Idiopathic pulmonary fibrosis (IPF) is a progressive fibrotic lung disease and characterized by abnormal growth of fibroblasts and lung scarring. While the pathogenesis of IPF is not clearly understood, activation of transforming growth factor-β (TGF-β) and disruption of alveolar basement membrane seem to play important roles in leading to excess disruption of the matrix, which is associated with activated matrix metalloproteinase (MMP) and aberrant proliferation of myofibroblasts. The Wnt/β-catenin pathway is an important regulator of cellular proliferation and differentiation and abnormal activation of Wnt/β-catenin signal was observed in IPF. We examined whether inhibition of the Wnt/β-catenin pathway could attenuate pulmonary fibrosis in a bleomycin-induced murine model of pulmonary fibrosis. Pulmonary fibrosis was induced in C57BL/6N mice by intratracheal instillation of bleomycin. To inhibit the Wnt/β-catenin pathway, small interfering RNA (siRNA) for β-catenin was administered into trachea 2 h before bleomycin instillation and every 48 h afterward until sacrifice on day 14. The level of β-catenin expression was increased in the epithelial cells of bleomycin-administered mice. Intratracheal treatment with β-catenin siRNA significantly reduced β-catenin expression, pulmonary fibrosis and collagen synthesis in bleomycin-administered mice compared with controls, with no significant effect on the inflammatory response. The β-catenin-targeted siRNA also significantly decreased the levels of MMP-2 (P < 0.01) and TGF-β (P < 0.01) expression in the lung tissue. Blockade of the Wnt/β-catenin pathway by β-catenin siRNA decreased bleomycin-induced pulmonary fibrosis in the murine model. These findings suggest that targeting Wnt/β-catenin signaling may be an effective therapeutic approach in the treatment of IPF.

Keywords: pulmonary fibrosis; bleomycin; Wnt/β-catenin pathway; siRNA; animal model

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ulation of Wnt signaling in adult tissue causes various diseases. The Wnt family constitutes a large family of secreted cysteine-rich glycoproteins that act as short-range ligands to locally activate receptor-mediated signaling pathways (Moon et al. 2004; Königshoff and Eickelberg 2010).

The Wnt signaling pathway can be divided into three distinct pathways, the best-characterized of which is the “canonical” β-catenin-dependent Wnt signaling pathway (Königshoff and Eickelberg 2010). This pathway involves binding of Wnt ligand to cell surface receptors, leading to cytosolic stabilization and nuclear translocation of β-catenin for regulation of target gene expression. This pathway participates in the development, regeneration, and remodeling of the lung. Wnt/β-catenin pathway is also thought to play a major role in pulmonary fibrosis. Abnormal activation of the Wnt/β-catenin pathway has been demonstrated in lung tissue of patients with IPF (Chilosi et al. 2003) and in an experimental model of bleomycin-induced pulmonary fibrosis (Liu et al. 2009). Several molecules known as important mediators associated with fibrosis including matrix metalloproteinases (MMPs) and TGF-β are controlled by Wnt signaling. MMP-2, 7 and 9 have been known as Wnt/β-catenin target genes (Brabletz et al. 1999; Zuo et al. 2002, Wu et al. 2006), and siRNA targeting shown to block protein production in the lung (Matsuyama 1998). Intratracheal administration of siRNA has been used to reduce proteinases (MMPs) and TGF-β mediators associated with fibrosis including matrix metalloproteinases (MMPs) and TGF-β is known to suppress β-catenin expression (Verma 2001; Verma et al. 2007). Recently, Henderson et al. (2010) reported that blocking Wnt signaling with Wnt/β-catenin/CREB-binding protein inhibited the bleomycin-induced lung fibrosis and gene expression of TGF-β.

In the present study, we investigated whether inhibition of the Wnt/β-catenin signaling pathway could attenuate lung fibrosis in a murine model of bleomycin-induced pulmonary fibrosis. We used a synthetic small interfering RNA (siRNA) to suppress β-catenin expression (Fire et al. 1998). Intratracheal administration of siRNA has been shown to block protein production in the lung (Matsuyama et al. 2006), and siRNA targeting β-catenin has been reported to reduce the level of β-catenin expression (Verma et al. 2003). We observed that intratracheal instillation of β-catenin siRNA effectively suppressed β-catenin expression and attenuated pulmonary fibrosis by reducing TGF-β and MMP-2 expression in the lung.

