Lung Fibroblast Repair Functions in Patients with Chronic Obstructive Pulmonary Disease Are Altered by Multiple Mechanisms

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Rationale: Fibroblasts are believed to be the major cells responsible for the production and maintenance of extracellular matrix. Alterations in fibroblast functional capacity, therefore, could play a role in the pathogenesis of pulmonary emphysema, which is characterized by inadequate maintenance of tissue structure.

Objectives: To evaluate the hypothesis that deficient fibroblast repair characterizes cells obtained from individuals with chronic obstructive pulmonary disease (COPD) compared with control subjects.

Methods: Fibroblasts were cultured from lung tissue obtained from individuals undergoing thoracotomy and were characterized *in vitro*. **Measurements and Main Results:** Fibroblasts from individuals with COPD, defined by reduced FEV₁, manifested reduced chemotaxis toward fibronectin and reduced contraction of three-dimensional collagen gels, two bioassays associated with fibroblast repair function. At least two mechanisms appear to account for these differences. Prostaglandin E (PGE), a known inhibitor of fibroblast repair functions, was produced in increased amount by fibroblasts from subjects with COPD, which also expressed increased amounts of the receptors EP2 and EP4, both of which signal through cyclic AMP. Incubation of fibroblasts with indomethacin or with the PKA inhibitor KT-5720 partially restored COPD subject fibroblast function. In addition, fibroblasts from subjects with COPD produced more transforming growth factor (TGF)- β 1, but manifested reduced response to TGF- β 1. The functional alterations in fibroblasts correlated with both lung function assessed by FEV₁ and, for the data available, with severity of emphysema assessed by DLCO.

Conclusions: Fibroblasts from individuals with COPD have reduced capability to sustain tissue repair, which suggests that this may be one mechanism that contributes to the development of emphysema.

Keywords: fibroblasts; prostaglandin E; transforming growth factor- β ; chemotaxis; contraction

Chronic obstructive pulmonary disease (COPD), currently the fourth leading cause of death in the United States, is characterized by reduction in expiratory airflow that is not completely reversible (1). Several anatomic lesions contribute to reduced airflow. These include accumulation of mucous secretions, peribronchiolar fibrosis and narrowing of small airways, and destruction of alveolar walls, which is the defining characteristic of

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Altered tissue structure is a key factor in chronic obstructive pulmonary disease (COPD).

What This Study Adds to the Field

Lung fibroblasts from patients with COPD demonstrated less activity in several *in vitro* measures associated with tissue repair. These appeared to be mediated by both increased production of prostaglandin E and decreased sensitivity to transforming growth factor- β .

emphysema (2–4). With regard to the latter, alveolar walls are normally elastic. As a result, lung expansion with normal inspiration generates elastic recoil, which serves to provide the pressure gradient driving gas from the distal alveoli into the proximal airways. This pressure gradient also generates the intraluminal pressure in the small airways, which prevents alveolar collapse during forced exhalation. Destruction of alveolar walls also reduces the tethering effect provided by alveoli inserting into small airways, which also maintains airway patency during forced exhalation. Together, these result in collapse of small airways in exhalation and decreased expiratory airflow.

Cigarette smoking is the most common cause of COPD (5). Current concepts suggest that cigarette smoke leads to an abnormal inflammatory response in the lower respiratory tract that, in turn, leads to tissue damage and destruction (6). The peribronchiolar fibrosis, which develops in the small airways, is believed to be a response to this injury (7). Destruction of alveolar walls, which characterizes emphysema, is believed to result from tissue destruction in excess of the capacity of the lung to repair cigarette smoke-induced damage (8).

Although cigarette smoking is clearly recognized as the major cause of COPD, smokers vary in their susceptibility (4). The mechanisms for this remain incompletely defined. The current study was designed to evaluate the hypothesis that individuals who develop COPD will differ in their ability to sustain tissue repair compared with individuals who do not develop COPD. The study took advantage of the fact that many smokers, both with and without COPD, undergo thoracotomy and removal of lung tissue for lesions suspected to be lung cancers. Fibroblasts, which are believed to be cells crucial to the maintenance of extracellular matrix within lung tissue (9, 10), were cultured from surgical specimens, and repair functions of fibroblasts were assessed using two established bioassays: fibroblast chemotaxis and fibroblast contraction of three-dimensional collagen gels, both of which are

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believed to model aspects of the repair response (11, 12). Finally, the mechanisms that underlie differences in repair responses were explored by evaluating paracrine signaling pathways known to regulate fibroblast repair responses. These results support the concept that individuals with COPD have deficient repair responses that could contribute to the development of emphysema.

METHODS

Patients

Primary lung fibroblasts from 12 subjects with moderate to severe COPD and 10 subjects without clinical or functional signs of COPD (controls) were included in the study (Table 1). One control subject, whose affected lung was without ventilation, had restrictive physiology. Eighteen subjects were undergoing surgery for lung tumor resection; four of the subjects with COPD were undergoing volume reduction surgery. The study was approved by the Human Studies Committee of the Medical Board of the State of Schleswig-Holstein and the Ethics Committee at the Karolinska Institute where samples were collected, and all subjects provided written, informed consent for the acquisition of material for research.

Lung Fibroblasts

Only lungs from patients without visible or palpable lung metastases were used. Pleura-free parenchymal specimens were excised after careful macroscopic evaluation from peripheral areas of the lobe as far away from the tumor site as possible. The tissue was immediately transferred into explant culture (Dulbecco's modified Eagle medium [DMEM], 10% fetal calf serum [FCS]). Most cells were assessed in passage 2 after primary culture and displayed the typical fibroblast morphology and were positive for vimentin and negative for cytokeratin staining as described previously (13). Fibroblast cultures were initiated at clinical sites (Germany and Sweden), cells were expanded *in vitro*, and frozen aliquots were shipped to Omaha, Nebraska, where the remainder of the experiments was performed.

Materials

Native type I collagen (rat tail tendon collagen) was extracted from rat tail tendons by a previously published method (14). Commercially available reagents were obtained as follows: transforming growth factor (TGF)- β 1 and biotinylated anti-TGF- β 1 were from R&D Systems (Minneapolis, MN); collagenase, 3,3',5,5'-tetramethyl benzidine, monoclonal anti-human fibronectin antibody, polyclonal anti-human fibronectin antibody, and indomethacin were from Sigma (St. Louis, MO); KT-5720 was purchased from Calbiochem (La Jolla, CA); DMEM and FCS were from Invitrogen Life Technologies (Grand

Island, NY); AH6809 was from Cayman Chemical (Ann Arbor, MI); L-161892 was the gift of Merck-Frosst, Montreal, Canada.

