

Protective Effect of Type I Collagen Antisense Oligonucleotides on Bleomycin Induced Pulmonary Fibrosis

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Abstract: *Background:* Pulmonary fibrosis is a chronic and usually untreatable fatal lung disease. It can result from many endogenous or exogenous causes, such as infection, chronic inflammatory diseases, environmental toxins, chemical poisoning, radiation and chemotherapy. The accumulation of collagen, largely type I collagen rich extracellular matrix (ECM) is the hallmark of lung fibrosis. Current therapies for pulmonary fibrosis utilizing corticosteroids and immunosuppressants have demonstrated only marginal effectiveness.

Methods: In the present study, we tested the efficacy of the mouse type I collagen antisense oligodeoxynucleotides (AS61-ODN, a 20 mer) in bleomycin induced pulmonary fibrosis in mice. Bleomycin was instilled transtracheally by direct tracheal cut down in mice, and oligonucleotides (ODN) were injected through the tail vein. Hematoxylin and eosin (H & E) and Masson's trichrome staining were done to determine the cellular architecture of the lungs after bleomycin and oligonucleotide treatment. Type I procollagen gene *COL1A1* expression was determined by Northern blot analysis. Type I collagen protein expression was determined by Western blot analysis and immunofluorescence.

Results: There was much less disruption of the lung architecture as indicated by significant decrease in the Ashcroft score, when mice were injected with AS61-ODN following bleomycin instillation. AS61-ODN significantly reduced the mRNA and protein expression of type I collagen by 45.2 % ($p < 0.015$) and 35.0 % ($p < 0.004$) respectively 21 days after treatment in bleomycin induced fibrotic lung tissue. Immunofluorescence of bleomycin induced mouse lung tissue treated with AS61-ODN showed a specific reduction in collagen expression and fibrosis.

Conclusion: Human type I collagen antisense oligodeoxynucleotides (AS60-ODN, a 20 mer), which is 99 percent homologous with mouse AS61-ODN, might be useful as a potential therapeutic agent to treat patients with pulmonary fibrosis.

Keywords: Type I collagen, COL1A1, antisense oligonucleotides, AS61-ODN, pulmonary fibrosis, bleomycin.

INTRODUCTION

Pulmonary fibrosis is a condition in which the lungs of a patient become scarred and fibrous. It is characterized by fibroblast proliferation and excessive synthesis and deposition of collagen and other extracellular matrix proteins [1-4], which result in irreversible distortion of the lung's architecture [5]. Gradually, the alveoli of the lungs are replaced by fibrotic tissue. The scarred lung tissue becomes thicker causing an often irreversible loss of the tissue's ability to transfer oxygen into the bloodstream, resulting in respiratory failure. Pulmonary fibrosis is a common problem of end-stage lung disease caused by environmental toxins, radiation, or chemotherapy treatments for cancer or many chronic inflammatory diseases [6-8]. Environmental exposure has been shown to associate with the development of idiopathic pulmonary fibrosis (IPF), the most serious interstitial lung disease (ILD), which is considered to be triggered by chronic epithelial injury [9, 10]. The present report focuses on the chemotherapeutic agent bleomycin

inducing pulmonary fibrosis in mice as a model for clinical pulmonary fibrosis in humans.

Bleomycin is an antibiotic with antitumor activity [11], highly effective against many malignant diseases [12, 13]. The application of bleomycin is complicated by the occurrence of sometimes the fatal side effects [14]. Among its side effects, however, the most important is interstitial pneumonia occurring during administration of the drug, followed by chronic fibrotic changes in the lung [15]. Lung injury induced by bleomycin is characterized by an increased deposition of interstitial extracellular matrix proteins in the alveolar wall that compromises respiratory function [2]. A recent study by Carver *et al.* [16] on the incidence of long-term cardiac or pulmonary toxicity secondary to chemotherapy and radiotherapy concluded that there is an increased incidence of cardiac and/or pulmonary dysfunction in cancer survivors.

At present, there is no animal model of pulmonary fibrosis to mimic all the cardinal features of the human disease. However, the bleomycin induced pulmonary fibrosis is largely and most frequently used, since it produces inflammation and fibrosis as seen in human lung fibrosis

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[15-19]. It has been well established that a single injection of bleomycin *via* the intratracheal route consistently produces lung injury and resultant fibrosis in rodents. Therefore, the intratracheal instillation of bleomycin has been a leading model of pulmonary fibrosis for many years [17], and in use today [15].

Several drugs have been tested in animal models to suppress inflammation and to treat lung scarring, but have not led to a useful clinical treatment [18, 19]. Hence there is a critical need for effective and safe therapies. We have previously demonstrated that the type 1 collagen antisense oligodeoxynucleotide, AS60-ODN (a human analog of mouse AS61-ODN) reduced the mRNA and protein expression of type 1 collagen in a novel *ex vivo* human wound injury model [20].

