Connective tissue growth factor mRNA expression is upregulated in bleomycin-induced lung fibrosis

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Lasky, Joseph A., Luis A. Ortiz, Boihoang Tonthat, Gary W. Hoyle, Miriam Corti, Grace Athas, Giuseppe Lungarella, Arnold Brody, and Mitchell Friedman. Connective tissue growth factor mRNA expression is upregulated in bleomycin-induced lung fibrosis. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 18): L365–L371, 1998.—Connective tissue growth factor (CTGF) is a newly described 38-kDa cysteine-rich peptide mitogen for fibroblasts and a promoter of connective tissue deposition in the skin. The CTGF gene promoter contains a transforming growth factor-β1 (TGF-β1) response element. Because TGF-β1 expression is upregulated in several models of fibrogenic lung disease, we asked whether CTGF is also upregulated in a murine lung fiberosis model and whether CTGF could mediate some of the fibrogenic effects associated with TGF-β1. A portion of the rat CTGF gene was cloned and used to show that primary isolates of both murine and human lung fibroblasts express CTGF mRNA in vitro. There was a greater than twofold increase in CTGF expression in both human and murine lung fibroblasts 2, 4, and 24 h after the addition of TGF-β1 in vitro. A bleomycin-sensitive mouse strain (C57BL/6) and a bleomycin-resistant mouse strain (BALB/c) were given bleomycin, a known lung fibrogenic agent. CTGF mRNA expression was upregulated in the sensitive, but not in the resistant, mouse strain after administration of bleomycin. In vivo differences in the CTGF expression between the two mouse strains were not due to an inherent inability of BALB/c lung fibroblasts to respond to TGF-β1 because fibroblasts from untreated BALB/c mouse lung upregulated their CTGF message when treated with TGF-β1 in vitro. These data demonstrate that CTGF is expressed in lung fibroblasts and may play a role in the pathogenesis of lung fibrosis.

transforming growth factor-β1; collagen; mice

CONNECTIVE TISSUE GROWTH FACTOR (CTGF) is a newly described fibroblast mitogen and promoter of collagen deposition. CTGF was first isolated from human umbilical artery endothelial cells (4) and subsequently was found to be expressed by human foreskin and dermal fibroblasts (12) but not by other cell types such as epithelial cells or leukocytes (15). It is a 38-kDa, cysteine-rich peptide (12) and shares homology with several other peptides, including CEFC-10 (24), Cyr61 (16) and Fisp-12 (21). Transforming growth factor-β1 (TGF-β1) has been shown to upregulate CTGF expression in vitro (8).

There is evidence that CTGF may be involved in the pathogenesis of dermal fibrosis. This is not unexpected, because TGF-β1 is known to be increased in fibrogenic dermal diseases, including scleroderma, and a positive correlation between CTGF expression and skin sclerosis has been reported (10, 11). In vitro studies have demonstrated that TGF-β1 is the only known inducer of CTGF mRNA expression (12). Dermal fibroblast CTGF expression is upregulated in response to TGF-β1 through a novel TGF-β1 response element in the CTGF promoter (8, 15). With the use of a rat skin wound model, it has been shown that upregulation of CTGF mRNA expression follows increases in TGF-β1 mRNA expression (12), suggesting that CTGF is also induced by the increased expression of TGF-β1 in vivo. Because dermal fibroblasts synthesize CTGF and also proliferate in response to CTGF, others (7) have proposed that CTGF may act as an autocrine mitogen induced by TGF-β1.

A prior publication has shown that fisp-12 mRNA, a murine gene homologous to CTGF, is expressed in murine lung (21). TGF-β1 expression is upregulated in many models of fibrotic lung disease, including murine models of bleomycin-induced lung fibrosis (18, 19). We postulated that if CTGF expression plays an important role in lung fibrogenesis, it would be increased during development of the disease. To test this hypothesis, we exposed bleomycin-sensitive (C57BL/6) and bleomycin-resistant (BALB/c) strains of mice to bleomycin via intratracheal injection and probed for CTGF mRNA expression at 8, 16, and 30 days after bleomycin administration. In addition, we showed that CTGF was upregulated in both human and mouse lung fibroblasts after treatment with TGF-β1 in vitro.