Cell Culture

Fibroblasts were isolated from surgical specimens of the lung as previously described (13). Briefly, portions of lung parenchymal tissue that were as distal from any tumor as possible, were free of the pleural surface, and did not contain any cartilaginous airways were dissected under sterile conditions, and minced and placed in culture. Cells from the outgrowth of these cells, termed "P0" were either frozen or passaged. Frozen cells at P0–P2 were shipped to Omaha where they were thawed, expanded, and evaluated. Human lung fibroblasts were cultured on tissue culture dishes (Falcon; Becton-Dickinson Labware, Lincoln Park, NJ) with DMEM supplemented with 10% FCS, 100 μ g/ml penicillin, 250 μ g/ml streptomycin, and 2.5 μ g/ml fungizone. Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ and passaged every 4–5 days at a cell density of 1×10^5 /ml. Thawed human lung fibroblast cells were used between the third and sixth subsequent passages.

Collagen Gel Contraction Assay

Collagen gels were prepared as described previously (15). Gel contraction was quantified using an Optomax V image analyzer (Optomax, Burlington, MA) daily. Data were expressed as percentage of the original gel size.

Measurement of Prostaglandin E₂, TGF- β 1, and Fibronectin Release

TGF- β 1 and prostaglandin E₂ (PGE₂) in the media of collagen gel culture or monolayer culture were determined by ELISA. Details of the cell preparation and ELISA methodology are provided in the online supplement. PGE₂ production from cells was determined by enzyme immunoassay (EIA; Cayman Chemical) following the manufacturer's instructions.

Chemotaxis Assay

Cell migration was assessed using the Boyden blindwell chamber (Neuroprobe, Inc., Gaithersburg, MD) as previously described. Chemotaxis was assessed by counting the number of cells in five high-power fields. This was accomplished by selecting a field in the center and in each quadrant of each of the spots. Wells with serum free (SF)-DMEM were used as negative controls.

Western Blotting

To standardize the culture conditions, cells were passaged at a cell density of 2×10^5 /ml and cultured for 48 hours, after which whole cell

TABLE 1. CLINICAL AND DEMOGRAPHIC FEATURES OF THE SUBJECTS

	No. of Control Subjects	Mean $\pm$ SD	No. of Subjects with COPD	Mean $\pm$ SD
Age, yr	10	65.7 $\pm$ 2.1 (55–76)	12	66.0 $\pm$ 2.3 (49–75)
Height, cm	10	170 $\pm$ 2.6 (155–181)	12	171 $\pm$ 2.0 (161–180)
Weight, kg	10	73.0 $\pm$ 4.6 (51–103)	12	76.4 $\pm$ 2.7 (64–88)
Sex, no. male/female	6/4		11/1	
Pack-years	8	45.3 $\pm$ 12.0 (15–90)	10	47.5 $\pm$ 6.3 (25–75)
GOLD 0/I/II/III/IV	10/0/0/0/0		0/0/5/4/3	
Using ICS	0/10		6/12	
FEV _{1.0} %	10	98.5 $\pm$ 6.8 (58.0–138.5)	12	43.7 $\pm$ 4.6 (19.0–66.6)*
VC%	10	102.4 $\pm$ 6.2 (60.0–122.4)	12	86.3 $\pm$ 5.5 (62.0–124.4)*
FEV/VC%	10	96.3 $\pm$ 2.9 (85.0–111.8)	12	55.9 $\pm$ 3.9 (39.5–73.0)*
TLC%	10	115.5 $\pm$ 5.7 (89.0–142.6)	12	176.6 $\pm$ 12.5 (130.9–240.8)*
RV, L	10	2.91 $\pm$ 0.44 (1.86–6.70)	12	5.02 $\pm$ 0.43 (3.28–7.31)*
RV/TLC	10	43.5 $\pm$ 3.7 (31.3–72.1)	12	58.5 $\pm$ 2.6 (44.6–73.9) [†]
D _{LCO}	4	93.5 $\pm$ 9.5 (79.4–121.5)	8	57.7 $\pm$ 7.2 (26.8–77.9)

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; D_{LCO} = diffusion capacity of carbon monoxide; ICS = inhaled corticosteroids; GOLD = Global Initiative for Chronic Obstructive Lung Disease.

* $P < 0.005$ compared with control.

[†] $P < 0.02$ compared with control.

Ten non-COPD (control) and 12 patients with COPD were enrolled into the current study.

lysates were prepared. To evaluate the response to TGF- β 1, cells were cultured as above. Media were then changed to DMEM without serum for 24 hours, after which cells were treated with and without 100 pM TGF- β 1 for 24 hours. Details of cell processing are provided in the online supplement. Primary antibodies were obtained as follows: anti-Smad2 and 3, anti-phospho-Smad2, anti-Smad7 (Zymed Laboratories, San Francisco, CA), anti-phospho-Smad3 (Cell Signaling Technology, Beverly, MA), anti-cyclooxygenase (COX) 1 and 2, anti-TGFR1 and II (Santa Cruz Biotechnology, Santa Cruz, CA), anti-prostaglandin E receptor (EP) 1, 2, 3, and 4 (Cayman Chemical), and anti- β -actin and anti- α -smooth muscle actin (Sigma). Bound antibodies were visualized using peroxidase-conjugated second antibodies and enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK) with a Typhoon Scanner (Amersham Biosciences).

Statistical Analysis

For summary and descriptive purposes, data are expressed as means $\pm$ SEM. Because the distribution of data is not known to be normal, nonparametric tests were used for comparisons. Comparisons between the two groups of subjects were performed using the unpaired, two-tailed Mann-Whitney test. For evaluation of experimental studies within a group where paired samples were available, the Wilcoxon test was used. For these comparisons, each subject was considered as an individual point. The evaluation of each subject included multiple replicates within an experiment that was repeated on multiple occasions. The mean value for each subject was then used to make statistical comparisons. For the primary functional outcomes, chemotaxis and contraction of three-dimensional collagen gels, all 22 subjects were evaluated. For other parameters, all available subjects were included in the analysis. However, because cells from older individuals and from individuals with COPD in particular grow slowly, sufficient materials were not available to evaluate all subjects for all mechanistic parameters. No available data were excluded from the statistical analysis or from any of the presented figures. *P* values less than 0.05 were considered significant.

RESULTS

Clinical and Demographic Features

The clinical and demographic features of the subjects are presented in Table 1. The two groups were similar in age and smoking status. Six of 10 non-COPD subjects and 11 of 12 subjects with COPD were males. The two groups differed significantly in lung function. As expected, the subjects with COPD had lower FEV₁ and diffusion capacity of carbon monoxide (DL_{CO}). In addition, subjects with COPD had increased total lung capacity, increased residual volume, reduced diffusion capacity, and evidence of emphysema on computed tomography scanning.

