Central Role of Cellular Senescence in TSLP-Induced Airway Remodeling in Asthma

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Abstract

Background: Airway remodeling is a repair process that occurs after injury resulting in increased airway hyper-responsiveness in asthma. Thymic stromal lymphopoietin (TSLP), a vital cytokine, plays a critical role in orchestrating, perpetuating and amplifying the inflammatory response in asthma. TSLP is also a critical factor in airway remodeling in asthma.

Objectives: To examine the role of TSLP-induced cellular senescence in airway remodeling of asthma in vitro and in vivo.

Methods: Cellular senescence and airway remodeling were examined in lung specimens from patients with asthma using immunohischemical analysis. Both small molecule and shRNA approaches that target the senescent signaling pathways were used to explore the role of cellular senescence in TSLP-induced airway remodeling in vitro. Senescence-Associated β-galactosidase (SA-β-Gal) staining, and BrdU assays were used to detect cellular senescence. In addition, the Stat3-targeted inhibitor, WP1066, was evaluated in an asthma mouse model to determine if inhibiting cellular senescence influences airway remodeling in asthma.

Results: Activation of cellular senescence as evidenced by checkpoint activation and cell cycle arrest was detected in airway epithelia samples from patients with asthma. Furthermore, TSLP-induced cellular senescence was required for airway remodeling in vitro. In addition, a mouse asthma model indicates that inhibiting cellular senescence blocks airway remodeling and relieves airway resistance.

Conclusion: TSLP stimulation can induce cellular senescence during airway remodeling in asthma. Inhibiting the signaling pathways of cellular senescence overcomes TSLP-induced airway remodeling.

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Introduction

Asthma is characterized by chronic inflammation and structural alterations in the airways [1] and airway remodeling is a common feature of asthma [2]. Remodeling is caused by repetitive injury to the airway wall arising from cycles of inflammation and repair [3]. Histological characteristics of airway remodeling include thickening of the lamina reticularis, epithelial shedding, subepithelial fibrosis, inflammatory cell infiltration, goblet cell hyperplasia, myofibroblast proliferation, smooth muscle hyperplasia and hypertrophy, and neovascularization of the airway wall [4]. These features and airway hyper-responsiveness (AHR) are common in severe lung diseases such as asthma [5].

Airway epithelia produce some inflammatory factors that play critical roles in regulating metabolic and immunologic responses within airways. These inflammatory factors are often involved in epithelial damage in patients with asthma [6]. TSLP, an IL-7-like inflammatory factor [7], is secreted by bronchial and lung epithelial cells [8–10]. Several groups have demonstrated that TSLP levels are increased in asthmatic epithelia [11,12]. TSLP protein levels are also increased in the
airway epithelia and bronchoalveolar lavage fluids (BALF) collected from patients with asthma [8,13]. Recently, several independent genome-wide association studies have demonstrated that TSLP is a susceptibility locus for asthma [14,15].

Senescent cells are frequently found at sites of chronic disease. Many older patients have histological and symptomatic features of chronic obstructive pulmonary disease (COPD) and asthma [16,17]. Alveolar and airway cell senescence from cigarette smoke and aging contributes to the destruction of alveoli by limiting the proliferative capacity necessary for tissue repair and promoting chronic inflammation. Both of these processes are hallmarks of COPD [18]. In addition, cigarette smoke can induce expression of senescence markers, such as SA-β-gal in lung epithelial cells and fibroblasts [19,20]. Certain cytokines are crucial in airway remodeling and cellular senescence. For example, paracrine IL-6 and IL-8 both induce maintenance of the senescent phenotype [18,21–23].

To determine whether inhibiting cytokine-induced cellular senescence can overcome airway remodeling in asthma, we explored the role of senescence in TSLP-induced airway remodeling in asthma in vitro and in vivo. We found that 1) senescent signaling pathways are activated in airway epithelium in patients with asthma, 2) TSLP stimulation induced cellular senescence and silencing of cellular senescence pathways inhibits TSLP-induced airway remodeling in vitro and 3) Stat3-targeted therapies overcome TSLP-induced airway remodeling by inhibiting cellular senescence in vitro and in vivo.