METHODS

Rat CTGF cloning and sequencing. RNA was isolated from rat lung fibroblasts (RLFs) with an acid-phenol extraction reagent (Ultraspec). A reverse transcription-polymerase chain reaction (RT-PCR) was performed on 0.5 µg of total RLF RNA with an RT-PCR kit (Promega). Primers were designed using sequences from GenBank and the MacMolly software program. Identical 19-base regions of the human CTGF and mouse fisp-12 genes separated by 199 bases were or...
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Isolation of human, rat, and mouse lung fibroblasts. RLFs were isolated from 8-wk-old male Sprague-Dawley rats exactly as previously described (3). Human lung fibroblasts (HLFs) were isolated from sections of grossly normal lung, which were resected for anatomic considerations during the course of curative surgical therapy for lung cancer. Primary HLFs were obtained through mechanical and enzymatic dissociation using a modification of the method used in obtaining RLF isolates. Briefly, a portion of the resected lobe distant from the cancerous lung nodule was resected and then parenchyma from peripheral lung tissue (within 2 cm of the pleura to avoid large vessels and bronchi) was diced into 1-mm pieces and rinsed several times with PBS to remove blood. The tissue was then placed in Dulbecco's modified Eagle's medium (DMEM) with 0.05% trypsin, 0.008% collagenase, and 0.005% DNase (Sigma) and stirred for 30 min at 37°C before being filtered through 100-µm nylon mesh. Filtered cells were washed twice in DMEM with 10% fetal bovine serum (FBS), Fungizone, penicillin (100 U/ml), and streptomycin (25 mg/ml). Cells were seeded at 10^6 cells/175-cm^2 flask and washed twice to remove nonadherent cells after 1 day of incubation at 37°C in 95% air-5% CO_2. When cultures were subconfluent, they were passaged for staining characterization and subcultured or cryopreserved in DMEM with 10% FBS and placed in a bacteriological plate for 35 min at 37°C to allow unwanted macrophages to attach. The unattached cells were aspirated and discarded. The remaining pellet was suspended in 2 ml of 4.2 M GTC with the Tissue Tearor (Biospec Products). The samples were transferred to Corex tubes and centrifuged at 10,000 g for 35 min at 37°C to allow unwanted macropaghes to attach. The unattached cells were aspirated and plated in tissue culture plates in DMEM with 10% FBS, and the medium was changed every 3 days.

Second-passage HLFs and MLFs obtained in this manner. The following primary antibodies were employed: anti-vimentin (Sigma); anti-α-smooth muscle actin (dow 1A4, Sigma), anti-desmin (clone 33, Dakopatt), anti-cytokeratin (clone CAM 5.2, Becton Dickinson), anti-macrophase (clone Mac378, Dakopatt), and anti-CD32 (rat anti-mouse, which recognizes natural killer cells, monocytes, macrophages, granulocytes, mast cells, and B lymphocytes; PharMingen). Mouse IgG was also used as a control for the mouse monoclonal primary antibodies. The secondary antibodies used for immunohistochemical identification were biotinylated goat anti-rat and goat anti-mouse (Jackson Immunologicals). One hundred percent of the HLFs and MLFs stained positively for vimentin, 100% of the MLFs and 89% of the HLFs stained for α-smooth muscle actin, 22% of the MLFs and 5% of the HLFs stained for desmin, less than 1% of the MLFs stained for CD32, none of the HLFs were positive for macrophase staining, and neither the MLF nor the HLF isolates stained for keratin. Therefore, we believe that the human and murine lung cells isolated were predominantly myofibroblasts.

Bleomycin exposure. C57BL/6 and BALB/c mice (25 g; obtained from Charles River) were rendered insensitive with trimethoanethol intraperitoneally before sterile tracheal cut-down surgery. Four units per kilogram of bleomycin (Bristol-Meyers) in a volume of 0.05 ml of 0.9% NaCl were administered into the tracheal lumen. Preliminary studies demonstrated that this dose would result in fibrosis with a low mortality. A similar volume of sterile 0.9% NaCl was instilled into control mice. After administration of bleomycin, the neck wound was closed with a clip, and the animals were allowed to recover from anesthesia on a warming plate.