Functional Fibroblast Phenotypes in Control and COPD Fibroblasts

Two functional phenotypes were evaluated in fibroblasts obtained from control subjects and subjects with COPD. First, the ability of fibroblasts to contract three-dimensional collagen gels was assessed. Although there was some overlap, as a group, fibroblasts from subjects with COPD contracted less than did cells from control subjects (Figure 1A). After 2 days of culture, fibroblasts from control subjects embedded in three-dimensional collagen gels had contracted the gels to $43.5 \pm 2.3\%$ of the original gel size. In contrast, fibroblasts from subjects with COPD contracted the gels to $63.6 \pm 2.4\%$ of the original size ($P = 0.0002$). At the end of the incubation, there were no differences in cell numbers in the gels in either group assessed by MTT assay. Fibroblasts from subjects with COPD were also less active in migration toward the chemoattractant fibronectin (Figure 1B). On average, from control subjects, 155 ± 26 fibroblasts migrated per five high-power fields, in contrast to fibroblasts from subjects with COPD which, on average, migrated 58 ± 14 fibroblasts per five high-power fields ($P = 0.005$). Although there were too few subjects for subset analyses, there was no obvious difference in the functional activity of cells obtained from subjects who underwent surgery for volume reduction compared with those who underwent surgery for suspected cancer, or between males and females (Figure 1).

Fibroblast Release of PGE₂, TGF- β 1, and Fibronectin

Because fibroblasts are known to release PGE₂, TGF- β 1, and fibronectin, all of which can function as autocrine/paracrine mediators that have the potential to modify both three-dimensional collagen gel contraction and chemotaxis, the release of these mediators was directly assessed. Fibroblasts from control subjects released significantly less PGE₂ than did fibroblasts obtained from subjects with COPD both in three-dimensional collagen gel culture (260.0 ± 51.5 vs. 641.5 ± 118.5 pg/10⁶ cells/ml, $P = 0.0069$) and in monolayer culture (22.2 ± 2.7 vs. 35.1 ± 4.2 pg/10⁵ cells/ml, $P = 0.041$) (Figures 2A and 2B). Fibroblasts from control subjects also released significantly less TGF- β 1 compared with fibroblasts from subjects with COPD when maintained in monolayer culture (12.6 ± 1.9 vs. 24.7 ± 2.9 pg/10⁵ cells/ml, $P = 0.0062$), and showed a trend of decrease in three-dimensional culture (169.8 ± 24.9 vs. 232.8 ± 16.3 pg/10⁶ cells/ml, $P = 0.0604$) (Figures 2C and 2D). No differences were observed between the groups in the release of fibronectin under either culture condition (Figures 2E and 2F).

Figure 1. Fibroblast function: contraction of three-dimensional collagen gels and chemotaxis. (A) Contraction of three-dimensional collagen gels. Fibroblasts from individual subjects were cultured and cast into three-dimensional collagen gels. These were maintained in suspension culture, and gel size measured daily (see METHODS). Vertical axis: gel size after 2 days expressed as a percentage of original size. (B) Chemotaxis. Fibroblasts from each subject were grown in monolayer culture, trypsinized, and chemotactic activity toward fibronectin (20 μ g/ml) was assessed (see METHODS). Vertical axis: number of

migrated cells per five high-power fields (5HPF). Controls are indicated by the squares and subjects with chronic obstructive pulmonary disease (COPD) by the triangles. Larger symbols indicate subjects who underwent surgery for volume reduction. Open symbols represent women and closed symbols represent men. The control subject with restrictive physiology is depicted by gray symbols. Each symbol represents an individual study subject and represents the mean of at least three separate experiments, each of which included three replicates. * $P < 0.02$, ** $P < 0.0005$.

Figure 2. Release of prostaglandin E₂ (PGE₂), transforming growth factor (TGF)-β1, and fibronectin by fibroblasts cultured from control subjects and subjects with chronic obstructive pulmonary disease (COPD). Media were harvested from three-dimensional collagen gel cultures and from monolayer cultures and evaluated for PGE₂, TGF-β1, and fibronectin by immunoassay (see METHODS). Control subjects are indicated by the squares and subjects with COPD by the triangles. Larger symbols indicate subjects who underwent surgery for volume reduction. Open symbols represent women and closed symbols represent men. The control subject with restrictive physiology is depicted by gray symbols. (A) PGE₂, three-dimensional gel culture; (B) PGE₂, monolayer culture; (C) TGF-β1, three-dimensional gel culture; (D) TGF-β1, monolayer culture; (E) fibronectin, three-dimensional gel culture; (F) fibronectin, monolayer culture. Vertical axes: mediator production expressed as amount per cells/day. **p* < 0.05, ***p* < 0.005.

Effect of Indomethacin on Fibroblast Contraction of Three-Dimensional Collagen Gels and Chemotaxis in Control and COPD Fibroblasts

PGE₂ is known to inhibit both fibroblast-mediated contraction of three-dimensional collagen gels and fibroblast chemotaxis toward fibronectin. Because PGE₂ release from fibroblasts from subjects with COPD was increased compared with that of control fibroblasts, this suggested that PGE₂ might, by an autocrine or paracrine mechanism, contribute to the reduced activity for both

functions noted in fibroblasts from subjects with COPD. To evaluate this potential mechanism, both three-dimensional collagen gel contraction and chemotaxis were evaluated in fibroblasts that had been treated with indomethacin to block endogenous PGE production. In the presence of indomethacin, there was a modest but significant increase in the contraction of three-dimensional collagen gels mediated by fibroblasts from subjects with COPD in the seven subjects tested. After 3 days, gel size was 53.7 ± 3.2 versus 52.3 ± 3.4 of initial size, *P* = 0.04 (Figure 3A). In

Figure 3. Effect of indomethacin (Indo) on contraction of three-dimensional collagen gels and chemotaxis. Fibroblasts from control subjects and subjects with chronic obstructive pulmonary disease (COPD) were cultured in the presence and absence of indomethacin, and contraction of three-dimensional collagen gels and chemotaxis were assayed (see METHODS). (A) Contraction of three-dimensional collagen gels. Vertical axis: gel size measured after 3 days of contraction expressed as percentage of original gel area. (B) Fibroblast chemotaxis.

Vertical axis: number of migrated fibroblasts/five high-power fields (5HPF). Each subject evaluated is expressed as an individual symbol, which represents the mean of at least two experiments each conducted in triplicate. The lines connect the values for individual subjects with and without indomethacin. **p* < 0.05, ***p* < 0.005.

contrast, there was no effect of indomethacin on contraction mediated by fibroblasts from control subjects. Although indomethacin increased contraction, even in its presence the contraction mediated by fibroblasts from subjects with COPD was less than that mediated by control subjects.