Materials and Methods

Ethics Statement

The study was approved by the Ethics Review Committee for Human Studies at Qilu Hospital, Shandong University. Each subject provided written, informed consent. Animal experiments were performed in accordance with the Institutional Animal Care and Use Committee of Shandong University.

The protocol was approved by the Committee on the Ethics of Animal Experiments of Shandong University. Each mouse was anesthetized with intraperitoneal ketamine (35 mg/kg). Cervical dislocation was used to euthanize mice. All efforts were made to minimize pain and discomfort.

Patients

The study was approved by the Ethics Review Committee for Human Studies at Qilu Hospital, Shandong University. Each subject provided written, informed consent. Endobronchial biopsies from 10 asthma patients were obtained from Shandong Provincial Chest Hospital (Jinan, Shandong, China). Healthy control specimens were obtained from Qilu Hospital of Shandong University (Jinan, Shandong, China), and were from pneumoaresections. Subject characteristics are shown in Table 1.

| Healthy control subjects | Asthmatic patients |
|--------------------------|--------------------|
| Sex (M/F) | 5/5 | 6/4 |
| Age (y) | 27.2 ± 5.31 | 29.7 ± 4.14 |
| FEV1(L) | 4.17 ± 0.69 | 3.68 ± 0.84 |
| FEV1(%) | 99.6 ± 7.15 | 91.8 ± 19.85 |
| PC20(mg/mL) | 145.17±20.12 | 1.99 ± 0.84 |

Histological analysis

Immunohistochemical staining was performed as described [24]. The immunohistochemical scoring (H score) was determined by multiplying the staining intensity by the percentage of positive tumor cells [25,26]. These primary antibodies were used: anti-p16 (f-12, SC-1661), anti-p21 (C-19, SC397, anti-TSLP (M-20, SC19178), anti-α-smooth muscle actin (α-SMA) (1A4, ab7817), anti-collagen I (ab34710) and anti-Ki67 (M-19, SC7846). All these antibodies were purchased from Santa Cruz Bio-technology, Inc. (Santa Cruz, CA, USA) or Abcam Inc. (Cambridge, MA, USA).

Cells and reagents

The human bronchial epithelial cell line, BEAS-2B, was derived from human bronchial epithelia and was purchased from ATCC (Manassas, VA). TSLP was purchased from R&D Systems (Minneapolis, MN, USA). WP1066 was purchased from Santa Cruz Bio-technology, Inc. (Santa Cruz, CA, USA).

Staining for SA-β-Galactosidase

BEAS-2B cells were fixed in 2% formaldehyde and 0.2% glutaraldehyde for 10 minutes at room temperature, washed in PBS, and incubated for 24 h at 37 °C with SA-β-Gal (Sigma-Aldrich, St. Louis, MO, USA) in staining solution (40 mM sodium citrate (pH 6), 150 mM, NaCl, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl₂ and 1 mg/ml bromo-4-chloro-3-indolyl-β-D-galactoside).

BrdU Analysis

BrdU Cell Proliferation Assay Kit was purchased from Chemicon International (Temecula, CA, USA) and used following manufacturer’s protocol.

Western Blotting

These primary antibodies were used: anti-p16 (f-12, SC-1661), anti-p21(C-19, SC397), anti-TSLP (M-20, SC19178), anti-α-SMA (1A4, ab7817), anti-collagen I (ab34710) and α - tubulin (B3, ab11324). All these antibodies were purchased from Santa Cruz Bio-technology, Inc. (Santa Cruz, CA, USA) or Abcam Inc. (Cambridge, MA, USA).
Mouse Asthma Model and WP1066 Treatment

Mice (female, 6-week-old, 18 - 20g each) were either control, untreated, OVA (Sigma-Aldrich, St. Louis, MO)-induced asthma model or WP1066-treated (n=10). Mice were sensitized with OVA as described previously [28]. WP1066-treatment mice were treated once daily with 40mg/kg WP1066 by intraperitoneal injection 1h before the OVA challenge. Mice were injected with saline, OVA or OVA+WP1066 during airway challenge. Mice were then examined for airway remodeling 48h after the last challenge and lung tissues were collected for immunohistochemistry analysis.

