSE induces accelerated senescence in MRC5 cells, evidenced by reduced population doublings (Fig. 6C), increased Sen-β-Gal activity (Fig. 6D), and reduction in proliferation marker Ki67 (Fig. 6D). Senescence is characterized by increased secretion of bioac-
A. DAPI, Telo-FISH, γH2A.X merge

B. Control and COPD Telomere-associated foci (TAF)

C. Telomere length

D. p16 and SIRT1 expression scores in Control and COPD

E. Mean number of TAF in Control and COPD

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tive, proinflammatory peptides; the so-called senescence-associated secretory phenotype (SASP). We first conducted a cytokine array analysis of 20 proinflammatory mediators and found that CSE exposure for 39 days leads to increased secretion of IL-6, IL-8, growth-related oncoprotein, monocyte chemotactic protein-1, and vascular endothelial growth factor (Fig. 6E). Most other cytokines or growth factors analyzed were secreted below detection level. This is consistent with previous reports suggesting that proinflammatory cytokines IL-6 and IL-8 are major components of the SASP and have been shown to contribute to the induction and maintenance of senescence in autocrine and paracrine fashions (1, 2). ELISAs for IL-6 and IL-8 detection independently confirmed the findings of the cytokine array (Fig. 6E). This rise in cytokine secretion following CSE exposure was first observed at day 13, but became enriched after 39 days in culture when cells reached premature senescence.

Consistent with a potential role for telomere dysfunction in the process, immuno-FISH revealed a significant increase in the percentage of cells containing TAF and in the mean number of TAF in both p16-positive and p16-negative cells per individual, with the horizontal line representing group median. AU, arbitrary units.

Fig. 1. Chronic obstructive pulmonary disease (COPD) patients show increased telomere-associated DNA damage foci (TAF) in small airway epithelial cells without significant telomere shortening. Explant lung tissue sections from patients with COPD and lung resection specimens from control subjects containing small airway material were analyzed for expression of senescence-associated markers by immunofluorescence in situ hybridization (immuno-FISH) and immunohistochemistry. A: representative images of immuno-FISH staining for γ-histone protein 2A.X (γH2AX; green) and telomeres (red) in small airway epithelial cells from patients with COPD and controls captured using ×100 oil objective and following Huygens (SVI) deconvolution. Arrows point to γH2AX foci colocalizing with telomeres (TAF), depicted by associated histograms and shown at higher magnification on the right (images are from one single Z-plane). B: immuno-FISH images of small airway epithelium in patients with COPD and controls color-coded according to number of TAF (blue: low number; red: high number). C: quantitative FISH (Q-FISH) images of small airway epithelium in patients with COPD and controls color-coded according to telomere length (blue: short; red: long). Dot plots represent percentage of cells positive for TAF and mean telomere length for each individual generated by quantifying Z-stacks of at least 50 cells per subject. The horizontal line represents group median. D: representative images of immunostaining for p16 and SIRT1 (brown) in small airway epithelial cells captured using ×40 objective. Arrows point to positive cells. Bar graphs represent the level of positive staining in the airway epithelium quantified using a semiquantitative scoring method. Values are median + range. E: representative image of double immunofluorescence staining for γH2AX (green) and p16 (yellow) combined with Q-FISH (red), carried out on lung tissue samples from patients with COPD (n = 8) to determine whether TAF and p16 expression colocalizes. Dot plot represents mean number of TAF in both p16-positive and p16-negative cells per individual, with the horizontal line representing group median. AU, arbitrary units. Statistics: Mann-Whitney U-test: *P < 0.05, **P < 0.01.
of TAF following CSE exposure, preceding other markers of senescence (Fig. 6, F and G). At day 67 in culture, all cells were positive for TAF, but mean number of TAF was increased in CSE-exposed cells (Fig. 6F).

Cigarette smoke-induced proinflammatory phenotype is accelerated by ROS-dependent telomere dysfunction. Mechanistically, it is unclear how CSE induces telomere dysfunction. Telomeres are highly sensitive to oxidative stress compared with the bulk of the genome and less efficiently repaired when subjected to single-strand breaks and DSBs (25, 42). Moreover, it has been shown that exposure to CSE increases ROS and oxidative stress markers in human fibroblasts and airway epithelial cells (9, 16). To determine whether TAF were induced by oxidative stress, we cultured MRC5 fibroblasts under low oxygen pressure (3% O2) and exposed them for 25 days to CSE. Culturing cells at low oxygen pressure is an accepted method of reducing intracellular ROS, and under these conditions we and others have previously reported reduced mitochondrial and cellular oxidative stress (39, 40, 43), which resulted in reduced DNA damage and delayed onset of senescence. We found that CSE significantly increased mitochondrial-derived ROS (measured by MitoSOX fluorescence), which was suppressed when culturing cells under 3% O2 (Fig. 7A). Consistent with a causal role for oxidative stress in TAF induction, we found that low oxygen was able to suppress smoke-induced increases in TAF (Fig. 7B). Similarly, treatment of primary small airway epithelial cells with antioxidants N-acetyl-cysteine significantly reduced short-term CSE-induced increases in TAF (Fig. 7C). As shown previously, CSE exposure increased percentage of cells positive for Sen-β-Gal, however, cultivation of cells at 3% O2 drastically reduced frequencies of Sen-β-Gal-positive cells (Fig. 7D). Furthermore, we found that CSE-driven growth arrest was suppressed upon cultivation of MRC5 fibroblasts at low oxygen (not shown).