In the presence of indomethacin, there was also an increase in chemotactic activity toward fibronectin in nine subjects with COPD tested (134 ± 27 vs. 61 ± 15 , $P = 0.0077$) (Figure 3B). Among control subjects, there was a trend toward an increase in chemotaxis in the presence of indomethacin in six of eight subjects tested (242 ± 36 vs. 172 ± 28) that did not achieve statistical significance ($P = 0.0687$) (Figure 3B). As with contraction of three-dimensional collagen gels, the increase in chemotaxis observed in the presence of indomethacin did not restore the chemotactic activity in fibroblasts from control subjects to that observed from subjects with COPD.

COX1 and COX2 Expression in Fibroblasts from Control Subjects and Subjects with COPD

Because fibroblasts from subjects with COPD produced increased amounts of PGE₂, which appeared to contribute to their functional alteration, the expression of COX1 and COX2, enzymes responsible for initiating the cascade that leads to PGE₂ production, was evaluated. Expression of COX1 and COX2 protein was assessed by Western blotting in fibroblasts maintained in monolayer culture. Although there was some overlap, fibroblasts from subjects with COPD expressed more COX2 than did fibroblasts from control subjects (Figure 4). Although several of the subjects with COPD had COX1 levels that exceeded the normal range, as a group the difference was not significant.

PGE Mediates Functional Inhibition through the EP2 and EP4 Receptors

PGE₂ can act on four distinct receptors that can activate a number of signaling pathways. To determine which receptors might contribute to PGE inhibition of fibroblast contraction and chemotaxis, EP receptor expression was evaluated by Western blot analysis. All four EP receptors were expressed (Figure E1 of the online supplement). As a group, although the differences observed were small, fibroblasts from subjects with COPD expressed significantly more EP2 receptor and EP4 receptor than did fibroblasts from control subjects (Figures E1B and E1D). In contrast, expression of the EP1 and EP3 receptors was not different between the groups (Figures E1A and E1C).

Both the EP2 and EP4 receptors signal by stimulating adenylyl cyclase, leading to an increase in intracellular cyclic AMP. Because increases in cyclic AMP can inhibit both fibroblast chemotaxis and contraction of three-dimensional collagen gels, signaling by these receptors through this pathway could contribute to the reduced activity noted in fibroblasts from subjects with COPD. To evaluate this possibility, two series of experiments were performed. First, the ability of inhibitors of EP2 and EP4 to affect fibroblast-mediated collagen gel contraction and chemotaxis was assessed. Second, the ability of the protein kinase A (PKA) antagonist KT5720 to modulate fibroblast activity was determined.

Both the EP2 antagonist AH6809 and the EP4 antagonist L-161892 resulted in augmented contraction of both control and COPD fibroblasts (Table 2). When added together, the two inhibitors resulted in a further augmentation of contraction in fibroblasts obtained from both groups of subjects. When added alone, the relative effect on contraction was slightly greater in fibroblasts from subjects with COPD—that is, the addition of the antagonists resulted in smaller gels, but the effect was greater in subjects with COPD than in control subjects. When added together, the relative increase in contraction observed in fibroblasts obtained from subjects with COPD was statistically significantly greater than that observed in fibroblasts obtained from control subjects (Table 2).

When added alone, both the EP2 antagonist AH6809 and the EP4 antagonist L-161892 resulted in augmented chemotaxis of fibroblasts from both control subjects and subjects with COPD. In contrast to the effect on contraction, the effect on chemotaxis was similar in both groups. When added together, however, the two groups responded differently. Augmented contraction was observed in both groups, but the relative effect of the two antagonists together was less than that observed with either one separately in fibroblasts obtained from control subjects. In contrast, the two antagonists added together had a greater effect in augmenting chemotaxis in subjects with COPD. As a result, the relative effect in augmenting chemotaxis was significantly greater for the two antagonists added together when comparing fibroblasts from subjects with COPD with fibroblasts from control subjects.

In addition, the effect of the PKA inhibitor KT5720 on contraction of three-dimensional collagen gels and on chemotaxis of fibroblasts from control subjects and subjects with COPD was assessed. KT5720 had essentially no effect on contraction of three-dimensional collagen gels mediated by control subjects. In contrast, KT5720 resulted in augmented

Figure 4. Cyclooxygenase 1 and 2 (COX1 and COX2) expression in fibroblasts from control subjects and subjects with chronic obstructive pulmonary disease (COPD). Fibroblasts were cultured in monolayer culture, and proteins extracted and subjected to Western blot analysis (see METHODS). (A) COX1; (B) COX2. Vertical axes: expression of protein normalized to β-actin. Each symbol represents the mean value for an individual subject that was assessed in two separate experiments.

Control subjects are indicated by squares and subjects with COPD by triangles. Larger symbols indicate subjects who underwent surgery for volume reduction. Open symbols represent women and closed symbols represent men. The control subject with restrictive physiology is depicted by gray symbols.

* $P < 0.005$. The inserts are representative blots.

TABLE 2. EFFECT OF EP2 AND EP4 ANTAGONISTS ON FIBROBLAST CONTRACTION OF THREE-DIMENSIONAL COLLAGEN GELS AND CHEMOTAXIS

	Collagen Gel Contraction*		Chemotaxis†	
	Control	COPD	Control	COPD
AH6809	82.7 ± 3.9	71.9 ± 5.1	2.46 ± 0.41	2.57 ± 0.32
L-161892	86.2 ± 1.7	82.9 ± 2.0	2.37 ± 0.56	2.60 ± 0.33
AH6809 + L-161892	65.1 ± 1.7‡	52.8 ± 2.3§	1.69 ± 0.14	3.73 ± 0.53

Definition of abbreviation: COPD = chronic obstructive pulmonary disease.

Fibroblast contraction of three-dimensional collagen gels was assessed in the absence and presence of the EP2 receptor antagonist AH6809 (10^{-5} M), the EP4 antagonist L-161892 (10^{-5} M), and the combination of the two antagonists. Each strain of fibroblasts available for assay was evaluated in triplicate under all four conditions on two occasions. Because each strain had a different baseline contraction or chemotaxis, the effect of the antagonists is expressed as percentage of augmented contraction (% of gel size) and fold increase in chemotaxis in comparison to the no-reagent control.

* Collagen gel size was measured on Day 3 and expressed as % of initial gel size. Data are expressed as mean ± SEM.

† Chemotaxis was expressed as fold change in migrated cell number. Data are expressed as mean ± SEM.

‡ $P < 0.01$, EP2 + EP4 antagonists versus EP2 or EP4 antagonist alone in control.

§ $P < 0.01$, COPD ($n = 7$) versus control ($n = 7$).

|| $P < 0.001$, COPD versus control.

contraction in five strains of fibroblasts from subjects with COPD. After 3 days, gel size was $42.5 \pm 4.7\%$ versus $40.9 \pm 4.3\%$ of initial size, $P = 0.02$. On average, this represented a $3.45 \pm 0.96\%$ increase in contraction that was statistically significant (Figure 5A).