Materials and Methods

Animals

The experimental protocol was approved by the Institutional Animal Care and Use Committee. We used female C57BL/6 mice (aged 8 weeks, weight 18-20 g) raised under specific pathogen-free conditions. The mice were housed in humidity- and temperature-controlled rooms under a 12 h light:12 h dark cycle with ad libitum access to food and water.

Generation of bleomycin-induced pulmonary fibrosis in mice

For bleomycin instillation, the mice were anesthetized by intraperitoneal administration of 30 mg/kg of a combination of zolazepam and tiletamine (Zoletil® 50; Virbac Laboratories, Carros, France) and 10 mg/kg of xylazine (Rompun®; Bayer Korea, Seoul, Korea) just before treatment. The trachea was exposed via a transverse incision in the neck. Then, 2 U/kg of bleomycin (Bleocin®, Nippon Kayaku, Tokyo, Japan) were dissolved in 50 μL of sterile saline and instilled intratracheally with a 27-gauge needle at end-expiration. The liquid was withdrawn immediately by 300 μL of air twice to ensure delivery to the distal airways. Animals in the control group received intratracheal instillation of 50 μL of sterile phosphate-buffered saline (PBS) only, according to the same method. Each group consisted of five mice.

siRNA preparation and treatment

The following mouse β-catenin mRNA target sequences were taken from a previous report (Yun et al. 2005): 5′-AAGGCTTTT CCCAGTCTCTCA-3′ (300-320) and 5′-AAGATGATGGTG TGCCAAGTG-3′ (1,400-1,420). For in vivo gene silencing, 80 nmol/kg of β-catenin-targeted siRNA or scrambled-sequence control siRNA were dissolved in DEPC-treated water to a total volume of 40 μL. Mice were anesthetized by intraperitoneal administration of Zoletil® (15 mg/kg), and siRNA was administered via the trachea using a MicroSprayer® aerosolizer (Penn-Century, Philadelphia, PA). The instillation was performed 2 h before bleomycin instillation and was repeated every other day until sacrifice. To examine the specific inhibition of β-catenin expression by β-catenin siRNA, we used luciferase siRNA as a control. The luciferase siRNA target sequences were 5′-GGCAACUAUGACUGACATT-3′ and 5′-UAGGUC ACUAGGAAUCCTT-3′.

Surgical procedures

All mice were sacrificed on day 14 after bleomycin instillation. After adequate anesthesia, the trachea was cannulated with a 20-gauge needle, and bronchoalveolar lavage fluid (BALF) was obtained. The cells in the BALF were collected after centrifugation (1,000 × g, 10 min, 4°C), and the supernatants were stored at −80°C until further analysis. The left lung was ligated at the level of the main bronchus, excised at the hilum, and immediately frozen in liquid nitrogen for collagen assay. The right lung was fixed with 10% formalin in the neck. The mice were anesthetized by intraperitoneal administration of zoletil® and tiletamine (Zoletil® 50; Virbac Laboratories, Carros, France) and 10 mg/kg of xylazine (Rompun®; Bayer Korea, Seoul, Korea) just before treatment. The trachea was exposed via a transverse incision in the neck. Then, 2 U/kg of bleomycin (Bleocin®, Nippon Kayaku, Tokyo, Japan) were dissolved in 50 μL of sterile saline and instilled intratracheally with a 27-gauge needle at end-expiration. The liquid was washed immediately by 300 μL of air twice to ensure delivery to the distal airways. Animals in the control group received intratracheal instillation of 50 μL of sterile phosphate-buffered saline (PBS) only, according to the same method. Each group consisted of five mice.

Histological examination

The total lung area of the sections was used for microscopic fibrotic evaluation and scoring. Lung fibrosis was graded according to the method described previously by Ashcroft et al. (1988): grade 0, normal lung; grade 1, minimal fibrous thickening of alveolar or bronchiolar walls; grade 3, moderate thickening of walls without obvious damage to the lung architecture; grade 5, increased fibrosis with definite damage to the lung architecture and formation of fibrous bands or small fibrous masses; grade 7, severe distortion of the architecture and large fibrous area; grade 8, total fibrous obliteration of fields. The grade of lung fibrosis was scored on a scale from 0 to 8 by evaluating 10 randomly chosen regions per sample at × 200 magnification by two pathologists who were blinded to the treatment groups. The mean of the scores from all examinations was considered as the fibrotic score.