Lung tissue procurement. At designated time points after exposure (8, 16, and 30 days), the mice were anesthetized with an intraperitoneal injection of trimethoanethol. After ligation of the abdominal aorta, the chest cavity was exposed. The lungs and heart were exposed, and the lungs were resected for anatomic considerations during the course of curative surgical therapy for lung cancer. The right lung was perfused with 10% neutral Formalin and 85% of the HLFs stained for CD32, none of the HLFs were positive for macrophase staining, and neither the MLF nor the HLF isolates stained for keratin. Therefore, we believe that the human and murine lung cells isolated were predominantly myofibroblasts.

Mouse lung fibroblasts (MLFs) were isolated from 6- to 8-wk-old female C57BL/6 and BALB/c mice. Briefly, mice were rendered insensitive with trimethoanethol (Aladdin) intraperitoneally and exsanguinated via the abdominal aorta. The lungs and heart were exposed, and the lungs were perfused free of blood with 20 ml of normal saline injected into the right ventricular outflow tract. The trachea was cannulated, and 3 ml of warmed dispase were rapidly injected into the lungs. Then 0.5 ml of low-melting-point agarose, warmed to 37°C, was injected slowly into the trachea, and the chest cavity was covered with ice for 2 min. The lungs were then removed and placed in dispace at room temperature for 45 min. The lung parenchyma was gently teased from the large airways with forceps and incubated in DMEM containing DNase for 5 min on a stir plate. Large pieces of lung tissue were rendered insensitive with trimethoanethol (Aladdin) intraperitoneally and exsanguinated via the abdominal aorta. The right lung was perfused with 10% neutral Formalin through a tracheal cannula at a pressure of 15 cmH_2O for 15 min. The trachea was then clamped, and the right lung was removed from the chest cavity and placed in fresh fixative overnight at 4°C before processing in paraffin.

Hydroxyproline assay. Total hydroxyproline content of the left lung was measured as an assessment of lung collagen content. A spectrophotometric assay was used to quantify lung hydroxyproline (14). Briefly, left lungs were removed from the −70°C freezer and homogenized in 5% trichloroacetic acid (1:9 wt/vol). The homogenized samples were centrifuged for 10 min at 4,000 g, and the pellet was washed twice with distilled water, then hydrolyzed for 16 h at 100°C in 6 N HCl. Hydrolysates were assayed colorimetrically at 561 nm with dimethylaminobenzaldehyde to quantify hydroxyproline. Hydroxyproline content is expressed as micrograms hydroxyproline per left lung.

Northern analysis: RNA isolation. Murine lungs were removed from the −70°C freezer and immediately dissociated in 2 ml of 4.2 M GTC with the Tissue Tearor (Biospec Products). The samples were transferred to Corex tubes and centrifuged at 10,000 g to remove unwanted precipitate. The supernatant was gently layered over 1 ml of 5.7 M CsCl, and the samples were spun in a Beckman table top centrifuge at 89,000 rpm for 5 h at 21°C. The supernatant was carefully aspirated and discarded. The remaining pellet was sus-
pended in 200 µl of water. Thereafter, 8 µl of 5 M NaCl along with 500 µl of ice-cold ethanol were added to precipitate the RNA. The RNA was washed once with cold 80% ethanol. The pellet was resuspended in diethyl pyrocarbonate-treated water and stored at −70°C until use.

Twenty micrograms of total rat lung RNA in denaturation buffer were added to each well of a 1.2% formaldehyde-agarose gel and separated overnight via electrophoresis. RNA was transferred from the gel to a nylon membrane (Immobilon-N) by capillary action overnight. Prehybridization solution and hybridization diluent consisted of 6× saline-sodium phosphate-EDTA buffer, 5× Denhardt’s solution, 1% SDS, and 20 µg/ml of salmon sperm DNA. Prehybridization took place for 3–5 h at 62°C, and hybridization took place overnight at 62°C. cDNA templates were randomly primed with 32P-radiolabeled CTP and the Ready-To-Go DNA labeling kit (Pharmacia). The rat CTGF plasmid was cloned in our laboratory (as described in Rat CTGF cloning and sequencing). 36B4 (a human ribosomal phosphoprotein), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), or 18S rRNA, using a fragment from a murine 18S rRNA plasmid (American Type Culture Collection), were used as loading controls. Labeled probes were separated from unincorporated nucleotides with the TE Midi SELECT-D, G-50 spin columns (5 Prime, Boulder, CO). Hybridized membranes were washed with 2× SSC with 0.5% SDS, then 0.2× saline-sodium citrate buffer with with 0.5% SDS at 37 and 62°C. The hybridized autoradiographic signal was detected with Biomax Film (Kodak) and quantified on a densitometric scanner (Bio-Rad).