KT5720 stimulated chemotaxis in fibroblasts from control subjects (five of seven strains responding) and in fibroblasts from subjects with COPD (seven of seven strains responding). The effect of KT5720 was statistically significant in both groups. The relative magnitude of the effect was greater in fibroblasts from subjects with COPD ($212.9 \pm 19.9\%$ vs. $139.8 \pm 14.2\%$ increase, $P = 0.0127$), although the relatively lower baseline chemotaxis of the fibroblasts from the subjects with COPD may have amplified this relative difference.

Response to TGF- β

Increased expression of PGE could contribute to inhibition of both chemotaxis and contraction of three-dimensional collagen gels in cells cultured from subjects with emphysema. However, this mechanism appears to account for only part of the difference between control subjects and subjects with COPD. For this reason, other mechanisms were also explored. Because TGF- β stimulates both contraction and chemotaxis, the increased production of TGF- β by fibroblasts from subjects with COPD appeared at odds with the observed decrease fibroblast chemotaxis and contraction in subjects with COPD. For this

reason, the response of fibroblasts from control subjects and subjects with COPD to TGF- β was explored.

When exposed to exogenous TGF- β , fibroblasts from both control subjects and from subjects with COPD augmented both collagen gel contraction and chemotaxis (Table 3). The ability of TGF- β to augment both functional measures, however, was significantly greater in fibroblasts from control subjects than in fibroblasts from subjects with COPD (Table 3). Similarly, TGF- β -stimulated fibronectin release was greater in fibroblasts from control subjects than in fibroblasts from subjects with COPD (Table 3). In contrast, the ability of TGF- β to induce autocrine PGE₂ release was not different between the groups.

Because the effect of TGF- β on gel contraction is mediated, at least in part, by an increase in α -smooth muscle actin expression, the ability of TGF- β to augment the expression of α -smooth muscle actin was evaluated. Under baseline conditions, fibroblasts from subjects with COPD demonstrated a trend ($P = 0.058$) toward increased α -smooth muscle actin expression. In response to exogenous TGF- β , however, there was a statistically significant increase in α -smooth muscle actin expression in fibroblasts from control subjects, whereas, in contrast, there was no significant effect on α -smooth muscle actin expression in fibroblasts from subjects with COPD. The difference between the groups was statistically significant (Figure 6).

To determine if the reduced responsiveness to TGF- β was mediated by an alteration in TGF- β receptors, the expression of TGF- β R1 and TGF- β R2 was assessed by Western blotting. Relative to β -actin expression, fibroblasts from subjects with COPD expressed increased amounts of TGF- β R1 compared with fibroblasts from control subjects under baseline culture conditions (Figure E2A). In contrast, there was no difference in the baseline expression of TGF- β R2 in fibroblasts obtained from the two groups of subjects (Figure E2B).

Because activated TGF- β R1 leads to phosphorylation of Smad2 and Smad3, the phosphorylation of these proteins was assessed. Under baseline conditions, the expression of nuclear phosphorylated (p)-Smad2 and p-Smad3 was significantly less in fibroblasts cultured from subjects with COPD compared with control subjects (Figure 7). In the presence of exogenous TGF- β 1, Smad3 phosphorylation increased in fibroblasts from both control subjects and subjects with COPD (Figure 7B). Although the relative increase after TGF- β 1 stimulation was greater in fibroblasts from subjects with COPD, the intensity of p-Smad staining was still less than in the control subjects. p-Smad2 also increased after TGF- β 1 exposure, but the change observed in fibroblasts from control subjects was less than that observed in subjects with COPD (Figure 7A).

To further assess Smad signaling, the ratio of nuclear to cytoplasmic phosphorylated Smad was also assessed. The nuclear to cytoplasmic ratio of p-Smads was not evaluable

Figure 5. Effect of KT5720 on fibroblast-mediated contraction of three-dimensional collagen gels and chemotaxis. Fibroblasts from control subjects and subjects with chronic obstructive pulmonary disease (COPD) were evaluated for contraction of three-dimensional collagen gels and chemotaxis in the presence and absence of KT5720. (A) Gel contraction. Gel size is expressed as percent of original gel area. (B) Chemotaxis is expressed as migrated cells per five high-power fields (5HPF). * $P < 0.04$, ** $P < 0.002$.

TABLE 3. EFFECT OF EXOGENOUS TRANSFORMING GROWTH FACTOR- β 1 ON FIBROBLAST-MEDIATED COLLAGEN GEL CONTRACTION, CHEMOTAXIS, AND RELEASE OF FIBRONECTIN AND PROSTAGLANDIN E_2

	Control (<i>n</i> = 8)	COPD (<i>n</i> = 8)
Collagen gel contraction	0.73 $\pm$ 0.02	0.81 $\pm$ 0.16*
Chemotaxis	1.50 $\pm$ 0.09	1.20 $\pm$ 0.08*
Fibronectin (monolayer)	7.09 $\pm$ 3.81	2.91 $\pm$ 0.84
Fibronectin (gel)	3.60 $\pm$ 0.62	1.93 $\pm$ 0.34*
PGE ₂ (monolayer)	2.81 $\pm$ 0.36	3.57 $\pm$ 0.64
PGE ₂ (gel)	2.24 $\pm$ 0.31	2.11 $\pm$ 0.46

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; PGE = prostaglandin.

Data are expressed (mean $\pm$ SEM) as fold change with transforming growth factor (TGF)- β 1 stimulation. Monolayer = cells that were cultured two-dimensionally in dishes. Gel = cells were cultured in the three-dimensional collagen gels. Fibroblasts from control subjects and subjects with COPD were cultured in the presence of TGF- β and each strain of fibroblasts available for assay was evaluated in triplicate under all four conditions on two occasions. The effect of the TGF- β is expressed as fold increase, comparing each strain to the size observed in the absence of antagonists.

* P < 0.05, COPD versus control.

under baseline conditions, because cytoplasmic levels were undetectable. After TGF- β 1 exposure, both nuclear and cytoplasmic p-Smad2 and p-Smad3 were detected in fibroblasts from both groups of subjects. There was also no difference observed in the nuclear to cytoplasmic ratio of p-Smad2 between the two subject groups (Figure 8A). In contrast, compared with control subjects, fibroblasts from subjects with COPD demonstrated a significantly reduced nuclear to cytoplasmic ratio for p-Smad3 (Figure 8B). Finally, because Smad7 is known to inhibit TGF- β signaling, Smad7 levels were also assessed and were found to be significantly increased in fibroblasts obtained from subjects with COPD compared with control subjects (Figure 9).

