Involvement of selenoprotein P in the regulation of redox balance and myofibroblast viability in idiopathic pulmonary fibrosis

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Idiopathic pulmonary fibrosis (IPF), a chronic progressive lung disease of unknown etiology, is characterized by the expansion of myofibroblasts and abnormal deposition of extracellular matrix in the lung parenchyma. To elucidate the molecular mechanisms that lead to IPF, we analyzed myofibroblasts established from patients with IPF by oligonucleotide microarrays. Gene expression profiles clearly suggested that lipid peroxidation is enhanced in myofibroblasts, which was confirmed by measuring cellular lipid hydroperoxides. One of the most highly up-regulated proteins in myofibroblasts was selenoprotein P, an antioxidant protein not previously associated with IPF. Subsequent analysis demonstrated that selenoprotein P reduces lipid hydroperoxides and maintains the viability of myofibroblasts. Thus, our results reveal a novel pathophysiologic function of myofibroblasts as a generator of lipid hydroperoxides, and a self-defense mechanism against self-generated oxidative stress.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic interstitial lung disease of unknown etiology characterized by an accumulation of extracellular matrix, resulting in impaired pulmonary function (Gross & Hunninghake 2001). The outcome in most patients with IPF includes gradual progression to respiratory failure within 3–5 years (American Thoracic Society 2000). Unfortunately, treatment of IPF with immunosuppressive or anti-fibrotic agents has been disappointing so far, and no clearly effective therapies are available (Walter *et al.* 2006). This prompts the need for the identification of molecular determinants of this disease, particularly those useful for the development of new diagnostic and therapeutic options.

The pathogenesis of IPF is complex, and the precise molecular mechanisms underlying the development of fibrosis are not fully elucidated (White *et al.* 2003). It has been believed that IPF represents a disease induced by persistent lung inflammation, resulting in the fibrotic response. However, accumulating observations suggest that foci of proliferating fibroblasts/myofibroblasts are more prominent than evidence of inflammation, as well as the poor efficacy of anti-inflammatory agents in patients with IPF, has led to a focus of the fibrotic pathway itself (Selman & Pardo 2003). Currently, it is considered that IPF results from cycles of epithelial injury, and the activation and proliferation of mesenchymal cells with the formation of fibroblastic foci, leading to the accumulation of extracellular matrix and abnormal wound repair (Selman & Pardo 2003). The extent of fibroblastic foci in lung biopsies is strongly associated with disease progression and a worsening prognosis, suggesting that myofibroblasts are the key effector in the pathogenesis of IPF (King *et al.* 2001). Thus, understanding the pathophysiological role of myofibroblasts, as well as mechanisms for their proliferation and persistence in lung tissue, should shed deeper insight into the basis of disease progression.

Recently, a new possibility for the mechanism of IPF was presented based on the analysis of oxidant and antioxidant expression in IPF (Kinnula *et al.* 2005). Reported observations include the up-regulation of biomarkers of oxidative stress (hydrogen peroxide, 8-isoprostane and NO) in the expired breath condensate (EBC) and bronchoalveolar lavage fluid (BALF) from

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patients with IPF (Montuschi *et al.* 1998; Kharitonov & Barnes 2001; Psathakis *et al.* 2006). Other studies reported the down-regulation of anitioxidants such as reduced glutathione in the lungs of affected patients (Behr *et al.* 1995). In addition, a number of antioxidant enzymes have been reported to be up-regulated in IPF (Lakari *et al.* 2000, 2001; Tiitto *et al.* 2003; Peltoniemi *et al.* 2004). These results suggest that oxidant–antioxidant imbalances may play a key role in the pathogenesis of IPF. This is further supported by the results of randomized clinical trials of treatments for IPF patients with *N*-acetylcysteine (NAC), a precursor of the major antioxidant glutathione. Although additional studies are needed for routine therapy, the data presented suggest that NAC, either alone or in combination with immunosuppressive therapy, may have beneficial effects in patients with IPF (Behr *et al.* 1997; Demedts *et al.* 2005; Walter *et al.* 2006). Thus, it is clear that the cellular redox state is dysregulated, and the resultant generation of reactive oxygen species (ROS) plays a significant role in the pathogenesis of IPF. However, the precise mechanisms of ROS metabolism, and their functions in the progression of IPF remain largely elusive.

In this study, we examined the gene expression profiles of myofibroblasts established from patients with IPF. This analysis reveals the selective up-regulation of enzymes responsible for the metabolism of lipid hydroperoxides. We further demonstrate that selenoprotein P, one of the most highly up-regulated proteins, regulates the cellular lipid redox state and maintains cell viability. Thus, selenoprotein P represents a novel effector in the pathogenesis of IPF, which may contribute to the persistence of myofibroblasts in an oxidative environment.