Statistical Analysis. All data are reported as means ± SE. ANOVA was used for comparisons between treated and control groups for hydroxyproline data. A nonparametric Student’s t-test was used for comparison of densitometric values from Northern blots. P values < 0.05 were considered significant.

RESULTS

The sequence of the rat cDNA fragment that was amplified with RT-PCR and ligated into the Bluescript vector is shown in Fig. 1. The cloned fragment of rat CTGF is 96% homologous with that of human CTGF and 90% with that of mouse Fisp-12. The homology between the rat sequence and that of human CTGF and mouse Fisp-12 clearly identifies the cloned RT-PCR fragment as rat CTGF and validates its use as a probe template for Northern analysis.

Primary isolates of HLFs constitutively expressed CTGF mRNA in vitro as shown in Fig. 2A. After exposure to recombinant TGF-β1 (10 ng/ml), there was an upregulation of CTGF expression at the earliest time point tested (2 h). We chose to use 10 ng/ml of TGF-β1 because this concentration has been shown to induce expression of CTGF in human foreskin fibroblast cultures (7). The TGF-β1-induced increase in CTGF mRNA remained elevated while in the presence of TGF-β1 for at least 24 h. These Northern analysis results were duplicated with an HLF isolate from a second donor. The combined results of three in vitro experiments from two HLF isolates are shown in Fig. 2B. These data demonstrate a significant upregulation of CTGF message in HLFs exposed to TGF-β1 in vitro.

We also performed Northern analysis for CTGF expression in primary cultures of human epithelial cells and in A549 cells (a malignant human lung epithelial cell line) but found no CTGF expression in the presence or absence of TGF-β1 (data not shown). Figure 3 demonstrates that neither tumor necrosis factor (TNF-α; 10 ng/ml), interleukin-1β (IL-1β; 1 ng/ml), nor PDGF-AB...
(10 ng/ml) upregulates CTGF expression 4 h after exposure, whereas TGF-β1 (10 ng/ml) does. Thus, of the cytokines tested to date, only TGF-β1 is capable of upregulating CTGF mRNA expression.

Figure 4 demonstrates the difference in lung collagen accumulation, as assessed by hydroxyproline measurement, between groups of five C57BL/6 and BALB/c mice 8, 16, and 30 days after intratracheal administration of bleomycin. Significant increase in hydroxyproline in the C57BL/6 bleomycin-exposed mouse lungs, compared with both the C57BL/6 control groups and the BALB/c lungs treated with saline and the BALB/c lungs treated with bleomycin, *P < 0.05. There were no significant differences between the saline control C57BL/6 and BALB/c lung hydroxyproline contents at 16 and 30 days.
C57BL/6 and BALB/c mice at 16 and 30 days after treatment (16-day C57BL/6, 48.4 ± 3.6 µg; 16-day BALB/c, 39.7 ± 2.4 µg; 30-day C57BL/6, 62.9 ± 4.4 µg; 30-day BALB/c, 61.2 ± 3.2 µg). Both strains of mice had a small, but statistically significant, increase in lung hydroxyproline content in bleomycin-treated mice compared with their strain-specific saline control mice at 8 days. Histology of lung tissue sections from these two strains at 16 and 30 days after bleomycin exposure correlated with the hydroxyproline data and showed subpleural fibrosis in the C57BL/6 mice and near normal architecture in the BALB/c mice (data not shown).