Telomere-dysfunction and resulting DDR activation result in increased expression of IL-6 and IL-8 (44). Consistent with a role for ROS-dependent telomere dysfunction contributing to the SASP, we found that low oxygen significantly reduced IL-6 and IL-8 secretion in MRC5 fibroblasts, irrespective of smoke exposure (Fig. 7E).

Mechanistically, it has been shown that persistent ATM activation is necessary for induction of the SASP (44). To test the hypothesis that CSE-dependent activation of a DDR results in increased IL-6 and IL-8, we treated smoke-exposed primary human airway epithelial cells with antioxidants N-acetyl-cysteine and hydroxyurea. As shown previously, CSE exposure increased percentage of cells positive for Sen-β-Gal, however, cultivation of cells at 3% O2 drastically reduced frequencies of Sen-β-Gal-positive cells (Fig. 7D). Furthermore, we found that CSE-driven growth arrest was suppressed upon cultivation of MRC5 fibroblasts at low oxygen (not shown).

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Table 3. Clinical characteristics of patients with COPD and controls (small airway epithelial cells used for RT-PCR)

| Patients with COPD | Normal Controls |
|-------------------|-----------------|
| Sex, male/female  | 6/1             | 3/1             |
| Age, yr           | 54.6 ± 7        | 49.5 ± 7.59     |
| FEV1, liter       | 0.54 ± 0.17‡    | 3.75 ± 1.24     |
| FVC, %            | 15.6 ± 14.13§   | 111 ± 17.33     |
| FVC, liter        | 2.59 ± 0.95*    | 5.02 ± 1.96     |
| FVC, %            | 58.8 ± 19.79†   | 120.25 ± 26.36  |
| Smoking history, pack yr | 38.6 ± 21.67† | 0               |
| GOLD score, I/II/III/IV | 0/0/0/0/6 | 0/0/0/0/0      |

Values are means ± SD; n, no. of subjects. *P < 0.05, †**P < 0.01, ‡P < 0.001, §P < 0.0001 compared with controls.
posed MRC5 fibroblasts with an ATM inhibitor (KU55933). We first demonstrated that KU55933 suppresses phosphorylation of γH2A.X (a target of ATM kinase), confirming its role in DDR inhibition (Fig. 7F). At 14 days, we found that ATM inhibition represses smoke-induced TAF increase (not shown), as well as IL-6 and IL-8 secretion (Fig. 7F), supporting the hypothesis that smoked-induced DDR activation results in increased SASP.

Altogether, the results from MRC5 cells suggest that cigarette smoke exposure causes telomere dysfunction, possibly through increased oxidative stress, which leads to senescence induction and SASP activation.
Increased cellular senescence is a major feature of aging and has been implicated in COPD pathogenesis. Short telomeres, known activators of cellular senescence, have been implicated in COPD, mostly in circulating leukocytes (45).

Recent data has questioned the utility of telomere length in circulating leukocytes as a biomarker of aging. While some studies suggest that leukocyte telomere length may act as a proxy for telomere length in other somatic cell types, there is evidence suggesting that this is not true for some tissues (47). Furthermore, recent studies have suggested telomere dysfunction can be induced independently of length. In fact, data suggest that senescence can be induced by activation of a DDR at relatively long telomeres in human fibroblasts during stress-induced (20, 25), replicative (30), and oncogene-induced senescence in vitro (46) and in mice in vivo (25). While the mechanisms driving telomere dysfunction are still unclear, these data suggest that a “critical” telomere length may not be the sole determinant in the activation of a persistent DDR.