Having demonstrated that fibroblasts from subjects with COPD differ functionally compared with fibroblasts from control subjects and that several mechanisms may contribute to these differences, it was of interest to relate the fibroblast functional phenotypes to the severity of COPD. When COPD severity was rated categorically by GOLD (Global Initiative for Chronic Obstructive Lung Disease) classification or continuously by FEV₁, both fibroblast-mediated gel contraction and

fibroblast chemotaxis toward fibronectin were significantly related to COPD severity (Figure 10). Similarly, DL_{CO}, which reflects severity of emphysema, was available in 12 of the subjects and was also found to correlate with fibroblast-mediated gel contraction and chemotaxis (Figure 11).

DISCUSSION

The current study demonstrates that fibroblasts from patients with COPD are less potent in contracting three-dimensional collagen gels and less responsive in migrating toward the chemoattractant fibronectin, two bioassays that are characteristic of fibroblast repair responses. Increased production of PGE₂, a known inhibitor of both gel contraction and chemotaxis (16–18), accounts for part of the reduced activity of fibroblasts from subjects with COPD. Consistent with the increased production of PGE, fibroblasts from subjects with COPD have increased expression of COX1 and COX2, two enzymes that regulate the production of prostaglandins in fibroblasts. Fibroblasts from subjects with COPD also expressed increased amounts of EP2 and EP4 receptors, both of which mediate the inhibitory action of PGE. Both the EP2 and the EP4 receptor signal by stimulating production of cyclic AMP. Consistent with this, blockade of the cyclic AMP-activated PKA partially ameliorated the reduced activity in fibroblasts from subjects with COPD. Increased production of PGE, however, only partially accounted for the reduced activity of fibroblasts, and other mechanisms likely also play a role.

Although fibroblasts from subjects with COPD produced increased amounts of TGF- β , a known stimulator of both gel contraction and chemotaxis (19, 20), fibroblasts from patients with emphysema were less responsive to the effects of TGF- β . This was not due to alteration in the expression of TGF- β receptors, although TGF- β induced a greater reduction in T β RII expression in fibroblasts from subjects with COPD than in those from control subjects. Increased levels of the inhibitory protein Smad7, which can inhibit TGF- β signaling pathways, were present in fibroblasts from subjects with COPD. Consistent with this, baseline levels of nuclear p-Smad proteins were less in fibroblasts from subjects with COPD. When exogenous TGF- β was added, there was an increase in phosphorylation of Smad2 and Smad3 in fibroblasts from subjects with COPD, but the levels and the relative amount of Smad3 translocated to the

Figure 6. α -Smooth muscle actin (α -SMA) expression. Fibroblasts from control subjects and subjects with chronic obstructive pulmonary disease (COPD) were cultured in the absence and presence of transforming growth factor (TGF)- β 1, and α -SMA expression was assessed by Western blotting. (A) Baseline α -SMA expression. Vertical axis: α -SMA expressed relative to β -actin. (B) Effect of TGF- β 1 on α -SMA expression. Vertical axis: fold change in α -SMA expression after incubation with TGF- β . Each

point represents data from an individual subject evaluated on two separate occasions. Control subjects are indicated by squares and subjects with COPD by triangles. Larger symbols indicate subjects who underwent surgery for volume reduction. Open symbols represent women and closed symbols represent men. The control subject with restrictive physiology is depicted by gray symbols. * P < 0.05. The inserts are representative blots. β -act = β -actin.

Figure 7. Phosphorylation (p)-Smad expression. Fibroblasts from control subjects and subjects with chronic obstructive pulmonary disease (COPD) were cultured in the presence and absence of exogenous transforming growth factor (TGF)-β1. Nuclear extracts were prepared and subjected to Western blotting for p-Smad2 and p-Smad3. (A) p-Smad2. Vertical axis: Band intensity expressed relative to nuclear protein. (B) p-Smad3. Vertical axis: Band intensity expressed relative to nuclear protein. Each pair of symbols connected by a line represents data from an individual subject evaluated on two separate blots. **P* < 0.05. The inserts are representative blots.

nucleus were reduced in fibroblasts from subjects with COPD, consistent with reduced TGF-β signal transduction in fibroblasts from subjects with COPD. α-Smooth muscle actin, which mediates gel contraction and is known to be induced by many stimuli including TGF-β (21), was increased in COPD fibroblasts at baseline. However, exogenous TGF-β did not augment α-smooth muscle actin expression in COPD fibroblasts, in contrast to its action on control fibroblasts.

Finally, the functional reductions in contraction of collagen gels and chemotaxis toward fibronectin observed in fibroblasts from subjects with COPD correlated with the severity of the COPD assessed by FEV₁ and with the severity of emphysema as assessed by DL_{CO}. Taken together, these data demonstrate that fibroblasts from patients with COPD have diminished capacity to mediate several repair responses. Moreover, these diminished activities appear to result from at least two mechanisms: increased production of inhibitory PGE₂ and decreased responsiveness to the stimulatory effects of TGF-β1. This study, therefore, is consistent with the concept that, because of reduced repair capacity, patients with COPD may not be able to maintain normal tissue repair functions, a defect that could contribute to the development of emphysema.

The current study evaluated fibroblasts cultured from lung tissue removed at the time of a surgical procedure. The sur-

geries were performed for suspected lung cancer in smokers with varying degrees of COPD and in four subjects for volume reduction. Every effort was made to culture only portions of parenchymal lung tissue that were distant from the potential malignant lesions, were distal from the pleural surface, and did not contain large airways (13). Like all studies of human lung fibroblasts, the cells studied, however, were fibroblasts that were expanded *in vitro*, and the precise origin of these cells is undetermined. In addition, COPD is heterogeneous within the lung. Histology was not available from the sites that were used to initiate cultures; thus, it is not possible to determine to what degree differences in tissue pathology contribute to differences observed. Finally, we cannot totally exclude the possibility that the differences observed in the current study reflect different responses of the groups to cell culture conditions or reflect other pathologic processes in the individuals who donated tissue. Most of the specimens used in the present study were obtained at a single center (Grosshansdorf, Germany), but several were obtained at a second site (Stockholm, Sweden). Samples from both sites showed similar results, which at least suggests that systematic bias during harvest of the tissues does not account for the differences observed.

The control subjects in the current study were undergoing surgical resection for suspected lung cancer, which is why the lung

Figure 8. p-Smad nuclear translocation after transforming growth factor (TGF)-β1 exposure. Fibroblasts from control subjects and subjects with chronic obstructive pulmonary disease (COPD) were cultured in the presence of TGF-β1, and nuclear and cytoplasmic extracts were prepared and subjected to Western blotting for p-Smad2 and p-Smad3. 10 μg protein for cytoplasm Smad detection and 5 μg protein for nuclear Smad detection were added in this assay. (A) p-Smad2; (B) p-Smad3. Vertical axes: ratio of nuclear to cytoplasmic

band intensities. Each symbol represents data from an individual subject evaluated on two separate blots. Control subjects are indicated by squares and subjects with COPD by triangles. Larger symbols indicate subjects who underwent surgery for volume reduction. Open symbols represent women and closed symbols represent men. The control subject with restrictive physiology is depicted by gray symbols. **P* < 0.05. The inserts are representative blots.