Antisense oligodeoxynucleotides (ASO) are now being widely evaluated for the treatment of many diseases, including muscular dystrophy [21], hypertension [22, 23], viral infections [24], cancer [25] and pulmonary fibrosis [26]. In addition, other studies have shown that ASOs to different collagen subtypes have been successful in reducing the expression of the targeted mRNA in different cell lines [27, 28]. Compared with viral vectors, ASOs are biodegradable, have lower toxicity, and are nonimmunogenic. Despite concerns about nonspecific effects, however, ASOs have very acceptable toxicity profiles [23, 29].

Here, we report that type I collagen expression is reduced by delivering AS61-ODN in bleomycin induced murine pulmonary fibrosis. Northern and Western blots, and immunohistochemical analysis showed that this novel AS61-ODN was effective in reducing type I collagen mRNA and protein expression and fibrosis in bleomycin treated mouse lung tissue. Human AS60, which is 99 percent homologous with mouse AS61-ODN has the potential to be used as a therapeutic agent for treating patients with pulmonary fibrosis.

MATERIALS & METHODS

Oligonucleotide Synthesis

We have obtained purified phosphorothioate oligodeoxynucleotide, synthesized by AstraZeneca, Mereside, England, using the following protocol: Phosphorothioate oligodeoxynucleotide synthesis was carried out at a 1 μ m scale on PE Biosystems 394 DNA synthesizer using phosphoramidite chemistry with TETD/acetone nitrile sulphurizing reagent. Oligonucleotides were purified on Poly-Pak TM II cartridges (Glen Research), desalted on NAPTM 10 columns (Amersham Pharmacia Biotech) and using Dowex 50WX8-100 ion exchange resin [30]. Initially, two hundred and ten ASOs to *COL1A1* were designed and synthesized (AstraZeneca, Mereside, England). AS60/AS61-ODN was selected after an elaborate screening process. As a control, the sense oligonucleotide of this same sequence (S61-ODN), was generated and used in parallel with AS61-ODN.

The sequence of the oligonucleotides used in this study were, S61-ODN- 5' CGCATGGCCAAGAAGACAGT - 3'(sense), AS61-ODN- 5' ACTGTCTTCTTGCCATGCG - 3'(antisense).

Bleomycin Induced Pulmonary Fibrosis in Mice

Bleomycin Sulfate from Sigma-Aldrich (1.1U/kg) in 40 μ l of sterile physiological saline was instilled transtracheally by direct cut down into the tracheal lumen in specific pathogen free 10 week old male C57BL/6 mice, after anesthetizing with isoflurane. Mice were obtained from NCI-Charles River laboratories. After one week of treatment with bleomycin, sense and antisense type I collagen oligonucleotides were injected through the tail vein (2.5 mg/kg ODNs plus 5.0 mg/kg of PEI in 100 μ l saline) once every two days for a total of ten injections. We used cationic polymer polyethylenimine (PEI), which has been widely used for non-viral transfection *in vitro* and *in vivo* and has an advantage over other polycations in that it combines strong DNA compaction capacity with an intrinsic endosomolytic activity [31]. Injections were done under anesthesia with isoflurane. 21 days later, mice were sacrificed by rapid exsanguination and lungs were collected, weighed and frozen for pathology; gene and protein expression analysis. All the animal experiments were approved by the Laboratory Animal Care Committee, and conducted in accordance with the Guidelines for Animal Experimentation of the Baylor College of Medicine, Houston, Texas.

In Vivo Delivery of Biotin labeled AS61-ODN

Initially, we performed this experiment to verify that the oligonucleotides were delivered to the lung tissue of experimental mice. We administered fluorescent biotin labelled AS61-ODN (Jackson Immuno Research Laboratories, Inc.) with PEI- branched (Sigma-Aldrich) vehicle in 100 μ l saline (a: AS61 (2.5 mg/kg) plus PEI (5.0 mg/kg) and b: AS61-ODN (5.0 mg/kg) plus PEI (10.0 mg/kg) by i.v. injection. After 24 hours, mice were sacrificed and the lung tissues were harvested and frozen. Frozen lung tissues were sectioned using a cryostat microtome, mounted in Tissue-Tek OCT compound (Invitrogen) compound and stained with Texas Red (Jackson Immuno Research Laboratories, Inc). Sections were visualized under the fluorescent microscopy and pictured. Fig. (1) shows that the AS61-ODN was delivered to lung tissue efficiently.

Histopathology Examination - (H&E) and Masson's Trichrome Staining

Morphological analyses were performed on formalin fixed, paraffin-embedded lung tissue sections from normal saline control, bleomycin treated experimental, and various oligonucleotides (AS61 and S61-ODN) treated mice. Sections were stained with haematoxylin and eosin (H&E) and Masson's trichrome. Histopathologic evaluation of pulmonary fibrosis was performed using Ashcroft scoring method, and followed blind principle [32].

Immunofluorescence Staining for Type I Collagen

Lung tissues from normal control, bleomycin and oligonucleotide-treated mice were embedded in Tissue-Tek OCT compound (Invitrogen), and allowed to cool on dry ice.