Using Q-FISH and real-time PCR in small airway epithelial cells, we failed to detect robust differences in telomere length between controls and patients with COPD. This contrasts with previous reports where telomere shortening is described in other lung cells from patients with COPD, including alveolar type II cells and endothelial cells (5, 48). It is possible that our study failed to detect differences in telomere length due to a relatively small sample size. Decreased telomere length in smokers (49) and patients with COPD (5) has been described, using larger sample sizes than in our study. However, only small differences in telomere length have been reported (<15% in most cases). We observe significant increases in the frequency of cells positive for TAF in patients with COPD, even with smaller sample sizes. Moreover, we have found clear evidence for increased TAF in small airway epithelial cells in lung tissue from COPD patients, which are younger than controls, demonstrating that TAF are robust indicators of COPD-associated damage, despite the age discrepancy. Consistently, another study also failed to find differences in telomere length between lung fibroblasts isolated from patients with emphysema and aged-matched controls, despite increased expression of senescence-associated markers (37). In addition to increased TAF, we observed increased p16, which is considered a hallmark of senescence. Moreover, TAF content was greater in p16-positive cells, suggesting that TAF correlate with expression of senescence markers. We did not detect differences in p21 positivity between patients with COPD and controls. However, the p16-pRB pathway may be activated following activation of, or independent to, the p21 pathway (24).

Our study cannot eliminate telomere shortening as a mechanism driving COPD-associated telomere dysfunction because I) in age-matched isolated small airway epithelial cells, our

DISCUSSION

Fig. 5. Late-generation TERC−/− mice show increased TAF in small airway epithelial cells and early-onset emphysema. Immuno-FISH staining for γH2AX and telomeres was carried out on lung tissue sections from late-generation TERC−/− and WT C57Bl/6 mice at 6.5 mo old. A: immuno-FISH images of small airway epithelial cells from WT and late-generation (fourth generation (G4)) TERC−/− mice color-coded according to number of TAF (blue: low number; red: high number) and telomere length (blue: short; red: long). Dot plots represent the percentage of cells positive for TAF (B) and the mean telomere length (C) for each animal generated by quantifying Z-stacks of at least 100 cells per animal taken using ×100 oil objective. D: representative images of H&E staining of alveolar air spaces in WT and G4 TERC−/− mice captured using ×20 objective. E: dot plots represent the mean number of air spaces quantified per visual field from 10 random images taken for each animal. Values are the mean for individual animals, with the horizontal line representing group mean. F: correlation between percentage of small airway epithelial cells positive for TAF and number of air spaces. Statistics: independent samples t-test, Pearson’s correlation; *P < 0.05, ***P < 0.001.
sample number is relatively small; and 2) in lung tissue sections, where our numbers are greater, the controls were older. Nonetheless, comparison of individual telomere lengths relative to telomere length in lung tissue revealed no significant differences, suggesting that telomeres can signal a DDR, irrespective of length. These data are in accordance with several recent publications reporting telomere dysfunction, irrespective of length, in a variety of cells (20, 25). We have used γH2A.X immunoreactivity alone to determine damage both in genomic DNA and at telomere regions. It has previously been shown that γH2A.X immunoreactivity can be detected independent of DNA damage. For example, one study described γH2A.X foci colocalizing with telomeres (TAF), shown at higher magnification on the right (images are from single Z-plane). C: MRC5 cells (untreated or exposed to 5% CSE every 48 h) were subjected to repeated passaging and population doubling (PD) level calculated for each condition and plotted against number of days in culture. D: representative images of Sen-β-Gal staining in MRC5 cells after 25 days in culture, and representative images of Ki-67 staining in MRC5 cells after 3 and 25 days in culture. E: Left: secreted protein array (RayBiotech) for range of inflammatory cytokines. Values are means ± SE of n = 4 experiments. F: immuno-FISH staining for γH2A.X and telomeres was carried out on MRC5 cells from a number of time points. Percentage of cells positive for TAF (left) or mean number of TAF per cell (right) generated by quantifying Z-stacks of at least 30 cells per condition using ×100 oil objective are shown. Values are means ± SE. G: representative Z-stacks of immuno-FISH staining for γH2A.X (green) and telomeres (red) in MRC5 cells exposed to 5% CSE or untreated at 3 and 25 days in culture captured using ×100 oil objective and following Huygens (SVI) deconvolution. Arrows point to γH2A.X foci colocalizing with telomeres (TAF) and are shown at higher magnification on the right (images are from single Z-plane).
formed large foci that colocalize with DSB repair proteins, and another forming abundant small foci dissociated from repair proteins, which may have a role in the mitotic process (35). It is, therefore, possible that the presence of γH2A.X foci at telomeres that we observe occur independently of oxidative DNA damage and DSBs to the sequence. However, based on our data, we have reasons to believe that the TAF we observe are not those described small foci associated with mitosis. First, γH2A.X foci, which generally colocalize with telomeres, are the largest in size, both in small airway epithelial cells and in human fibroblasts (25). Second, the presence of TAF inversely correlates with decreased proliferation and downstream pathways of senescence in both fibroblasts and small airway epithelial cells. However, since we have not analyzed colocalization between γH2A.X, telomeres, and DSB repair proteins, it is possible that the TAF we observe may not be the outcome of DSBs, but due to activation of another signaling event.