Figure 9. Expression of Smad7. Fibroblasts were cultured and cytoplasmic extract subjected to Western blotting for Smad7. Vertical axis: Smad7 expression relative to β -actin. Control subjects are indicated by squares and subjects with chronic obstructive pulmonary disease (COPD) by triangles. Larger symbols indicate subjects who underwent surgery for volume reduction. Open symbols represent women and closed symbols represent men. The control subject with restrictive physiology is depicted by gray symbols. * $P < 0.05$. The inserts are representative blots. β -act = β -actin.

tissues were available for study. Although this represents a “convenience” sample, there are a number of advantages to this control group. Because the control subjects had a similar age, smoking history, and underlying diagnosis, except for COPD, compared with the subjects with COPD, it is unlikely that these variables contributed to the differences observed. Half of the subjects with COPD had been treated with inhaled corticosteroids. Although the numbers available do not allow demonstration of a treatment effect, it is possible that treatment for COPD, with inhaled corticosteroids or with other medications, could have contributed to some of the differences observed. Similarly, no differences were obvious based on sex or on reason

for surgery (i.e., potential cancer or volume reduction). However, the study was small, and failure to detect differences based on these parameters does not exclude an effect. Considering the increased risk for women to develop emphysema (22, 23), rigorous study of cells based on sex would be of interest.

The current study evaluated two bioassays. Contraction of three-dimensional collagen gels has been used for several decades as a model of the tissue contraction that characterizes resolving granulation tissue and the development of fibrosis (19, 21, 24). In this process, mesenchymal cells attach to collagen fibers through integrin-dependent mechanisms and generate mechanical tension. This mechanical tension results in tissue contraction. As a result, wound size can be reduced. In addition, contraction has been associated with promoting apoptosis and therefore may play a role in the resolution phase of normal healing (25–28). Chemotaxis, which is the ability of cells to migrate in response to a chemotactic gradient, is believed to be a mechanism by which cells are recruited to sites of injury. Chemotactic responses may account for the recruitment of fibroblasts from local populations or, alternatively, from circulating precursor/stem cells (29, 30). Both bioassays are model systems that have been used to evaluate cell functions *in vitro*. Both are believed to be related to fibroblast response to injury and thus to model tissue repair and perhaps the excessive repair that occurs in fibrosis. In the context of the current study, emphysema could result from the inability of the lung to respond to injury. It is also possible that both functions could be related to normal tissue maintenance and that a deficient maintenance program in the lung could contribute to the development of emphysema. Although both have been used extensively, the *in vivo* significance of the *in vitro* findings remains incompletely defined. Nevertheless, reduced activity in both assays suggests that the fibroblasts present in the lung parenchyma of patients with COPD may have a diminished capacity to mediate repair, which could contribute to the development of emphysema. Other fibroblast functions not assessed in the current study, including the production of growth factors for other cells, production of extracellular matrix

Figure 10. Relationship between fibroblast-mediated gel contraction and chemotaxis to fibronectin to the severity of chronic obstructive pulmonary disease (COPD). Subjects were grouped into COPD severity categories, according to GOLD stage (A, B) or by FEV₁ (C, D). These were then related to either fibroblast-mediated gel contraction (A, C) or to fibroblast chemotaxis (B, D). * $P < 0.05$ compared with control (A, B). Control subjects are indicated by squares and subjects with COPD by triangles. Larger symbols indicate subjects who underwent surgery for volume reduction. Open symbols represent women and closed symbols represent men. The control subject with restrictive physiology is depicted by gray symbols. GOLD = Global Initiative for Chronic Obstructive Lung Disease.

Figure 11. Relationship between fibroblast-mediated gel contraction and chemotaxis to fibronectin and diffusing capacity of carbon monoxide (DL_{CO}). (A) Gel contraction. Vertical axis: Gel size as % of original; horizontal axis: DL_{CO} as % predicted. (B) Fibroblast chemotaxis toward fibronectin. Vertical axis: Chemotaxis as cells/five high-power fields (5HPF); horizontal axis: DL_{CO} as % predicted. Control subjects are indicated by squares and subjects with COPD by triangles. Larger symbols indicate subjects who underwent surgery for volume reduction. Open symbols represent women and closed symbols represent men.

macromolecules, and release of inflammatory mediators, could also affect the development of emphysema.

Emphysema is a process in which alveolar wall is destroyed. Current concepts suggest that the production of inflammatory mediators, most commonly as a result of cigarette smoking, contributes to destruction of alveolar wall through the production of toxic oxygen species (31, 32), potent proteases (9, 33, 34), and toxic peptides capable of inducing cellular apoptosis (35, 36). It is likely, however, that the development of emphysema represents a balance between tissue destruction and the ability of the lung to repair after injury. In this context, the development of emphysema resembles the development of osteoporosis (37, 38), which is also a balance between tissue destruction and tissue repair. The demonstration in the current study, that fibroblasts from the lung parenchyma of patients with COPD are deficient in selected repair functions, is consistent with the concept that some individuals have an increased susceptibility to the development of emphysema. Cellular proliferation is yet another function of fibroblasts associated with repair. In a previous report, which included some subjects whose cells were used in the current study, deficient proliferation of fibroblasts from patients with COPD was reported (13), further supporting the concept of deficient repair in lung fibroblasts in patients with COPD. Cause-and-effect relationships, however, are not proven by these studies, as it is possible that the altered repair functions observed are a consequence of the development of COPD.

The mechanisms that account for the differences in fibroblast function in the subjects with COPD compared with the control subjects are not yet defined. Acute exposure of fibroblasts to cigarette smoke has been reported to inhibit fibroblast repair functions (12, 39). However, cigarette smoke exposure is unlikely to account for the differences, because the two groups had similar smoking histories.

Accelerated senescence has been suggested as a mechanism for emphysema. Morla and colleagues reported reduced telomere length in peripheral blood lymphocytes in patients with emphysema (40). Similarly, in a study that also included some of the currently evaluated subjects, Muller and coworkers reported an increase in several markers of senescence in fibroblasts from subjects with COPD, although telomere length was unchanged (41). Potential mechanisms for accelerated senescence in COPD are undefined. Moreover, fibroblasts from aged individuals have also been reported to have reduced repair functions but, in the current study, the subjects with COPD and control subjects were similar in age. Age, *per se*, is thus not likely to account for the difference between the groups.