Frozen tissue blocks were stored at -80°C until use. Tissues were thawed to - 20°C and cut on a cryostat (Leica CM 1800) in 6 μ m thick slices and fixed with 3.7% paraformaldehyde. All subsequent labeling steps were performed at room temperature unless indicated otherwise.

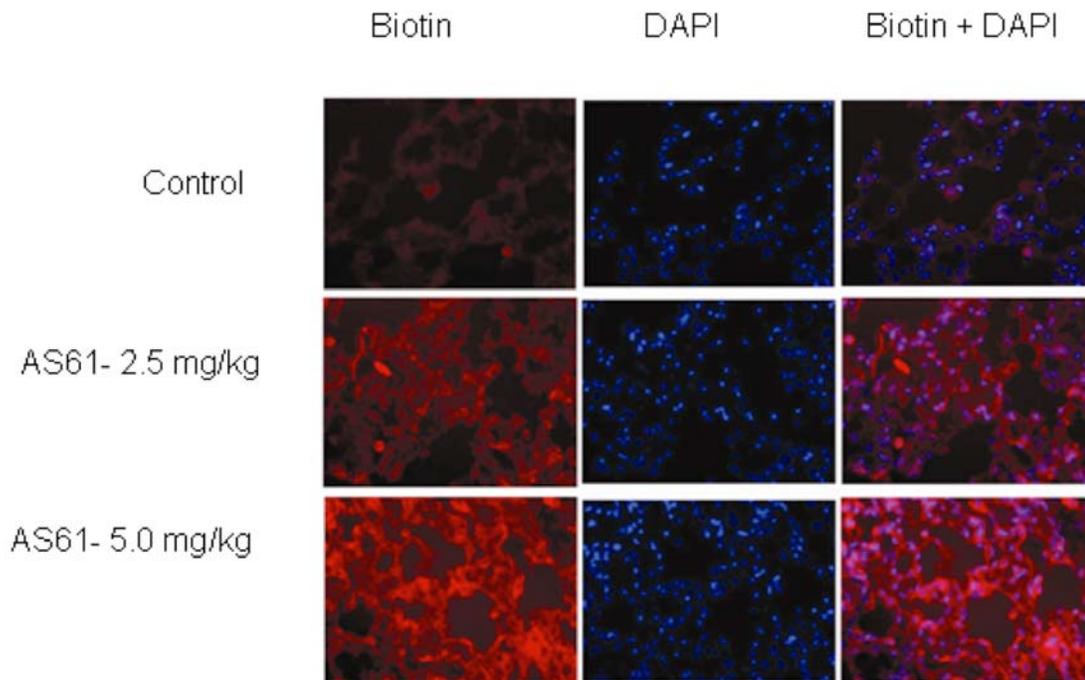


Fig. (1). Fluorescence of lung samples from normal saline control, and fluorescent labelled biotin AS61-ODN injected mice. Tissue sections were counterstained with nuclear stain DAPI; viewed under the fluorescence microscope (magnification $\times 40$).

Slides were washed 3 times for 10 minutes in PBS and treated with 0.5% Triton X-100 in PBS for 5 minutes. After slides were washed again as described above, they were blocked for 2 hours with 1% BSA (Sigma Chemical Co), then incubated overnight with a rabbit anti-human collagen type I antibody (Chemicon International) diluted 1:100 in PBS plus 1% BSA. Tissue sections were washed in PBS and incubated in PBS plus 1% BSA with a biotin-conjugated goat anti-rabbit antibody (1:1000, Jackson ImmunoResearch) for 15 minutes. Sections were washed and incubated for 15 minutes with avidin Texas Red ($1\mu\text{g}/\text{mL}$; Jackson ImmunoResearch). Sections were then counterstained for 2 minutes with DAPI. All slides were viewed with a fluorescence Nikon E600 microscope, and images were captured on a Digital camera (Kodak).

Northern Blot Analysis

Northern blot analysis was performed as described by Focht & Adams 1984 [33]. High quality total RNA was prepared from lung tissue from normal, bleomycin treated and bleomycin plus oligonucleotide treated mice by glyoxalation. Equal amounts of total RNA were separated by electrophoresis on 1.5 % agarose RNA gels and blotted to Gene Screen membranes (PerkinElmer, Wellesley, MA). The membranes were cross linked and then hybridized with a ^{32}P -radiolabelled *COL1A1* cDNA probe (A gift from Dr. Decrombruegge, MD Anderson Cancer Center). The intensity of the specific bands was quantitated by laser densitometry and Labworks4 gel documentation software (UVP Inc., Upland, CA).