Immunohistochemistry

Lung tissues were examined by immunohistochemical staining of β-catenin with a rabbit anti-β-catenin antibody (ABR-Affinity BioReagents, Golden, CO), using a previously reported dianinoben-
zidine method (Matsuyama et al. 2000). Briefly, sections of 4-μm thickness were mounted on poly-l-lysine-coated slides, dewaxed, and washed in Tris-buffered saline for 10 min. Endogenous peroxidase activity was inhibited using 3% hydrogen peroxide solution in methanol for 10 min. After three washes in PBS with 1% saponin, non-specific sites were blocked. Then, the sections were incubated with a solution of primary antibody diluted 1:50 diluted for 2 h at room temperature. Negative control slides were incubated with rabbit IgG (ABR-Affinity BioReagents). Secondary biotinylated anti-Ig antibody (Dako, Carpinteria, CA) was added, and the slides were incubated for 30 min at room temperature. After rinsing, the slides were incubated with horseradish peroxidase-conjugated streptavidin (Dako) and then washed with deionized water. Diaminobenzidine substrate solution was added, followed by incubation for 10 min. A brown reaction product represented a positive result.

Transfection of mammalian cells

To evaluate the suppressive effects of the siRNA, we performed the experiments using murine lung epithelial (MLE-12) cell lines (ATCC® CRL-2110™, MD). We administered bleomycin in the culture media of MLE-12 cells. With the treatment of bleomycin, MLE-12 cells were transfection with or without a construct expressing β-catenin-targeted siRNA or control siRNA using Lipofectamine™2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol.

Western blot analysis

To detect MMP-2, we lysed the cells in 1 mg of lung tissue from each mouse in 1 ml of lysis buffer containing 50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 10% glycerol, and a protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO) using a hypersonic homogenizer (IKA-Werk, Staufen, Germany). Western blotting was performed using rabbit IgG anti-MMP-2 antibody or mouse IgG anti-β-catenin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (Yang et al. 2003).

Collagen assay

Lung collagen content was determined by assaying total soluble collagen using the Sircol™ soluble collagen assay (Biocolor, Carrickfergus, Co Antrim, UK) as described previously (Chung et al. 2003).

RT-PCR

RNA was extracted from frozen mouse lung tissue or cultured cell extract using TRI Reagent® (Sigma-Aldrich) according to the manufacturer’s instructions. A total of 5 μg of template RNA was reverse transcribed with an RT-PCR PreMix Kit™ (iNtRON Biotechnology, Seongnam, Korea). The following primers were used: MMP-2, 5′-ATGCCATCCCTGATAAACCCTG-3′ and 5′-TTGTCGACGCTATGGAAGATGAT-3′; β-catenin, 5′-GCCTGAGAACATAATGGC-3′; GAPDH, 5′-GAGCAGCTTTCATTGAC-3′; and GAPDH, 5′-AACGACCCCTTCATTGAC-3′.

ELISA

The protein concentrations of TGF-β and IL-6 in BALF supernatants were measured using an ELISA kit (Quantikine®; R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Statistical analyses

The data are expressed as means ± SEM. The two-tailed Student’s t-test was used to evaluate group differences. Statistical analyses were performed using SPSS (version 17.0; SPSS, Chicago, IL), and P values < 0.05 were taken to indicate statistical significance.

Results

Bleomycin administration increased β-catenin expression in bronchial epithelium

Immunohistochemical staining showed a markedly elevated level of β-catenin expression mainly in the bronchial epithelial cells of bleomycin-instilled mice compared with controls (Fig. 1A). In addition, β-catenin expression in lung tissue was significantly induced after instillation of bleomycin (Fig. 1B).

β-Catenin siRNA effectively inhibited β-catenin expression in vitro

After treatment of bleomycin, the expression of β-catenin was greatly increased in the MLE-12 cell lines. Significant inhibition of β-catenin mRNA expression was observed in MLE-12 cells transfected with β-catenin-targeted siRNA (150 pM) (Fig. 2). Control siRNA did not inhibit β-catenin expression, but did inhibit the expression of luciferase. These findings show that β-catenin siRNA selectively and effectively suppressed the expression of β-catenin.