It is possible that genetic differences account for the differential repair functions (42, 43). In this context, individuals who have defective repair responses would, with aging and

cigarette smoke exposure, be more likely to develop emphysema. However, it seems unlikely that a single genetic defect in either of the pathways described, PGE signaling or TGF- β signaling, could account for the multiplicity of effects observed. A single genetic defect in a control pathway, for example in a transcription factor that regulates the expression of many downstream genes, could account for the multiple mechanisms of defective repair observed in the current study. An acquired alteration is also possible and could occur either with or without a concurrent genetic defect (44). For example, cigarette smoke is well known to induce somatic cell mutations (45). The accumulation of somatic cell mutations in lung cells could lead to acquired repair defects.

In the current study, overproduction of PGE was found to be a mechanism for the reduced repair responses in fibroblasts from subjects with COPD. PGE is a well-established inhibitor of several fibroblast repair responses, including chemotaxis (18, 46), proliferation (47, 48), production of extracellular matrix macromolecules (48), and contraction of three-dimensional collagen gels (16, 17). The current study evaluated only chemotaxis and contraction, but it seems likely that PGE may also contribute to the reduced proliferation noted in fibroblasts from patients with COPD. Whether fibroblasts from COPD produce reduced amounts of extracellular matrix macromolecules and whether this is also attributable, at least in part, to overproduction of PGE remains undetermined but would be consistent with reduced repair through this mechanism.

Several studies support the concept that PGE levels *in vivo* may regulate fibroblast repair (49, 50). First, PGE has been quantified in bronchoalveolar lavage fluid, and the concentrations in lung lining fluid have been estimated to be in the range in which either increases or decreases would be expected to have physiologic effects (51). Consistent with this model, reduced levels of PGE have been suggested to play a role in the augmented fibrosis that characterizes interstitial lung diseases (52, 53). Conversely, increased levels of PGE have been reported in the exhaled breath condensate of patients with COPD (54), consistent with the results reported in the current study.

PGE signals through at least four G protein-coupled receptors. Of these, EP2 and EP4 are both known to signal by activating Gs, leading to increases in cyclic AMP, which is believed to be the mechanism by which PGE inhibits fibroblast responses. Signaling through the EP1 and EP3 receptors, in contrast, activates alternate signaling pathways (55). The current study demonstrated an increase in EP2 and EP4 receptor expression in fibroblasts from patients with COPD. Inhibition of EP2 and EP4 potentiated both contraction and chemotaxis consistent with PGE-mediated inhibition acting through both of these receptors. Interestingly, the effect of these inhibitors was

greater than that of indomethacin. Indomethacin blocks all prostaglandin synthesis. The difference in effects, therefore, could be due to indomethacin blocking additional prostaglandins. Alternatively, PGE can also act through the EP1 and EP3 receptors. Stimulatory actions of PGE on these receptors could account for the net greater effect of the receptor antagonists compared with indomethacin. Whether alteration in receptor level expression contributes to the pathogenesis of disease remains to be determined. Western blot analysis demonstrated increased expression of EP2 and EP4, although definitive demonstration of increased receptor expression would require additional studies using other methods such as receptor binding studies. However, the presence of EP2 and EP4 receptors, which would be expected to signal through cyclic AMP and inhibit repair, is consistent with a mechanism for reduced repair mediated by PGE in fibroblasts from patients with COPD.

In addition to inhibition by PGE, the current study also demonstrates that fibroblasts from patients with COPD have diminished response to TGF- β . The response of Smad3 to exogenous TGF- β appeared to be more diminished than that of Smad2. This is of interest because the stimulatory effect of TGF- β on gel contraction and on α -smooth muscle actin expression is mediated through Smad3 (21). The signaling pathway that leads to increased chemotaxis is, as yet, undefined. The mechanism for reduced signaling through Smad3 in fibroblasts from subjects with COPD is also undefined. However, Smad3 has several sites that can be phosphorylated in addition to those that are targets of the T β RI (56). Phosphorylation of Smad3 by protein kinase C (PKC), for example, has been suggested to reduce signaling by decreasing nuclear translocation (57).

The role of TGF- β and PGE in the maintenance of normal lung structure remains to be determined. Mice deficient in Smad3 develop lungs normally but, with age, develop progressive emphysema (58, 59). This is consistent with the concept that defective TGF- β -mediated repair can lead to the development of emphysema. COPD is also characterized by chronic inflammation of the lower lungs (6). TGF- β has a suppressive effect on inflammation, and TGF- β 1-deficient mice develop fatal postnatal lung inflammation (60). The reduced responsiveness of fibroblasts from subjects with COPD, despite the increased TGF- β levels, may be a mechanism that contributes to the increased inflammation that characterizes these individuals.

Subjects in the current study were divided into two groups based on lung function (i.e., whether COPD was present or absent). Neither DL_{CO} nor high-resolution computed tomography scans were performed on all subjects. Although limited data were available, the severity of emphysema as assessed by DL_{CO} was related to the fibroblast function. This is consistent with reduced fibroblast function contributing to the development of emphysema. A similar mechanism has been suggested to account for the emphysema that develops in the Smad3-deficient mouse.

COPD is characterized not only by loss of alveolar structures but, in many individuals, by the development of peribronchiolar fibrosis as well. The current study assessed function of fibroblasts cultured from the pulmonary parenchyma and therefore is most likely relevant to emphysema rather than airway disease. Several studies suggest that the functional phenotype of fibroblasts in the airways may differ from that of the pulmonary parenchyma (61, 62). Inhibition of fibrotic responses has been suggested as a means to prevent the progression of disease in the small airways. The current study, however, suggests that such a strategy has the potential to contribute to the development of emphysema.

In conclusion, the current study demonstrates that fibroblasts from patients with COPD have diminished repair responses compared with similarly aged control subjects with similar

smoking histories. The diminished repair capacity of fibroblasts from patients with COPD could contribute to the development of emphysema in these subjects.

Conflict of Interest Statement: S.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. O.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. X.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. H.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. X.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. Y.A. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.F. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. C.M.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.C.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. D.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. L.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. H.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. H.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.I.R. has participated as a speaker at programs organized by AstraZeneca, Boehringer Ingelheim, GlaxoSmithKline, Otsuka, and Pfizer; he serves on advisory boards for Altana, AstraZeneca, Dey, GlaxoSmithKline, Novartis, Schering-Plough, and Talecris; he has conducted clinical trials for Almirall, Altana, Astellas, Centocor, GlaxoSmithKline, Nabi, Novartis, and Pfizer; he has served as a consultant for Adams, Almirall, Altana, AstraZeneca, Bend, Biolipox, Centocor, Critical Therapeutics, GlaxoSmithKline, ICOS, Johnson & Johnson, Novartis, Ono, Parengenix, Pfizer, Roche, Sankyo, Sanofi, and Schering-Plough. A patent is pending on a method for stem cell differentiation; S.I.R. is a coinventor of the patent owned by the University of Nebraska.

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