Mouse lung RNA was isolated from both the C57BL/6 and BALB/c strains 8, 16, and 30 days after the administration of bleomycin or an equal volume of saline as a negative control. As shown in Fig. 5, there was an upregulation in the CTGF mRNA expression (3.85 ± 2.03-fold saline control) in the C57BL/6 lungs at the earliest time point tested (8 days) that preceded histological evidence of collagen deposition. CTGF mRNA expression was statistically elevated in bleomycin-treated C57BL/6 lungs compared with C57BL/6 saline controls at 16 and 30 days after exposure (the bleomycin-treated and saline-treated ratios were 2.45 ± 0.19- and 2.46 ± 0.46-fold saline control, respectively). In contrast, CTGF mRNA expression was not upregulated in the BALB/c strain at any of the time points tested after bleomycin exposure. The 18S ribosomal RNA band was used as a loading control.

Because there was a difference in the expression of CTGF mRNA between the two strains in vivo, we investigated whether or not there would be a difference in CTGF mRNA expression exhibited in MLFs isolated from the two strains in vitro after the addition of TGF-β1 (10 ng/ml). Figure 6 shows that both C57BL/6 and BALB/c mouse strains upregulate their CTGF mRNA expression to a similar magnitude and over a similar time frame in response to TGF-β1.

**DISCUSSION**

The data presented in this paper represent the first studies designed to determine whether human and murine lung fibroblasts express CTGF, as reported previously for dermal fibroblasts (12). We found that both human and mouse lung mesenchymal cells express CTGF message constitutively in vitro (Figs. 2 and 5). Our finding that TGF-β1 upregulates CTGF mRNA expression in murine and human lung fibroblasts in vitro is similar to findings others have described with human dermal fibroblasts. We found that other proinflammatory cytokines released early in lung injury, e.g., TNF-α and IL-1β, did not upregulate CTGF under the conditions we employed. The lack of effect of early inflammatory cytokines (TNF-α and IL-1β) on CTGF expression has not, to our knowledge, been shown previously using any source of fibroblasts. Thus TGF-β1 remains the only known inducer of CTGF mRNA expression. A novel TGF-β1 motif has been described in the CTGF promoter (8). Once anti-CTGF antibodies are commercially available, studies that demonstrate, using Western analysis, that CTGF peptide expression is increased along with CTGF mRNA would be important.

Like TGF-β1, CTGF is capable of inducing normal rat kidney (NRK) fibroblast proliferation under serum-free conditions in vitro (15). However, unlike TGF-β1, CTGF is not capable of inducing anchorage-indepen-

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**Fig. 5.** A: Northern analysis of CTGF mRNA and 18S ribosomal RNA (loading control) expression in lungs of bleomycin-sensitive (C57BL/6) and bleomycin-resistant (BALB/c) mice killed at 8, 16, and 30 days after bleomycin exposure. S, saline treated; B, bleomycin treated. B: multiples of change ± SE of CTGF mRNA expression in bleomycin-treated C57BL/6 and BALB/c mouse lungs (n = 3) compared with saline controls at 8, 16, and 30 days after treatment, adjusted for RNA loading with the 18S ribosomal RNA band intensity (*P < 0.05 between the 2 groups).
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PDGF-independent CTGF-mediated pathway.

TGF-β1 has been shown to be a mitogen for smooth muscle cells and human foreskin fibroblasts in vitro through a PDGF-dependent pathway (13, 25). At low concentrations, TGF-β1 stimulates proliferation through induction of PDGF-A chain synthesis. At higher concentrations, TGF-β1 may inhibit mesenchymal cell proliferation by decreasing transcription of the PDGF-α receptor, to which PDGF-A chain binds (2). Investigators have studied the mitogenic effect of TGF-β1 on 3T3 cells from mouse embryos with a homozygous deletion of the PDGF-α receptor (23). They found that PDGF-α receptor-deficient 3T3 cells still proliferate in response to TGF-β1, although the magnitude of the mitogenic response is reduced by about one-half. Other investigators have shown that at least 70% of the mitogenic activity of TGF-β1 for cells can be blocked with an anti-PDGF antibody (2). It is possible that these studies may be reconciled by the observation that a polyclonal anti-PDGF antibody is capable of recognizing CTGF on Western analysis, although there is minimal homology between CTGF and PDGF (11, 26). Alternatively, there may be inherent differences in response to various mitogens between embryonic 3T3 cell lines and more differentiated smooth muscle and fibroblast isolates. At present, TGF-β1 appears to act as a mitogen through both a PDGF-A chain-dependent and a PDGF-independent CTGF-mediated pathway.