β-Catenin siRNA inhibited the expression of β-catenin in vivo and attenuated pulmonary fibrosis

Next, we examined the effects of β-catenin siRNA on bleomycin-induced pulmonary fibrosis in mice. Bleomycin administration induced leukocyte infiltration and fibrotic changes in the lungs. Pulmonary fibrosis was significantly attenuated by intratracheal administration of β-catenin siRNA, as determined by histological examination (Fig. 3A) and pathological scoring (Fig. 3B). In addition, a Sircol collagen assay showed markedly reduced collagen synthesis in mice treated with β-catenin siRNA (Fig. 3C). Luciferase siRNA had no effect on pulmonary fibrosis in vivo according to the results of histological examination, pathological scoring, and collagen deposition. Based on immunohistochemical staining, treatment with β-catenin siRNA also effectively suppressed bleomycin-induced β-catenin expression in vivo (Fig. 3D). These findings suggest that the suppressive effect of β-catenin siRNA on pulmonary fibrosis was directly related to the inhibition of the Wnt/β-catenin pathway. Next, to determine whether the anti-fibrotic effect of β-catenin siRNA was mediated by an anti-inflammatory effect, we examined the inflammatory cells and inflammatory cytokines in BALF. On day 14, marked increases in the total inflammatory cell count and macrophage count in BALF were found in bleomycin-treated mice; however, β-catenin siRNA treatment did not attenuate the inflammatory cell count in BALF (Fig. 4A). In addition, TNF-α and IL-6 protein production were not inhibited by siRNA targeting β-catenin (Fig. 4B, 4C).
Fig. 1. Expression of β-catenin in the lung tissue of bleomycin-instilled mice. (A) Lung tissue sections were stained with anti-β-catenin antibody. Arrows indicate the expression of β-catenin mainly in the bronchial epithelial cells. (B) The level of β-catenin expression in lung tissue was determined by western blot. Data are means ± SEM. *P < 0.01 compared with the control, by Student’s t test. These data are representative of at least three experiments (n = 5-7 mice per group for each experiment).

Fig. 2. Effects of siRNA targeting β-catenin in MLE-12 cells. Bleomycin was administered to MLE-12 cell cultures with or without β-catenin siRNA or control luciferase siRNA. The results of western blot for β-catenin and GAPDH are shown. From left: PBS-treated cells, bleomycin-treated cells, β-catenin siRNA transfection into bleomycin-treated cells, and control siRNA transfection into bleomycin-treated cells.
These results imply that the effects of β-catenin siRNA treatment on pulmonary fibrosis were not mediated by anti-inflammatory actions.

**β-Catenin-targeted siRNA inhibited MMP-2 and TGF-β expression in vivo after bleomycin treatment**

As MMPs play important roles in the pathogenesis of IPF, we examined MMP-2, -7, and -9 expression in lung tissue homogenate by immunoblotting. The MMP-2 level was markedly increased in bleomycin-treated mice, and treatment with β-catenin siRNA significantly reduced the MMP-2 expression in lung tissues (Fig. 5A). However, the expression levels of MMP-7 and -9 were unaffected by β-catenin siRNA treatment. An analysis of TGF-β in whole-lung tissue indicated a marked increase in TGF-β expression in bleomycin-injured lung tissue, and β-catenin siRNA significantly inhibited TGF-β expression (Fig. 5B). These findings suggest that the anti-fibrotic effect of β-catenin siRNA was mediated by the inhibition of MMP-2 and TGF-β expression.
Discussion

Although the pathogenesis of IPF has not been explained in detail, recent studies have suggested that the Wnt/β-catenin pathway may be involved in the development of IPF. The results of the present study indicate that blockade of the Wnt/β-catenin pathway by siRNA targeting β-catenin attenuated bleomycin-induced pulmonary fibrosis in a murine model. β-Catenin siRNA effectively inhibited the expression of β-catenin both in vitro and in vivo. Inhibition of the Wnt/β-catenin pathway suppressed pulmonary fibrosis mediated by decreases in the expression of MMP-2 and TGF-β. To our knowledge, this is the first report that local treatment using siRNA targeting β-catenin diminishes pulmonary fibrosis in an experimental animal model.