Western Blot Analysis

Type I collagen protein expression was measured by Western blot analysis. Lung tissue from normal control, bleomycin, and bleomycin plus oligonucleotide-treated mice

was homogenized in ice cold lysis buffer (Leupeptin 10 mg/ml, Pepstatin 10 mg/ml, Antipain 5mg/ml, PMSF 34.84mg/ml Trypsin 10mg/ml, Benzamidin 10mg/ml, Aprotinin 5-10 TIU/ml all sigma in PBS). Lung tissue was cut into small pieces and placed in tubes with 5 mL of ice cold lysis buffer. Tissues were placed in an ice bath and homogenized with a Polytron tissue disruptor for 2 minutes. Lysates were centrifuged for 15 minutes at 12,000g at 4°C and the supernatants frozen at -80°C. Total protein was determined by Bradford assay (Bio-Rad). Lysates were subjected to Western blot analysis. Equal amounts of total protein (20 μg) were loaded in each lane. Blots were blocked and incubated overnight at 4°C with a polyclonal rabbit anti-human collagen type I antibody (Chemicon International, Temecula, CA) diluted 1:100 in 5% non fat milk. After washing in PBS plus 0.05% Tween 20, blots were incubated with a ECL anti-rabbit IgG, Horseradish Peroxidase linked whole antibody (from donkey), (GE Healthcare UK Limited Little Chalfont Buckinghamshire HP7 9NA UK) 1:2000 for 1h. After wash, Type I collagen protein expression was detected with the ECL Western blotting detection kit (Pharmacia Biosciences, Piscataway, NJ) and quantitated by laser densitometry and Labworks4 gel documentation software (UVP Inc).

Statistical Analysis

Paired Student's t-tests were conducted using Microsoft Excel 2003 (Redmond, WA) The p values were two-tailed and considered significant if less than or equal to 0.05.

RESULTS

Histo-Morphological Analysis

As it has been previously reported [17, 34-37] and as expected, bleomycin instillation caused a disruption of the

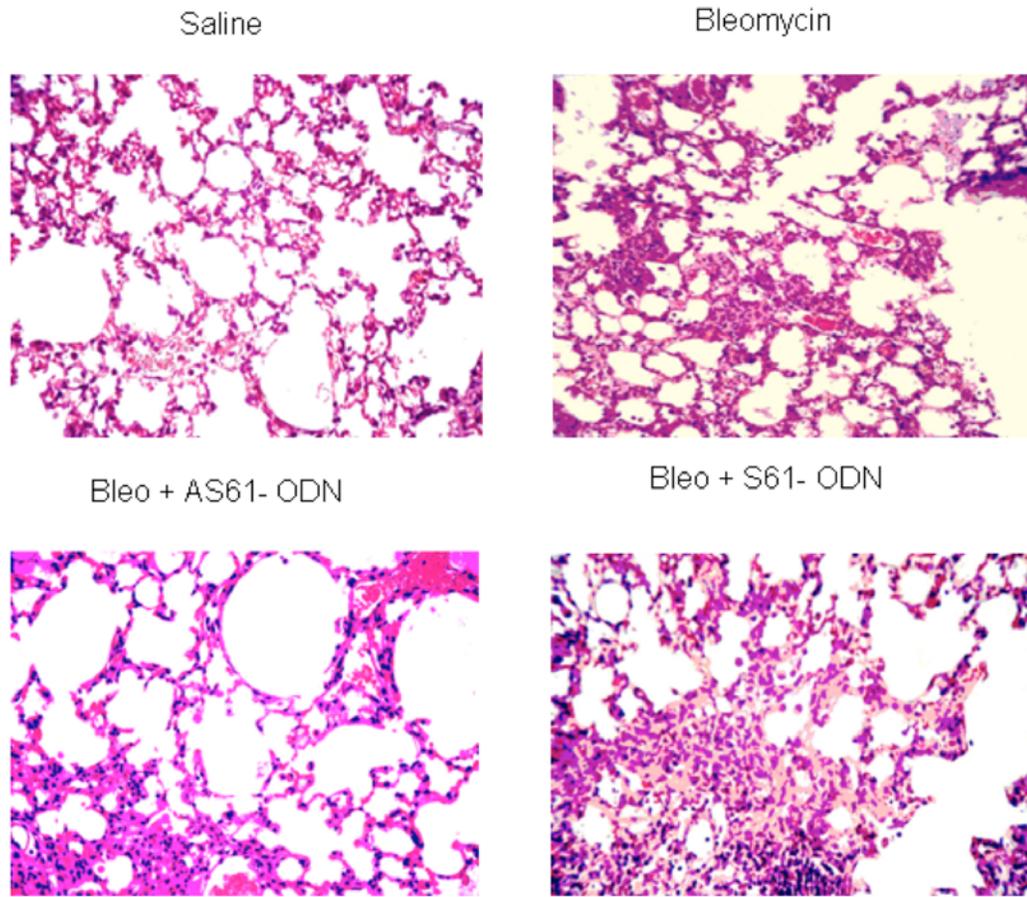


Fig. (2). Histo-morphological appearances of lung samples from normal saline control, bleomycin treated, and bleomycin plus AS61 and S61-ODN treated mice. Tissue sections were stained with hematoxylin-eosin and viewed under the light microscope (magnification $\times 40$).