Analysis of Airway Hyper responsiveness (AHR)

Methacholine was used at 0, 3, 6, 9, 12 g/L and a dose-response curve was constructed. Measurements of AHR were made 1 min after each dose and 2 min between doses. Results are expressed as the maximum resistance after each dose minus baseline (PBS alone) resistance.

Statistical analysis

Statistical analysis was performed using the SPSS 12.0 software package. Data were analyzed using Student’s t-test. Significance was defined for p<0.05.

Results

p16 and p21 protein levels are upregulated in airway epithelium of patients with asthma

Cellular senescence is a state of irreversible growth arrest and is induced by a wide variety of conditions, including telomere shortening (replicative senescence) and telomere-independent signals (stress-induced senescence) [29,30]. Previous reports have demonstrated that cigarette smoke induces airway epithelial cellular senescence [20,21] and senescent cells are readily detected in airway epithelia of patients with COPD [31]. Given the common features of asthma and COPD, specifically, airway remodeling, we examined cellular senescence in airway remodeling in asthma. We first asked if senescent signaling pathways are activated in airway epithelia of asthma. Two major signaling pathways are involved in senescence: the p19ARF/p53/p21 pathway and the p16INK4a/CDK/pRb pathway [32]. Several groups have found that p21 mRNA and protein levels are increased in asthma airways, and expression levels correlate with asthma severity [33–35]. In addition, thioredoxin (TRX) can repress the expression of p21 and block airway remodeling in an asthma mouse model [36]. We examined p21 and p16 protein levels in bronchial epithelia collected from patients with asthma and found increases in p16 and p21 protein levels in epithelial cells from asthma patients (Figure 1B & C). To determine if upregulation of p16 and p21 correlates with lower levels of proliferation, we examined the expression of Ki67 in asthma epithelia samples. As expected, the expression of Ki67 protein was repressed in epithelia samples from asthma patients (Figure 1B & C). To examine whether senescent pathway activation correlates with airway remodeling, we examined markers of airway remodeling in asthma patient samples by looking at collagen I and α-SMA protein levels. As expected, increased expression levels of collagen I were found in asthma patient epithelia (Figure 1B & C). In addition, α-SMA was highly expressed in the basement membrane of thickened airway walls of asthma samples (Figure 1B & C). These results suggest that pathways of cellular senescence and airway remodeling are activated in airway epithelia from patients with asthma.

Cellular senescence is induced by TSLP in human airway epithelial cells in vitro

To further explore the role of cellular senescence in airway remodeling of asthma, we tested whether TSLP, a critical cytokine in airway remodeling in asthma, induces senescence in human airway epithelial cells. Previous studies demonstrated that the neutralization of TSLP could inhibit airway remodeling in a murine model of allergic asthma induced by dust mites [37]. In our experiment, the airway epithelial cell line, BEAS-2B, was used [38]. BEAS-2B cells were treated with TSLP (1.5ng/ml) and SA-β-gal staining was performed to detect senescent cells. β-galactosidase, the lysosomal hydrolase, is
Figure 1. Protein expressions of p16 and p21 in human asthmatic airway epithelium tissue. (A) Micrographs of histological sections of the asthmatic human airway showing the loss of epithelial integrity and wall thickening by H and E staining. Magnification 400X. (B) TSLP, p16, p21, Ki67, α-SMA and collagen I expression. Arrows indicate areas of positive expression. Magnification, 400×. (C) Bimodal H score distribution of TSLP, p16, p21, Ki67, α-SMA and collagen I immunoperoxidase reactions.

