Glycogen Synthase Kinase-3β Inhibition Attenuates the Progression of Pulmonary Fibrosis.

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ABSTRACT

RATIONALE: Idiopathic Pulmonary Fibrosis (IPF) is a progressive interstitial lung disease with a median survival of 3 years after diagnosis. Although the etiology of IPF is unknown, it is characterized by the extensive death of alveolar epithelial cells and the increased appearance of myofibroblasts. While the source of these myofibroblasts is diverse, these cells are believed to play an important role in disease progression. Because current treatments only slow the progression of the disease, identification of new more, efficacious targets is still warranted.
METHODS: Immunofluorescence, phase contrast microscopy, western blotting, quantitative PCR, adenoviral transduction, small molecule inhibition of cell signaling pathways, and other cell biology techniques. Intratracheal model of bleomycin mediated pulmonary fibrosis. Pulmonary function testing and CT scanning for volume analysis.
RESULTS: We first found that GSK-3β expression was enhanced in lungs isolated from bleomycin-injured mice compared to saline-treated controls. We next confirmed that TGF-β, Factor Xa, thrombin, plasmin and uPA induced α-SMA and Col-1 expression in normal and IPF fibroblasts. These same mediators enhanced GSK-3β activation via phosphorylation of tyrosine-216 (p-Y216) and induced GSK-3β localization to the nucleus. Down-regulation of GSK-3β (>75%) using a shRNA adenoviral vector, significantly reduced markers of fibroblast-myofibroblast differentiation by TGF-β. Inhibition of GSK-3β signaling with the novel inhibitor 9ING41 blocked the induction of myofibroblast markers, α-SMA and Col-1, and reduced morphological changes demonstrated by transitioning fibroblasts. Thus, GSK-3β inhibition reversed markers of established myofibroblast differentiation; α-SMA and Col-1. In *in vivo* studies, the progression of bleomycin mediated pulmonary fibrosis was significantly attenuated by GSK-3β inhibition with 9ING41, intraperitoneally administered 14d post injury. Specifically, 9ING41 treatment significantly improved lung function (compliance) and lung volumes ($p<0.05$) of injured mice compared to untreated and vehicle-treated controls. Further, myofibroblast markers (α-SMA and collagen) were reduced in 9ING41 treated mice compared to untreated and vehicle-treated controls. Fibrotic lesions were also reduced by 9ING41 treatment.
CONCLUSIONS: These are the first studies to demonstrate that the GSK-3β signaling pathway is critical for the induction of myofibroblast differentiation. These studies also show that the therapeutic targeting of GSK-3β attenuates the progression of pulmonary fibrosis. This work provides a rationale for continued investigation of the GSK-3β signaling pathway in the control of fibroblast-myofibroblast differentiation and outcomes of pulmonary fibrosis. The therapeutic targeting of GSK-3β appears to mitigate fibrotic pulmonary remodeling *in vivo*.

Introduction

Because most instances of pulmonary fibrosis (PF) are idiopathic in nature, idiopathic pulmonary fibrosis (IPF) is the most common form of fibrotic lung disease. The overall incidence and prevalence of IPF in the United States is 14.0 - 42.7 and 16.3 per 100,000 persons respectively. The median survival of IPF patients ranges from 2.5 to 3.5 years after diagnosis with a median age of 65 years. The mean age of presentation at initial diagnosis is 63 years with familial cases presenting earlier at 59.4 years. Over 30,000 people die from PF every year in the United States. Although several treatments such as pirfenidone and nintedanib have been identified, they only slow the progression of this deadly disease. These considerations justify the search for new targets and an effective intervention to mitigate mortality from PF.

Chronic infiltration by and proliferation of α-SMA expressing myofibroblasts results in progressive fibrosis. These cells promote the accumulation of matrix proteins, including collagen, that contribute to PF. This damage eventually culminates in destruction of alveolar architecture, formation of fibrotic foci, and compromise the ability of the lung to facilitate normal gas exchange. Upon activation, fibroblasts can transition into myofibroblasts that are largely responsible for the increased collagen and matrix synthesis and deposition found in PF.

GSK-3 is a serine/threonine kinase that was first identified as a regulator of glycogen metabolism and insulin signaling, through the regulation of glycogen synthase. GSK-3 has 2 isoforms, α and β. GSK-3β functions overlap with those of GSK-3α, however the converse is not true as GSK-3β knockout mice are embryonically lethal. Although GSK-3β is a constitutively active kinase, its activity is potentiated by phosphorylation of the tyrosine 216 residue (Y216). GSK-3β is also reported to regulate numerous transcription factors including NFκB, CREB, myocardin and myocardin-related transcription factors in different organs and systems. Aberrant GSK-3β activity contributes to pathological conditions including Alzheimer's disease, diabetes mellitus, and carcinogenesis.

While some studies have reported that inhibition of GSK-3β promotes myofibroblast differentiation and increased expression of mesenchymal markers, others show that GSK-3β inhibition blocks fibroblast activation and reduces indices of lung injury. Further, our laboratory showed that the novel GSK-3β inhibitor, 9ING41, reduced myofibroblast differentiation of pleural mesothelial cells in our empyema model and improved lung function and pleural injury outcomes. We present here our findings in normal and IPF fibroblasts and in our mouse model of pulmonary fibrosis.