normal lung architecture, especially collapsed alveoli, along with areas of inflammatory cell infiltrates and foci of fibroblasts, as determined by H&E staining of cryosections (Fig. 2). There was much less disruption of the lung architecture as indicated by significant decrease in the Ashcroft score, when mice were injected with AS61-ODN following bleomycin instillation (Table 1). H&E and Masson's trichrome staining showed that lung tissue samples in saline treated control mice had a normal pulmonary architecture and only a few alveolar macrophages, while the samples in bleomycin treated mice had typical fibrotic changes including infiltration with excessive inflammatory cells (Fig. 2), collagen depositions, large fibrous areas and collapsed alveolar spaces (Fig. 3).

Table 1. Effect of AS61 in Bleomycin-Induced Pulmonary Fibrosis

Mouse group	Ashcroft score
Bleomycin treated	6.4 ± 1.1
Antisense (AS61) treated	$4.0 \pm 1.2^*$
Sense (S61) treated	6.0 ± 0.96

Data represent mean \pm SD, n=6-7, * p < 0.01 vs BLM group.

Initially, we tested whether the AS61-ODN had been delivered to lungs of experimental mice by injecting (i.v)

fluorescent biotin labelled AS61-ODN (Fig. 1). Fluorescent biotin labelled AS61-ODN (2.5 and 5.0 mg/kg) was delivered efficiently as shown in Fig. (1).

The effects of AS61-ODN on $\alpha 1$ type 1 collagen (*COL1A1*) mRNA expression were determined by standard Northern blot techniques. Mice injected with bleomycin were treated with AS61 and S61-ODN (2.5 mg/kg) or saline. There was an increase (164 %) of *COL1A1* mRNA expression in bleomycin treated group of mice when compared with the saline treated control group, while *COL1A1* mRNA expression was 45.2 % (p < 0.015) lower in the AS61-ODN treated group than bleomycin treated group of mice. Total RNA was collected from each sample, and ethidium bromide staining ensured equal loading of the samples (Fig. 4B).

Western blots of lung tissues from saline control mice and mice injected with bleomycin experimental mice, and mice treated with AS61 and S61-ODN (2.5 mg/kg) were quantified. There was a 35.0 % (p < 0.004) decrease in detectable levels of type I collagen when mice were injected with AS61-ODN (Fig. 5A) compared to controls. Immunofluorescence analysis of the tissues confirmed that 2.5 mg/kg of AS61-ODN was the lowest concentration of oligonucleotide required to see a reduction in the total amount of type I collagen expression in mouse lung tissue (Fig. 6). In addition, the morphology of the cells upon DAPI

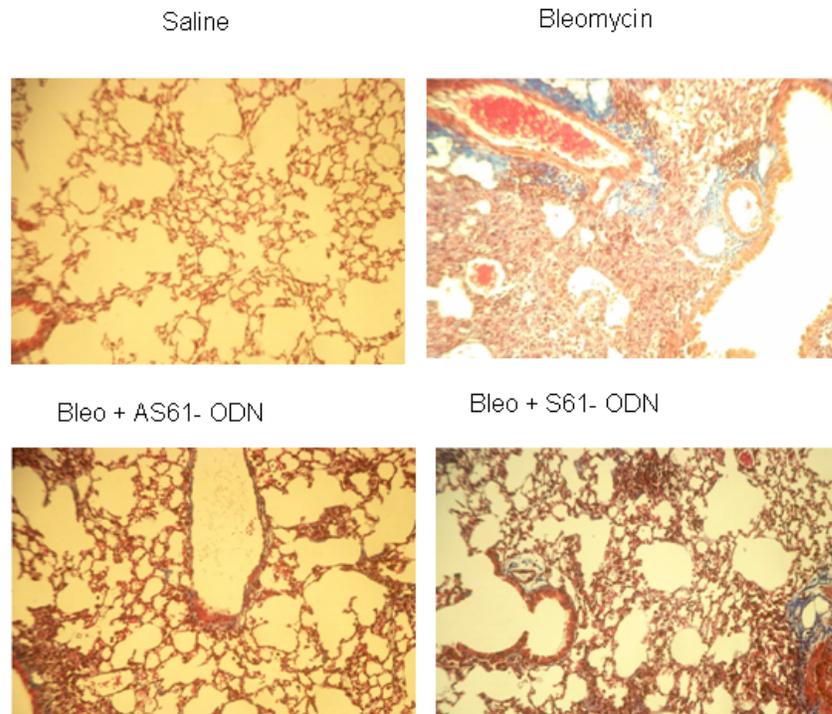


Fig. (3). Histo-morphological appearances of lung samples from normal saline control, bleomycin treated, and bleomycin plus AS61 and S61-ODN treated mice. Tissue sections were stained with Masson's trichrome and viewed under the light microscope. The blue staining represents collagen deposition. (magnification $\times 40$).