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active at pH 4, but SA-β-gal is active at pH 6 and is only present in senescent cells; allowing the two activities to be readily distinguished [39]. Phospho-Stat3, a downstream target of TSLP [40] served as a control to monitor the activation of TSLP signaling in BEAS-2B cells. We found that cellular senescence was induced by TSLP stimulation. Specifically, senescent cells increased >15 fold upon treatment with 1.5 ng/ml TSLP (Figure 2A). To confirm these findings, p16 and p21 expression analysis and BrdU labeling were also performed. As shown in Figure 2B, upregulation of p16 and p21 was detected from 3-24 hours after TSLP stimulation (Figure 2B). TSLP-induced p16 and p21 upregulation occurred in a TSLP dose-dependent manner (Figure 2A). Furthermore, cell proliferation analysis (BrdU labeling) revealed that TSLP stimulation significantly inhibited cell proliferation up to 24 hours after TSLP treatment (Figure 2C). In addition, there was increased expression of SA-β-gal and decreased Ki67 expression in BEAS-2B cells upon TSLP treatment. (Figure 2D & E). Furthermore, we also found TSLP stimulation induced TSLP secretion (Figure 2F). Taken together, these results indicate that TSLP stimulation induces cellular senescence in BEAS-2B cells.

Cellular senescence induction is required in TSLP-induced airway remodeling

We examined the role of cellular senescence in airway remodeling in asthma. For this analysis, we generated senescing cell lines of BEAS-2B cells by stably expressing shp16, and/or shp21. It has been shown that silencing of p16 and p21 signaling pathways together inhibits cellular senescence [41]. We examined TSLP-activated airway remodeling in p16 and p21 silenced cells (Figure 3A). We found that TSLP stimulation induces the activation of airway remodeling markers, including collagen I and α-SMA [24], [42]. This activation is inhibited when p16 and p21 are both silenced. As expected, silencing the p16 or p21 pathways alone does not inhibit TSLP-induced activation of airway remodeling in vitro (Figure 3A). To examine if both of p16 and p21 silencing can inhibit TSLP-induced cellular senescence, SA-β-gal expression analysis was performed and cell proliferation was tested using BrdU labeling and MTT analysis in TSLP-stimulated BEAS-2B cells with stable p16 and/or p21 silencing vectors. As expected, silencing of both p16 and p21 pathways inhibits TSLP-induced SA-β-gal activation (Figure 3B) and inhibits cell proliferation (Figure 3C & D). These results suggest that cellular senescence is required in TSLP-activated airway remodeling.

A Stat3 inhibitor suppresses senescence-associated airway remodeling in BEAS-2B cells

Previously, we demonstrated that exogenous TSLP activated the Stat3 signaling pathway in human lung fibroblasts [24] and we confirm these data here (Figure 4A). To further examine the involvement of Stat3 in TSLP-induced senescence in BEAS-2B cells, BEAS-2B cells were incubated with 10μM of the Stat3 inhibitor WP1066 for 2h and then treated with different concentrations of TSLP. Then, SA-β-gal, p21 and p16 expression and BrdU labeling analyses were performed. Collagen I and α-SMA expression were used to monitor airway remodeling. We found that WP1066 preincubation suppressed TSLP-induced senescence and airway remodeling in BEAS-2B cells (Figure 4B & C & D).

WP1066 treatment attenuates airway hyper-responsiveness (AHR) and airway remodeling in a mouse asthma model

To determine whether WP1066 treatment can relieve airway resistance in vivo, we generated a mouse asthma model by daily OVA challenge. Airway resistance was monitored using flexiVent. We found that WP1066 treatment significantly overcame airway resistance (Figure 5A). We also found that OVA-challenge activated cellular senescence in the mouse airway epithelium (Figure 5B & C). In addition, OVA-challenge induced the expression of α-SMA and collagen I in mouse airway epithelia (Figure 5B & C). These results indicate that chronic allergen exposure in mice promotes bronchial remodeling. In addition, we used p21, p16 and Ki67 as senescence markers in airway epithelium in vivo. We found that inhibiting Stat3 by WP1066 inhibited cellular senescence and resulted in the upregulation of α-SMA and collagen I in mouse airway epithelia (Figure 5B & C).