Methods

- Mouse Model of Pulmonary Fibrosis: All experiments involving animals were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at Tyler. C57Bl/6j mice (20 g, Jackson Laboratories) were anesthetized by ketamine/xylazine IP injection. Mice received bleomycin sulfate (0.8U/kg, Teva) via intratracheal administration. The control group received normal saline under the same conditions. Mice were periodically monitored following injury to record body weight, dehydration status, and general well-being.
- shRNA treatment of fibroblasts: Normal and IPF fibroblasts were infected with eGFP adenovirus or an adenoviral vector encoding GSK-3β shRNA (Vector Biolabs). Post infections, cells were incubated in normal complete media for 24h prior to serum-starvation in RPMI 1640.
- Agonist Treatment of normal and IPF fibroblasts: Serum-starved fibroblasts were treated with Factor Xa (7nM), thrombin (7nM), plasmin (6 nM) or uPA (20 nM) in RPMI-1640. TGF-β (5 ng/ml) was used as a positive inducer of myofibroblast differentiation. Collected conditioned media were analyzed by Western blot for changes in collagen-1 (Col-1, Biotinylated Southern Biotech). Cellular lysates were immunoblotted for alpha smooth muscle actin (α-SMA, Mab Sigma-Aldrich) and GSK-β (Cell Signaling). β-actin and Akt were used as loading controls.
- GSK-3β Inhibition: To inhibit GSK-3β, the selective inhibitor 9ING41 (Actuate Therapeutics, 10-0.5 μM) was added to serum free media for up to 48h. For blockade studies, 9ING41 was added 24h prior to addition of the agonists. For reversal studies, 9ING41 was added 24h after the agonist was applied.
- Fibroblasts: Up to 4 Normal and IPF fibroblasts lines were analyzed for the data presented herein. These fibroblast lines were a generous gift from Dr. Eric White, University of Michigan.

Results

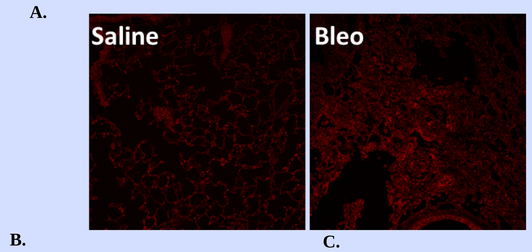

Figure 1. Various mediators induce myofibroblast differentiation of normal and IPF fibroblasts. A, Lung sections from saline and bleomycin treated mice were stained for GSK-3β. GSK-3β is increased in injured mice. Serum starved human fibroblasts (normal, B and D, and IPF, C and E) were treated with various mediators to induce myofibroblast differentiations(TGF-β, Factor Xa, thrombin, plasmin and uPA). Cell lysates and conditioned medias were then resolved by SDS-PAGE and Western blotted for α-SMA and collagen 1, markers of myofibroblast differentiation. All mediators induce α-SMA expression and GSK-3β expression. Collagen was most prominently induced by TGF-β and FXa. All tested mediators induced distinct phenotypic changes in fibroblast morphology (D and E).

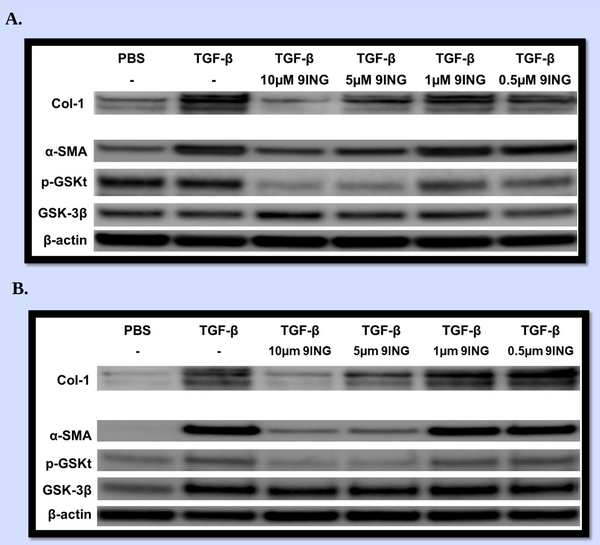

Figure 2 GSK-3β inhibition with 9ING41 reduces fibroblast activation. Fibroblasts (normal, A, and IPF, B) were treated with various doses of 9ING41 (10-0.5 μM) in serum free media. Cells were then treated with TGF-β for 48h. Conditioned medias and cell lysates were then resolved by SDS-PAGE and immunoblotted for Col-1 and α-SMA. TGF-β induced Col-1 and α-SMA protein in fibroblasts. 9ING41 (10 and 5μM) blocked TGF-β mediated induction of α-SMA and Col-1. Tyr-216 phosphorylation of GSK-3β (pGSKt) was likewise reduced by 9ING41. Similar results were found in qPCR analyses.