Our study suggests that cigarette smoke enhances oxidative stress and contributes to telomere dysfunction in vitro and in vivo. Data indicate that telomeres are particularly susceptible to oxidative stress compared with the rest of the genome (25, 42); however, the mechanisms are not completely understood. Telomere repeats contain guanine triplets, which are susceptible to oxidative modifications and are less efficiently repaired when subjected to different types of DNA damaging agents (20, 25). While cigarette smoke has been shown to induce...
γH2A.X (3), this is, to our knowledge, the first time TAF have been observed. The importance of this finding lies in the fact that, when a DDR is induced at telomeric regions, it is persistent and unresolved, which is characteristic of senescence (25). We did not determine whether cigarette smoke exposure increased levels of oxidative stress in vivo. However, it has been shown by other groups that both short- and long-term exposures to cigarette smoke increase markers of oxidative stress in the lungs of mice, including 8-hydroxy-2′-deoxyguanosine and 4-hydroxynonenal (6, 51, 52). The importance of oxidative DNA damage to the pathogenesis of COPD has been underscored by a number of studies showing that patients with COPD have different types of oxidative DNA damage in both the nuclear and mitochondrial genomes (7, 11, 33, 41). However, this is the first report, to our knowledge, describing irreparable damage is highly important in the context of senescence, since telomeres are particularly susceptible to oxidative damage and are irreparable.

Telomere length in COPD patients has been shown to inversely correlate with mRNA expression of inflammatory cytokines (5); however, it is still unclear whether there is a causal link between telomere dysfunction and the SASP as a result of cigarette smoke exposure. We demonstrate that 1) inhibition of ROS suppresses smoke-induced telomere dysfunction, along with decreased secretion of IL-6 and IL-8; and 2) inhibition of ATM, one of the main initiating factors of a DDR, diminishes CSE-induced IL-6 and IL-8 release. Altogether, these data suggest a causal link between ROS, activation of a DDR at telomeres, and the proinflammatory phenotype characteristic of senescence. However, it is not possible to delineate from these experiments whether telomeric damage specifically is responsible for ATM-dependent SASP induction, since ATM inhibition with KU55933 will have global effects. Technically, it would be very difficult to inhibit ATM activity only at telomere regions, but this would allow any causal link between telomere dysfunction and SASP activation to be identified. Moreover, it is not possible to extrapolate the findings from MRC5 cells to primary airway epithelial cells, as we were unable to culture these cells for longer than 5 days without development of epithelial-to-mesenchymal transition-related phenotypic changes, which is a limitation of our study.

Our data propose that TAF correlate with development of lung emphysema more strongly than telomere length in aging mice and could play a causal role in age-related lung degeneration, given that late-generation mTERC−/− mice show early onset of emphysematous-like changes. There is still uncertainty regarding the role of telomere length in emphysema: a previous study using G4 mTERC−/− mice failed to observe lung emphysema (4). However, the authors reported very small differences in telomere length of <15% between wild-type and G4 mTERC−/− mice, in contrast to an almost fourfold difference we observed. This may explain the discrepancies in the data, as another study using G4 mTERC−/− reported loss of alveolar integrity coupled with similar telomere signal loss, as observed in our mice (32).

In summary, while our data do not invalidate the role of telomere shortening in COPD-associated senescence, it suggests that TAF are a more robust marker of senescence in COPD, compared with telomere length. We observe increases in percentage of cells positive for TAF in COPD, despite relatively small samples sizes. Moreover, although we observed good associations between number of γH2A.X foci alone and mean number of TAF with age and in the context of cigarette smoke exposure, we consistently observe more significant increases in TAF. Telomeres occupy just 0.02% of the genome, and thus the probability of damage occurring at telomeres is extremely low. Despite this, we observed robust increases in TAF with age and even following short-term cigarette smoke exposure, suggesting that telomeres may have particular properties that render them susceptible to damage. In fact, it has been shown that telomeres accumulate more single-stranded breaks than the bulk of the genome in response to oxidative stress (42). It has been argued that this may be due to the fact that telomeric repeats contain guanine triplets, which are remarkably sensitive to oxidative modifications (22). These factors, coupled with the reported protection of telomeres from repair activities, may contribute to their specific targeting and persistent damage as a consequence of cigarette smoke exposure and during the aging process. Further work needs to be performed to establish whether TAF are associated with COPD susceptibility and severity or have any prognostic value. We propose that TAF may be causal to structural decline and increased inflammatory processes that occur during physiological lung aging and in COPD.

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DISCLAIMERS

The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, or the Department of Health.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

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