Discussion

Cellular senescence in airway remodeling of asthma

Senescence is an important fail-safe mechanism often arising in response to telomere erosion and stress. Chronic inflammation is a major histological characteristic of asthma and plays a critical role in airway remodeling. Chronic inflammation also represents tissue stress. Therefore, it is not surprising that cellular senescence is induced in airway epithelia of asthma. Senescence arises through checkpoint activation and cell cycle arrest and is a barrier to cell proliferation and tumorgenesis [42]. In fact, activation of cell cycle checkpoint proteins prevents the replication of genomically unstable cells [43]. Previous reports have demonstrated the critical role of cellular senescence in COPD and other respiratory diseases, such as pulmonary fibrosis and lung cancer [44–46]. Here we further demonstrate that cellular senescence is required in TSLP-induced airway remodeling in asthma. We confirm the upregulations of p16 and p21 and the repression of Ki67 in epithelia from asthma patients (Figure 1). We also found that upregulation of p16 and p21 is required in TSLP-induced airway remodeling (Figure 3). Both the p16 and p21 pathways are involved in senescence [32]. Uprogulation of p21 in asthma has been reported. Furthermore, previous reports demonstrate that the increased levels of p21 protein correlates with asthma severity [33–35]. The mechanism of p21 activation in asthma has been investigated and evidence indicates that some cytokines induce the expression of p21. In vivo studies demonstrate the therapeutic potential of p21-targeted therapy in asthma. For example, thioredoxin (TRX) reduces gene expression of TGF-β1, EGFR, and p21 to influence airway epithelia and prevent airway remodeling in a asthma mouse model [47].
Figure 2. Cellular senescence is induced by TSLP stimulation in vitro. 
(A) TSLP-induced p16 and p21 upregulation occurs in a TSLP dose-dependent manner in human bronchial epithelial BEAS-2B cells. BEAS-2B cells were stimulated with different doses of TSLP as indicated for 6h. Protein expressions of p16, p21 and phospho-Stat3 (Try705) were detected by western blotting. 
(B) BEAS-2B cells were stimulated by 1.5ng/ml TSLP and protein expressions of p16 and p21 were detected by western blotting. 
(C) BEAS-2B cells were stimulated with 1.5ng/ml TSLP then stained for BrdU. (*p< 0.05). 
(D) BEAS-2B cells were stimulated with 1.5ng/ml TSLP then stained for SA-β-gal activity at 6 and 24 hours post stimulation. 
(E) Ki67 staining. 
(F) Levels of TSLP in culture media were examined by ELISA.
Figure 3. Senescent inhibition overcomes TSLP-induced airway remodeling in vitro. BEAS-2B cells with stable shp16, shp21 or both were incubated with TSLP (1.5ng/ml) for 6 h. (A) Cells were collected and total proteins were extracted and analyzed by western blotting. (B) Cells were fixed and stained with SA-β-gal (upper panel) and then positive SA-β-gal cells were quantified (*p>0.05, **p < 0.05) (low panel). (C) Cells were stained with BrdU (*p>0.05, **p < 0.05). (D) Senescent inhibition overcomes TSLP-induced cell growth inhibition in vitro. The relative cell number was detected to evaluate cell growth at different time points using MTT assays.

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Figure 4. Inhibition of Stat3 overcomes TSLP-induced senescence and airway remodeling in BESA-2B cells. (A) TSLP-induced activation of Stat3 and airway remodeling. BESA-2B cells were stimulated with 1.5ng/ml TSLP and total protein was collected at different time points. Protein expressions of phospho-Stat3, Stat3, α-SMA and Collagen I were analyzed by western blotting along with α-tubulin, which serves as a loading control. BESA-2B cells were stimulated with 1.5ng/ml TSLP and 10 μM WP1066 as indicated. (B) Total protein was collected after 6 hour TSLP stimulation. Protein expressions of phospho-Stat3, Stat3, p21, p16, α-SMA and Collagen I were analyzed by western blotting. Expression of α-tubulin, serves as a loading control. (C) Cells were fixed and then stained with SA-β-gal (upper panel) and SA-β-gal positive cells were quantified (\(* p < 0.05\) (low panel). (D) Cells were stained with BrdU (\(* p < 0.05\)).

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Figure 5. Experimental therapies with WP1066 in OVA-challenged chronic asthmatic mice. (A) OVA-challenge was performed in BALB/c mice as described [28]. WP1066 was administered by intraperitoneal injection at doses of 40mg/kg 1h before the OVA-challenge. Airway resistance was measured using increasing concentration of methacholine and assessed using the flexiVent system. Results are expressed as the mean of experiments done in triplicate ± the standard error of the mean (SEM) (* p<0.05, **p>0.05 vs control). (B) TSLP, p16, p21, Ki-67, α-SMA and collagen I protein expressions were analyzed, 200×. (C) Bimodal H score distribution of TSLP, p16, p21, Ki67, α-SMA and collagen I immunoperoxidase reactions are presented.