Accumulating evidence supports a vital role of the Wnt pathway in the development of lung fibrosis. In several studies investigating putative IPF-related genes by gene profiling analysis (Kaminski and Rosas 2006; Yang et al. 2007), proteins in the Wnt pathway were shown to be highly expressed in IPF. Increased expression of β-catenin (Chilosi et al. 2003; Morrisey 2003; Liu et al. 2009) and other components of the canonical Wnt signaling pathway (Konigshoff et al. 2008; Konigshoff et al. 2009) has been observed in human IPF lung tissues and in experimental animal models of lung fibrosis. In addition, several Wnt target genes such as MMP-7 (matrilysin), osteopontin, and Wnt1-inducible signaling protein 1 were identified in experimental and idiopathic lung fibrosis (Konigshoff et al. 2009). Recently, activation of the canonical Wnt signaling pathway with increased expression of the Wnt target gene, WISP1, in hyperplastic ATII cells was demonstrated, and inhibition of WISP1 expression was shown to attenuate experimental lung fibrosis in vivo (Konigshoff et al. 2009). In addition, Han et al. (2009) reported that the siRNA targeting β-catenin prevented the α-defensin-induced increases in human lung fibroblast proliferation and collagen synthesis, and it was implicated in the pathogenesis of fibrotic lung diseases, including IPF. Therefore, the Wnt pathway may represent a powerful target for the treatment of pulmonary fibrosis.

Blockade of the Wnt pathway has also been tested in the regulation of fibrotic responses in tissues other than the

![Fig. 4. Effects of β-catenin siRNA on pulmonary inflammation in vivo. (A) Total inflammatory cell counts and differential cell counts in BALF 14 days after bleomycin administration. (B) Concentrations of TNF-α in BALF, assessed by ELISA. (C) Concentrations of IL-6 in BALF, assessed by ELISA. From left: PBS-administered mice, bleomycin-administered mice, β-catenin siRNA treatment in bleomycin-administered mice, and control siRNA treatment in bleomycin-administered mice. Data are means ± SEM. *P < 0.01 compared with the control, by Student’s t-test. NS, nonsignificant.](image-url)
Wnt/β-Catenin and Pulmonary Fibrosis

l lung (Cheng et al. 2008; He et al. 2009). When Dickkopf (Dkk), the best-characterized naturally secreted antagonist of the Wnt pathway, was administered locally in a naked plasmid vector (He et al. 2009) or systemically in an adenoviral vector (Cheng et al. 2008) for gene therapy, blockade of the canonical Wnt pathway inhibited renal interstitial fibrosis (He et al. 2009) or hepatic stellate cell activation and liver fibrosis (Cheng et al. 2008). In the present study, we used synthetic siRNA targeting β-catenin to block the Wnt pathway.

The complexity of the pathogenesis of pulmonary fibrosis suggests that combinatorial approaches would be required for treatment. With improvements in methodologies, simple pharmacological treatment has evolved into combinatorial treatment options, including newer types of treatments. The use of siRNA for transient silencing of mRNA expression has been suggested as a treatment option and has been tested for therapeutic application to treat a variety of genetic, viral, cancer-related, and metabolic diseases (Shrivastava and Srivastava 2008). Treatment using siRNA for blockade of key molecules by gene silencing in certain disease settings has shown significant effects, and there have been several preclinical and phase I or II clinical trials of siRNA for treatment of lung diseases, including viral infection and asthma (Shrivastava and Srivastava 2008). The major obstacle to the use of siRNA as a therapeutic option is delivery into tissues and then into the cytoplasm of cells. However, siRNA uptake is extremely efficient in the lung and occurs even in the absence of transfection reagent (Matsuyama et al. 2006). Therefore, it may be possible to develop a procedure to transiently block the expression of proteins using a noninvasive procedure for intrapulmonary delivery of aerosolized siRNA for specific purposes. There have been several reports regarding the use of siRNA as a treatment option for pulmonary respiratory syncytial virus (RSV) infection (Shrivastava and Srivastava 2008), influenza virus infection (Ge et al. 2003), asthma, and more recently, animal models of tuberculosis (Rosas-Taraco et al. 2009). Several trials using siRNA have also been conducted in the field of cancer treatment (Takeshita and Ochiya 2006). However, there have been no previous reports regarding the effects of siRNA targeting β-catenin in an in vivo model of pulmonary fibrosis. The results of the present study confirm the safety and efficacy of intratracheal β-catenin-targeted siRNA in a murine experimental fibrosis model. Recently, Henderson et al. (2010) reported similar findings, that inhibition of Wnt/β-catenin pathway reverses pulmonary fibrosis. They used ICG-001, a specific inhibitor of Wnt/β-catenin gene transcription in a cyclic AMP response-element binding protein binding protein (CBP)-dependent fashion. β-catenin is a common effector molecule in canonical Wnt pathway and intracellular β-catenin increases by binding Wnt proteins and frizzled receptors. Interaction of β-catenin and LEF/TCF transcriptional factors activate target gene transcription. ICG-001 selectively inhibits the β-catenin/TCF interaction in the nuclear transcription process. While Henderson et al. inhibited Wnt pathway at the nuclear transcription level, we blocked the effector molecule at cytoplasm level by use of siRNA for β-catenin.