CTGF may also be responsible for mediating the collagen deposition generally attributed to TGF-β1. The addition of CTGF to cultured dermal fibroblasts results in the upregulation of fibronectin and type I collagen transcription (7). Upregulation of CTGF mRNA preceded the phase of collagen deposition in a rat skin wound model (12) as well as in our model of bleomycin-induced lung fibrosis. To our knowledge, there are no published data regarding the effect of blocking CTGF on collagen deposition in vivo.

We sought to determine whether there is a difference in CTGF expression between the bleomycin-sensitive (C57BL/6) and bleomycin-resistant (BALB/c) mouse strains after bleomycin exposure. There were no differences in lung CTGF mRNA expression between the strains in unexposed animals. However, bleomycin-sensitive C57BL/6 mice showed an increase in whole lung CTGF message expression after bleomycin administration, whereas bleomycin-resistant BALB/c mice did not. The greater than twofold increase in CTGF expression observed in mice that developed fibrosis is likely to underestimate the induction of this gene by bleomycin, because the areas of lung fibrosis were focal and represented less than 25% of the lung parenchyma. These data are consistent with a role for CTGF in lung fibrogenesis.

There was no difference in the upregulation of CTGF mRNA expression between cultured lung C57BL/6 and BALB/c fibroblasts treated with TGF-β1. Thus the lack of CTGF induction in the BALB/c mice in vivo apparently is not due to an intrinsic inability of the BALB/c MLFs to upregulate CTGF expression in response to TGF-β1. However, others have described the upregulation of TGF-β1 mRNA expression in the lungs of these two murine strains after bleomycin exposure (1, 18, 19). TGF-β1 is upregulated in C57BL/6 mice 7–14 days after intratracheal bleomycin administration (18, 19). Baecher-Allan and Barth (1) have performed semiquantitative PCR on lung RNA from bleomycin-treated mice and found that TGF-β1 expression is upregulated in both C57BL/6 and BALB/c strains, although there was a sevenfold increase in the C57BL/6 mice compared with a threefold increase for the BALB/c mice.

It is important to recognize that TGF-β1 must be activated before it can bind to its receptor. TGF-β1 may be activated by acid hydrolysis or proteolytic cleavage (20). Therefore, TGF-β1 mRNA expression alone may not reflect the activity of TGF-β1 in lung injury models. We have previously shown that there is a difference in lung inflammation between C57BL/6 and BALB/c mice after bleomycin administration (17). The observed differences in lung inflammation between the two strains may be due to the lack of TNF-α upregulation in the BALB/c strain compared with the C57BL/6 strain after treatment with bleomycin (17). It is also possible that the lack of lung inflammation in the BALB/c strain may result in decreased conversion of TGF-β1 to its active form.

Earlier differences have also been described between mouse strains in the sequence of events that occur between exposure to bleomycin and the development of lung inflammation and eventual lung fibrosis. A greater than 2.5-fold increase in bleomycin hydrolase activity, an enzyme that reduces the efficiency of bleomycin-mediated oxygen radical formation to less than 1%, is present in the lungs of BALB/c mice compared with C57BL/6 mice (6). Also, a comparative lack of poly(ADP-ribose) polymerase activity, as a reflection of DNA breakage, has been described in BALB/c mice when contrasted to C57BL/6 mice after bleomycin exposure (9). A possibility to further explain differences between murine strains in response to bleomycin is that the BALB/c mice do not suffer the same degree of DNA damage in response to bleomycin as do C57BL/6 mice. Therefore, less TNF-α is released, resulting in less inflammation and TGF-β1 activation, which in turn leads to less CTGF expression and less fibrosis.

The concept of cytokine networks has been put forward to explain the pathogenesis of pulmonary fibrosis (5). The present study suggests that CTGF is upregulated by TGF-β1 and has the potential to mediate some of the mitogenic and collagen deposition effects attributed to TGF-β1 in the lung. The upregulation of CTGF in the bleomycin-sensitive strain, but not in the resistant strain, at time points preceding and
during the fibrotic phase of the lung injury adds credence to the hypothesis that CTGF is playing a significant role in the pathogenesis of bleomycin-induced lung fibrosis.

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