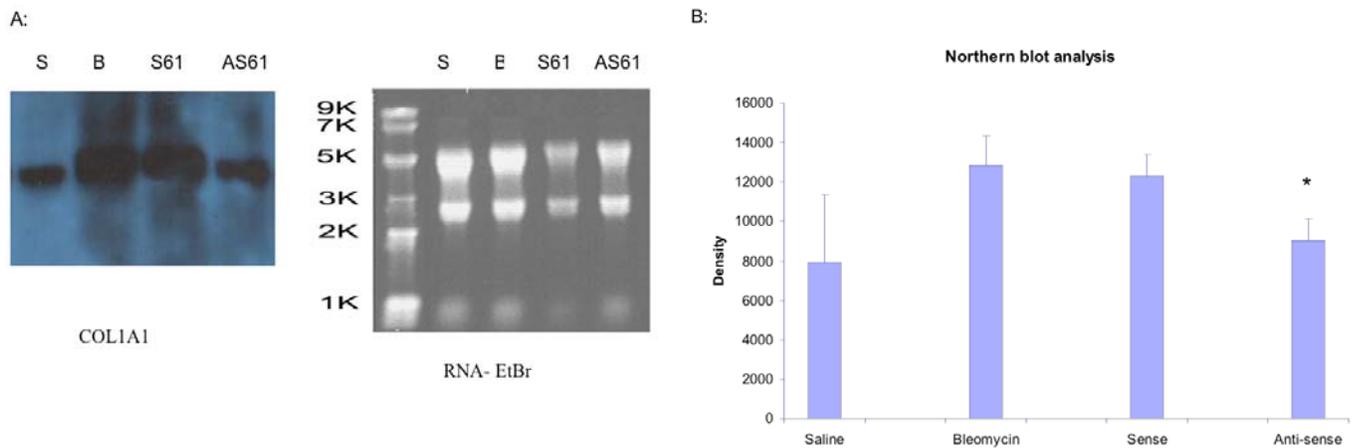


Fig. (4). **A:** Northern blot analysis of $\alpha 1$ type I collagen mRNA expression in lung tissue samples from saline (S), bleomycin treated (B), and bleomycin plus AS61 and S61-ODN treated mice. Reduced level of type I collagen mRNA expression from mice lung tissue treated with AS61-ODN (A). Total RNA from each sample was stained with ethidium bromide (B). Presence of 28S, 18S and 5S rRNA is indicated on each gel. Northern blots of the same samples were labeled with the ^{32}P -radiolabeled *COL1A1* cDNA probe.

B: Quantification of type I collagen mRNA expression. Autoradiographs were subjected to band intensity analysis. The densities were averaged from 3 separate experiments \pm SD, and P values were analyzed by Student's t-test. Levels of mRNA expression of the saline control experiments were set to 100%. Levels of *COL1A1* mRNA from mice lung tissues treated with bleomycin, anti-sense AS61 and sense S61-ODN are shown. * indicates a $P < 0.015$.

staining demonstrated that this dose (2.5 mg/kg) was not toxic to the tissues.

As expected in this study, expression of type I collagen mRNA and protein in the lung was increased during bleomycin-induced pulmonary fibrosis when compared to the saline treated control mice. AS61-ODN treatment

resulted in 45.2 % ($p < 0.015$) and 35.0 % decrease ($p < 0.004$) in type I collagen mRNA and protein expression respectively 21 days after treatment. Immunofluorescence of bleomycin induced mouse lung tissue treated with AS61-ODN also showed a specific reduction (41.3 %) in collagen expression. Our findings demonstrated that the intravenous introduction of antisense oligonucleotides against type I