Interestingly, in our study, β-catenin siRNA treatment did not affect parameters related to inflammation, such as

Fig. 5. Effects of β-catenin siRNA on MMP-2 expression in vivo. (A) Immunoblot data for MMP-2 expression in lung tissue homogenates. (B) Immunoblot data for TGF-β expression in lung tissue homogenates. From left: PBS-administered mice, bleomycin-administered mice, β-catenin siRNA treatment in bleomycin-administered mice, and control siRNA treatment in bleomycin-administered mice. Data are means ± SEM. *P < 0.01 compared with the control, by Student’s t-test.
total cell count, differential cell count, or cytokine levels in BALF. These observations indicate that the inhibition of the Wnt pathway at the transcriptional level from the early phase of lung injury did not affect lung inflammation caused by bleomycin. Inflammation has been suggested to have a very limited involvement in the pathogenesis of pulmonary fibrosis (Mapel et al. 1996; Selman et al. 2001), and the role of Wnt signaling in lung inflammation has largely been unexplored. Based on a comparison of the gene expression profiles in different mouse models of infection, allergy, and lung injury, Lewis et al. (2008) found the regulation of the Wnt signaling pathway in only one mouse model of bleomycin-induced lung fibrosis, and not in any other inflammatory lung disease model (Lewis et al. 2008). Another study using a mouse model of oxidant-induced injury with subsequent hyperoxia exposure demonstrated increased nuclear β-catenin levels during the fibroproliferative phase, but not during the acute lung injury period (Douglas et al. 2006). These studies strongly implicate Wnt signaling in the resolution and regeneration phases after lung injury, rather than the attenuation of lung inflammation. Previous trials of glucocorticoids indicated that even the strongest anti-inflammatory treatment could not attenuate the natural course of IPF in human patients (Mapel et al. 1996; Selman 2002). In these trials in human patients, the inflammation parameters were determined on day 14. However, the appropriate time to observe acute inflammation in the murine bleomycin injury model is day 7 after instillation. Although further investigation is required, we propose that the Wnt pathway plays a role in acute inflammation associated with bleomycin-induced lung injury in mice.