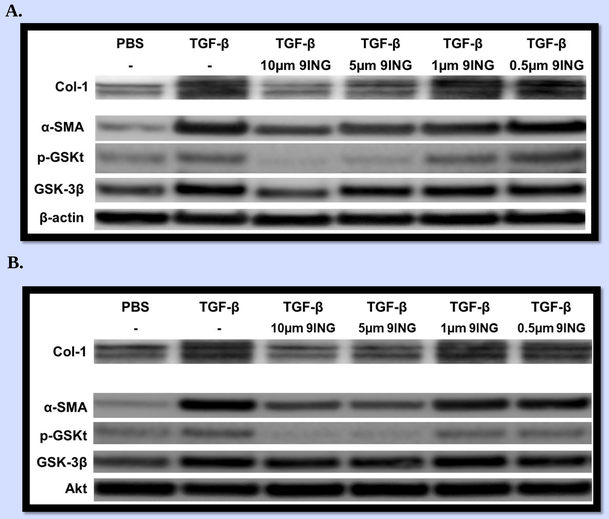

Figure 3 GSK-3β inhibition with 9ING41 reverses induction of fibroblast activation. Fibroblasts (normal, A, and IPF, B) were first treated with TGF-β for 24h in SFM. Cells were then treated with various doses of 9ING41 (10-0.5 μM) in serum free media for 24h. Conditioned medias and cell lysates were then resolved by SDS-PAGE and immunoblotted for Col-1 and α-SMA. TGF-β induced Col-1 and α-SMA protein in fibroblasts. 9ING41 (10 -1μM) attenuated α-SMA and Col-1 induction. We next treated fibroblasts with TGF-β for 24h, then added 9ING41 (10-1μM), and incubated for another 24h (B). 9ING41 (10 and 5 μM) attenuated increases in Col-1 and α-SMA. These findings were confirmed by qPCR analyses (data not shown). Tyr-216 phosphorylated GSK-3β was also reduced by 9ING41. 9ING41 reversed TGF-β mediated myofibroblast differentiation.

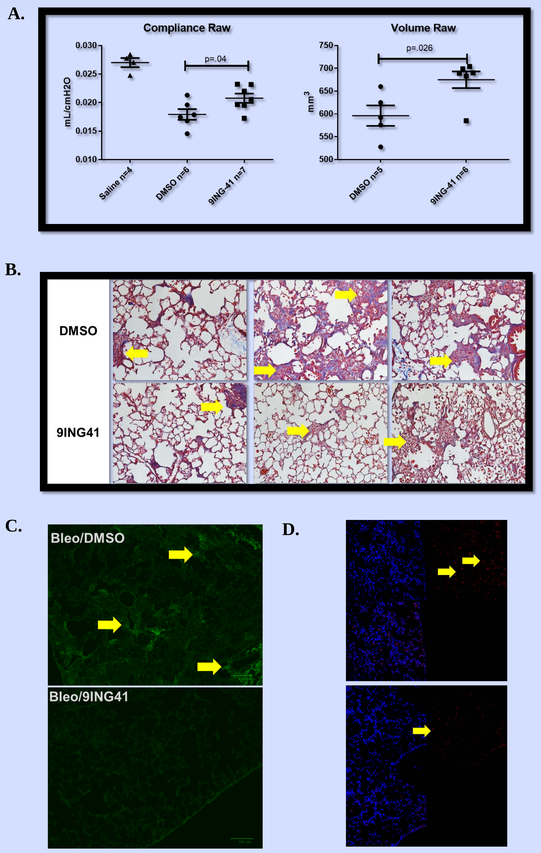

Figure 5. GSK-3β inhibition attenuates pulmonary fibrosis. C57Bl/6J mice were intratracheally administered bleomycin sulfate. After 14d, mice were treated with either vehicle (DMSO) or 9ING41 (30mg/kg) for 14d, A. At the completion of the 28d course, lung compliance was determined using the Scireq flexivent. Lung renditions were also collected by CT scan to determine lung volumes. B, Lung tissue sections from vehicle and 9ING41-treated mice were Trichrome stained and imaged at 20X. Images are representative of 30 fields/slide/mouse, n=6 animals per treatment. Lung sections from vehicle and 9ING41-treated mice were immunostained for Col-1 (C) or α-SMA (D) and imaged by confocal microscopy at 40X. 9ING41 reduced collagen and α-SMA expression in treated mice. Solid arrows indicate areas of collagen deposition (C) or α-SMA expression (D). Images are representative of 30 fields/mouse and n=3 mice/treatment.

Conclusions

1. GSK-3β expression is increased in a mouse model of pulmonary fibrosis.
2. Targeting of GSK-3β attenuates the induction of myofibroblast differentiation.
3. Phosphorylation of GSK-3β at Tyr-216 appears critical for fibroblast activation.
4. GSK-3β inhibition reduces the extent of pulmonary injury *in vivo*.
5. GSK-3β inhibition may represent a new therapeutic target for the treatment of pulmonary fibrosis.

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