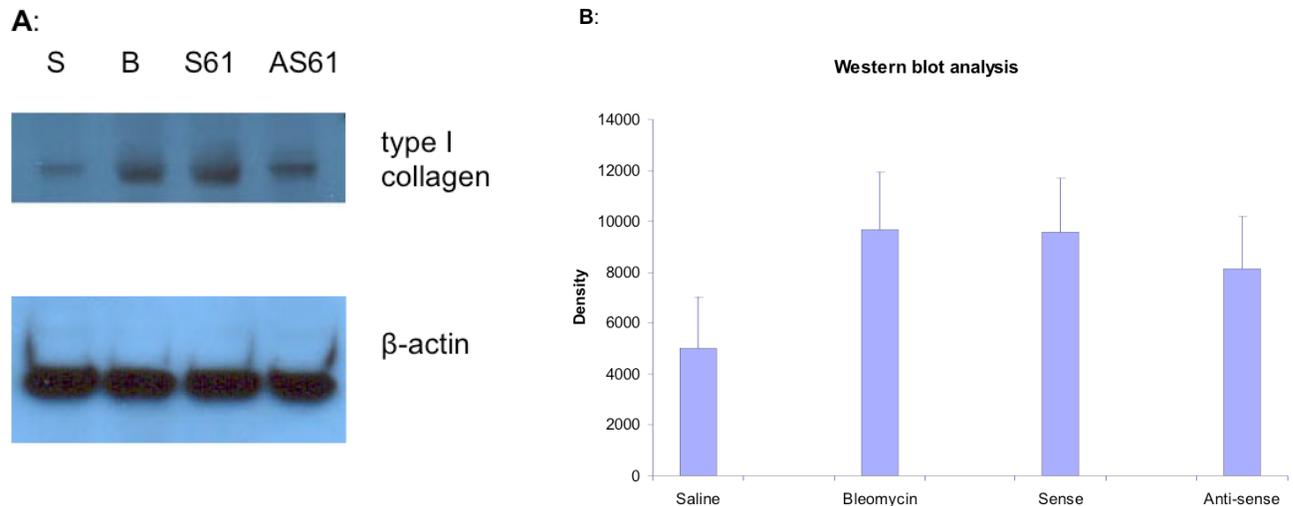


Fig. (5). **A:** Western blot analysis of type I collagen protein expression in lung tissue samples from saline (S), bleomycin treated (B), and bleomycin plus AS61 and S61-ODN treated mice lung tissue. Reduced expression of type I collagen protein from mice lung tissue treated with AS61-ODN (2.5 mg/kg). Western blots of cell-lysates were probed with a peroxidase labeled anti-mouse type I collagen antibody (A).

B: Quantification of type I collagen protein expression. Western blots were subjected to band intensity analysis. The densities were averaged from 3 separate experiments \pm SD, and P values were analyzed by Student's t-test. Levels of type I collagen from saline control experiments were set at 100%. Type I collagen protein expression from mice lung tissue treated with bleomycin, anti-sense AS61 and sense S61-ODN are shown. * indicates a $P < 0.004$.

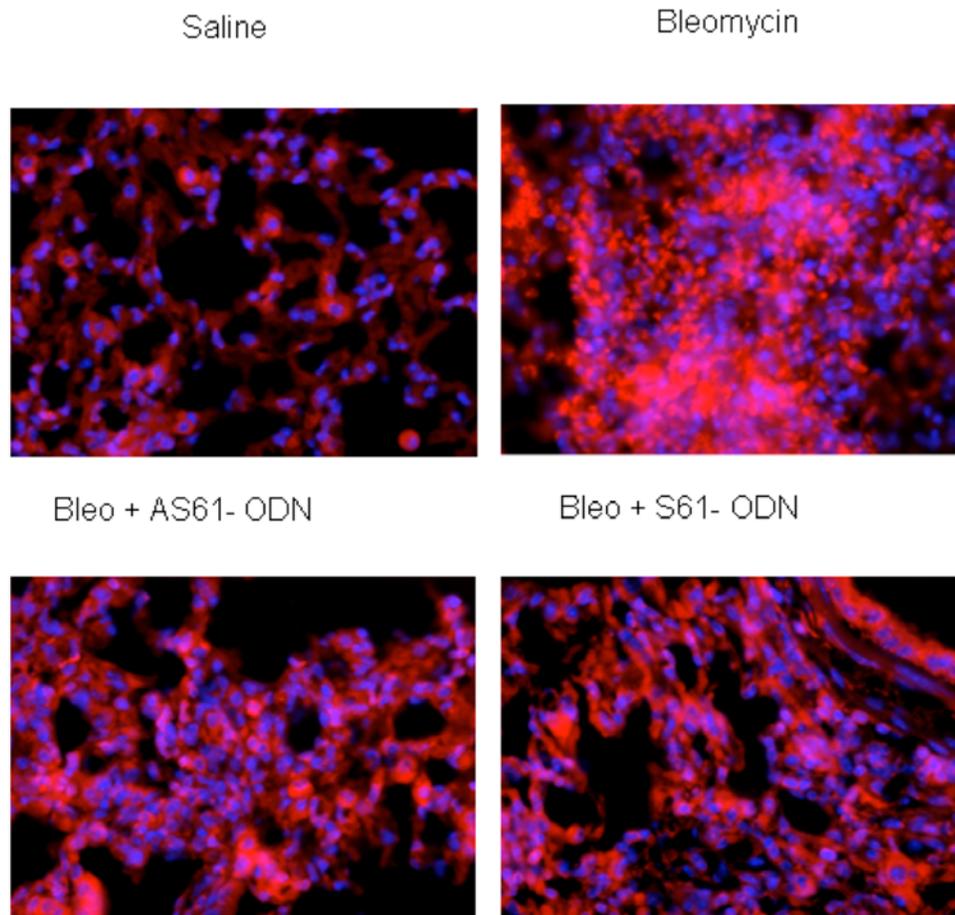


Fig. (6). Immunofluorescence analysis of type I collagen expression in lung tissue samples from normal saline (S), bleomycin treated (B), and bleomycin plus AS61, and S61-ODN treated mice. Tissue sections were stained for mouse anti-type I collagen, counterstained for nuclear stain DAPI; and viewed under the fluorescent microscope (magnification $\times 40$).

collagen effectively decreases both gene and protein levels of type I collagen and reduces fibrotic lesions. Thus, we demonstrated the protective effect of AS61-ODN on bleomycin induced pulmonary fibrosis in mice.