In contrast to the lack of a significant effect on inflammation, the blockade of β-catenin by siRNA decreased the expression of MMP-2 in the present study. Our results also suggest that in the absence of a definite effect on inflammation, an effect on the modulation of important gelatinases may lead to decreased accumulation of extracellular matrix and a resultant decrease in fibrosis. The accumulation of excessive amounts of matrix proteins, i.e., collagens (Corbel et al. 2002), is the main characteristic in the pathology of IPF. The deposition of excessive collagen results from an imbalance in collagen turnover, and MMPs are the key enzymes involved in ECM degradation in physiological and pathological conditions. MMPs have also been implicated in a range of pulmonary diseases characterized by alterations in alveolar structure, including interstitial fibrosis, acute respiratory distress syndrome, chronic obstructive pulmonary disease, asthma, and lung cancer (O’Connor and FitzGerald 1994). During the host defense response, in which inflammatory cells are attracted to the lung to combat microorganisms and other irritants deposited in the airways, potent proteolytic enzymes, including MMPs, are produced and facilitate the clearance of foreign and noxious agents. However, in excess, these enzymes can destroy the ECM environment, disrupt resident cells, and stimulate further inflammation (Tetley 1993). Among the members of the MMP family, MMP-9 (gelatinase B) and MMP-2 (gelatinase A) most specifically cleave gelatin and type N collagen, important constituents of the basement membrane. MMP-2 is produced by tissue structural cells, including fibroblasts, endothelial cells, and epithelial cells, whereas MMP-9 is produced by inflammatory cells such as macrophages, neutrophils, and eosinophils (Hoshino et al. 1998). In the present study, there were no significant differences in inflammatory parameters such as BALF total cell count, differential cell count, and inflammatory cytokine levels between β-catenin siRNA-treated and untreated bleomycin-administered mice, which may explain the similar level of MMP-9 between the two groups. In a model of chronic inflammation and fibrosis induced by repeated LPS exposure, the increased MMP-2 and MMP-9 levels were observed together; however, MMP-9 produced by inflammatory cells participated in the breakdown of excessive collagen deposition during the early inflammatory phase, and MMP-2 produced by mesenchymal cells took over for MMP-9 in the late phase (Corbel et al. 2001). MMP-2 and MMP-9 are normally expressed in bronchiolar epithelial cells and arteriolar smooth muscle cells, and are weakly expressed in a few alveolar macrophages and type II pneumocytes. However, in rats with intratracheal bleomycin instillation (Kim, J.Y. et al. 2009), the bronchiolar epithelial cells showed intense immunoreactivity for MMP-2 or MMP-9 throughout the entire course of lung injury and fibrosis, with more prominent expression in cells showing features of cellular injury, activation, and/or repair. The levels of MMP-2 and MMP-9 expression were markedly increased, with a peak on day 4; from day 5 to 14, expression seemed to be diminished, along with a decrease in number of inflammatory cells (Kim, J.Y. et al. 2009). Zymographic analysis of the BALF showed that MMP-2 expression in the lung had a tendency to decline less rapidly than MMP-9 and remained at relatively high levels on days 14 and 21 (Kim, J.Y. et al. 2009). We analyzed the effects of siRNA targeting β-catenin on day 14 after bleomycin treatment, representing the early phase of fibrosis and the end of acute inflammation, which corresponds well with the lack of difference in inflammation and MMP-9 expression. We also examined the expression of MMP-7 (data not shown), but its levels were too low for intensity measurements.

Strong β-catenin expression was observed in the bronchial epithelium of the lung in bleomycin-treated mice. This was very similar to the observations described in previous reports (Chilosi et al. 2003; Konigshoff et al. 2008) that indicated the nuclear localization of β-catenin in atypical type II epithelial cells (ATII cells) and interstitial fibroblasts in IPF lungs, especially in areas where abnormal remodeling of the lung architecture was evident (Chilosi et al. 2003). The canonical Wnt signaling components, including WNT ligands, β-catenin, and GSK-2β, are localized mainly to the bronchial and alveolar epithelium (Konigshoff et al. 2008). In the bleomycin-induced lung
injury model, MMP-2 is expressed mainly in epithelial cells and macrophages. We did not examine MMP-2 expression in the lung tissue by immunohistochemical staining. However, based on previous reports, we speculate that there may be a correlation between the expression of β-catenin and MMP-2. These molecules were shown to be expressed in the same cells, and the inhibition of β-catenin resulted in a decrease of the MMP-2 level. In addition, there have been several reports regarding the relationships between specific MMPs and molecules involved in the Wnt pathway. MMP-7 and MT1-MMP are known target molecules of the Wnt pathway (Crawford et al. 1999; Karow et al. 2009), and the expression of MT1-MMP, along with MMP-2 and MMP-9, was regulated by stimulation with a specific Wnt isoform, Wnt 3a, in mouse mesenchymal stem cells (Karow et al. 2009). In the angiogenic process of rat microvascular endothelial cells, β-catenin acts as a positive regulator of MMP-2 transcription following VEGF stimulation (Doyle and Haas 2009). Previous studies have provided no precise explanation regarding the mechanism by which inhibition of β-catenin decreases the MMP-2 level in the model of pulmonary fibrosis.

In summary, the Wnt pathway appears to be a potential therapeutic target for the treatment of pulmonary fibrosis. Using siRNA targeting β-catenin, an important component of the canonical Wnt pathway, we showed that blockade of the Wnt pathway attenuated bleomycin-induced lung remodeling and fibrosis in mice. The use of specific siRNA may be a safe and effective therapeutic modality for the treatment of pulmonary fibrosis.

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Conflicts of Interest

The authors report no conflicts of interest.

References