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TSLP-induced cellular senescence and airway remodeling

TSLP is considered a pivotal cytokine linking innate and adaptive immune disorders [48–50]. Environmental pollutants, including ambient particulate matter, diesel exhaust particles and tobacco smoke, upregulate TSLP expression in airway epithelial cells [51–53]. The TSLP-induced signaling pathway in epithelium has been demonstrated previously [54]. TSLP can induce multiple signaling pathways in asthma, including STAT6, IL-4 [55], IL-1β and TNFα [56], p38 and Jun kinase (JNK ). The central role of Stat3/5 in TSLP-signaling pathway has also been unveiled [57]. Here we further explored the signaling pathway in TSLP-induced airway remodeling in asthma. We found TSLP activates cellular senescent signaling pathways (including the p21 and p16 pathways) to activate airway remodeling in vivo and in vitro.

Currently, TSLP-targeted therapies are being explored in asthma. One recent report demonstrates that neutralization of TSLP can inhibit airway remodeling in a murine model of allergic asthma induced by house dust mites [37]. Antibodies AMG157 targeting TSLP are currently being evaluated for patients with mild atopic asthma in a Phase Ib clinical trial (ClinicalTrials.gov identifier: NCT01405963). However, other small molecules targeting TSLP deserve to be further studied.

Stat3-targeted therapy in asthma

Targeted therapy has been widely explored and used in asthma. The use of biologics in asthma, includes the IgE-targeted antibody omalizumab, the IL-5-targeted antibody mepolizumab, the IL-13-targeted antibody tralokinumab, the IL-9-targeted monoclonal antibody MEDI-528 and TNFα receptor fusion proteins [58]. Here we found that inhibiting senescent signaling pathways overcome TSLP-induced airway remodeling (Figure 3 & 5). In addition, we found that inhibiting Stat3 by WP1066 can inhibit airway remodeling (Figure 4).

Previous reports have demonstrated the central role of Stat3 signaling in TSLP-regulated inflammation and airway remodeling in asthma [24]. Specifically, TSLP receptor (TSLPR) activation in bronchial epithelial cells involves Stat3 phosphorylation and induces IL-13 production and cell proliferation [59]. Furthermore, we demonstrated that signaling pathway of Stat3 mediated TSLP-induced airway remodeling in asthma [24].

Small molecules that target Stat3 are well established [60]. Several small molecules targeting Stat3 are currently being evaluated for head and neck tumors in Phase I/II clinical trials (clinicaltrials.gov), including WP1066. WP1066 is a cell-permeable, AG 490 tyrosphostin analog that effectively inhibits the Jak2-Stat3 pathway [61] and subsequently inhibits the growth of multiple kinds of cells [61–63]. Here we demonstrate that WP1066 targets Stat3 to inhibit airway remodeling in vivo and in vivo (Figure 4, 5) and this inhibition is mediated by inhibiting the senescent p21 and p16 signaling pathways. Furthermore, we found WP1066 treatment can overcome AHR in an asthma mouse model (Figure 5A ). AHR is a useful marker of airway abnormality in asthma and has been used to predict the course of asthma [64]. These data confirm the effects of Stat3 targeted therapy in asthma and encourage clinical studies to evaluate the therapeutic potential of Stat3-targeted therapy in asthma and the development of other small molecules that target Stat3. However, it is a concern that inhibiting senescent signaling promotes carcinogenesis. It will be important to evaluate the effectiveness and carcinogenesis of any new senescence pathway inhibitor.

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Author Contributions

Conceived and designed the experiments: LD RC J. Wu. Performed the experiments: J. Wu J. Wang JZ WG MY. Analyzed the data: J. Wu FD. Contributed reagents/materials/analysis tools: RW FD J. Wu. Wrote the manuscript: FD J. Wu.

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