DISCUSSION

Fibrosis is an important cause of morbidity and mortality in a variety of lung diseases [38, 39]. According to a recent report from the Pulmonary Fibrosis Foundation (USA), there are five million people worldwide and around 200,000 patients in United States, that are affected by pulmonary fibrosis. Of these, more than 40,000 patients die annually. Currently, there is no effective treatment or a cure for pulmonary fibrosis. Excessive accumulation of collagen, specifically type I collagen during the development of fibrosis is a key factor contributing to this mortality [1, 3, 4, 38, 39]. Although a moderate degree of collagen synthesis and accumulation is acceptable in organ fibroses, it often accumulates in excessive amounts and impairs the normal function of the affected organ. Previous studies have shown that directly targeting collagen expression with ASO to collagen subtypes is successful in lowering collagen expression. For instance, treatment with ASOs to collagen type III or procollagen type V, important collagen subtypes in ligaments and tendons, results in reduced collagen expression in human patellar tendon fibroblasts [40, 41] and in animal model [42]. Lowering collagen expression in these tissues may have implications in improving their mechanical properties during the healing process of ligaments and tendons. Our own study demonstrated the efficacy of directly targeting the *COL1A1* collagen gene using antisense to type I collagen, reducing collagen expression in a human-skin organ model [20].

Type I collagen is a major constituent of the skin, ligaments, tendons, bone, and numerous interstitial connective tissues [43]. This fibrillar collagen constitutes about 85% of mature scar tissue and is a critical molecule in fibrotic processes [44]. It is a triple helical molecule composed of two $\alpha 1$ polypeptide chains from the *COL1A1* gene and one $\alpha 2$ polypeptide chain from the *COL1A2* gene [45]. Under normal conditions, the $\alpha 1(I)$ and $\alpha 2(I)$ genes are expressed at a 2:1 ratio [43].

Different approaches have been investigated to minimize or interfere with the fibrotic process by indirectly inhibiting the synthesis of type I collagen [38, 43, 46-55]. However, these pathways are complex and not fully characterized. Reduction of collagen levels may be achieved by the inhibition of molecules that activate collagen expression, including cytokines or connective tissue growth factor (CTGF). However, this method may be detrimental to the host due to nonspecific inhibition of additional downstream activities mediated by these molecules. Thus, a more specific reduction of type I collagen expression to reduce the effects of fibrosis is of significant interest.

It has been well documented that bleomycin administration leads to the accumulation of leukocytes, especially macrophages and fibroblasts, and deposition of collagen [56-58]. The development of fibrosis in intratracheal delivery of bleomycin has been shown biochemically and histologically by day 14 with maximal responses of collagen deposition and fibrosis during days

21–28 [36, 56]. 3 weeks after bleomycin injection, fibrosis progresses, which compromises respiratory function, and eventually causing significant morbidity to the animal [56].

In the current study, expression of type I collagen mRNA and protein in the lung tissue was increased during bleomycin-induced pulmonary fibrosis. Our study examined whether treatment with antisense oligonucleotides to type I collagen could reduce collagen deposits in a bleomycin model of pulmonary fibrosis. Results presented in this study demonstrate the efficacy of directly targeting the *COL1A1* collagen gene and thereby type I collagen protein expression.

Taken together, these results suggest that antisense inhibition of the type I collagen may provide a promising new approach to molecular therapy for pulmonary fibrosis.

COMPETING INTERESTS

Dr. Rahul K. Nath, MD holds patents for type I collagen antisense oligonucleotide (US patent number 10149352).

ACKNOWLEDGMENTS

This work was supported in part by the Texas Nerve and Paralysis Institute and Zeneca Pharmaceuticals Research Foundation. We would like to thank Dr. Xiaozhu Huang (University of California San Francisco, School of Medicine-Lung Biology Center) for providing the mouse model technical assistance, and Dr. Yiping Li (Department of Medicine, Baylor College of Medicine) for providing the animal protocol assistance and animal facility.

ABBREVIATIONS

ECM	=	extracellular matrix
AS61-ODN	=	mouse type I collagen antisense oligodeoxynucleotides
AS60-ODN	=	human type I collagen antisense oligodeoxynucleotides
ODN	=	oligonucleotides
ASO	=	Antisense oligodeoxynucleotides
H & E	=	hematoxylin and eosin
<i>COL1A</i>	=	type I procollagen gene
I.P	=	intraperitoneal
I.V	=	intravenous
S.C	=	subcutaneous
I.T	=	intratracheal
PEI	=	cationic polymer polyethylenimine
EtBr	=	Ethidium Bromide

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Received: April 12, 2010

Revised: May 24, 2010

Accepted: May 25, 2010

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