Results

Gene expression profiling of myofibroblasts isolated from patients with IPF

Two primary myofibroblast cell cultures (LF1, LF2) were isolated from individual patients with IPF (Supplementary Fig. S1). Microarray analysis of these cells was carried out to explore the pathophysiological function of myofibroblasts in IPF. We used Affymetrix HG Focus oligonucleotide microarrays comprising approximately 8700 human mRNAs. The data sets were compared with the profile of TIG7 cells, a human embryonic lung fibroblast cell line. Unsupervised hierarchical clustering revealed that the gene expression profiles of LF1 and LF2 cells are more similar to each other than to TIG7 cells (data not shown), implying that genes consistently up- or down-regulated in LF1 and LF2 cells may represent pathophysiological features of myofibroblasts in IPF. As expected, myofibroblast marker proteins were up-regulated in LF1 and LF2 cells (Table 1). Gene ontology analysis of the top 300 genes that were significantly up-regulated in both LF1 and LF2 cells ($P < 0.005$) reveals active features of myofibroblasts. Analysis of molecular function profiles shows an enrichment of genes associated with extracellular matrix structural constituents and proteoglycans, potentially reflecting active remodeling of the extracellular matrix in IPF (Fig. 1A). The profiling of biological

Table 1 Selected genes up-regulated in myofibroblast cells isolated from patients with IPF

		Normalized ratio (log ratio)	
GenBank ID	Gene name	LF1	LF2
Lipid hydroperoxide metabolism			
NM_005410	selenoprotein P	11.5	10.5
NM_000072	CD36	8.3	5.6
NM_001353	aldo-keto reductase family 1, member C1	2	2.7
U05598	aldo-keto reductase family 1, member C2	2.1	2.7
AB018580	aldo-keto reductase family 1, member C3	6.4	5.5
NM_000689	aldehyde dehydrogenase 1	6	3.5
AF065214	cytosolic phospholipase A2	2.6	1.8
Muscle markers			
NM_003289	tropomyosin 2	0.6	0.5
NM_004791	integrin, β-like 1	1.8	0.9
AK022548	integrin, α 7	0.6	1.3
NM_005965	myosin light polypeptide kinase	1.4	1
NM_006097	myosin regulatory light chain 2, smooth muscle isoform	0.9	0.4
NM_002961	S100 calcium-binding protein A4	3.1	4.7

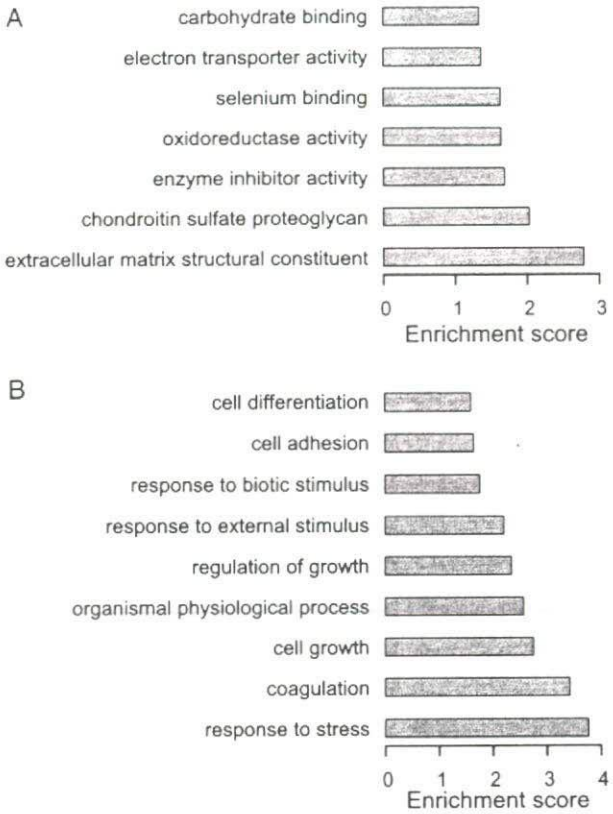

Figure 1 Gene expression profiling reveals redox regulation as a key feature of myofibroblasts in IPF. (A, B) Distribution of genes up-regulated in myofibroblasts in different functional categories. The top 300 genes up-regulated in myofibroblasts, all of which showed at least a 1.5-fold difference with a significance level of $P < 0.005$, were annotated using DAVID 2.0 software <http://david.abcc.ncifcrf.gov/> and EASE software based on their molecular function (A) and biological process (B). Data are expressed as enrichment score, the geometric mean (in -log scale) of *P*-values in a corresponding annotation. All enrichments are statistically significant (Fisher's exact score, $P < 0.05$).

processes showed enrichment of genes involved in stress responses (Fig. 1B). This result was further enhanced by the enrichment of genes related to the biology of ROS (Fig. 1A, oxidoreductase activity, selenium binding, electron transporter activity). Thus, the gene expression profiles reveal that redox regulation is one of the key features of myofibroblasts in IPF.

Selective up-regulation of genes associated with lipid hydroperoxide metabolism

A closer look on the list of the genes highly up-regulated in LF1 and LF2 cells provides further information on redox regulation in myofibroblasts. As shown in Table 1, significant up-regulation was observed in genes associated with the metabolism of lipid hydroperoxides. These include phospholipid hydroperoxide reductase (selenoprotein P), the scavenger receptor of phosphatidylcholine hydroperoxide (CD36), oxidoreductases of reactive aldehydes formed from the breakdown of lipid peroxidation (aldehyde dehydrogenase 1, aldo-keto reductase 1C1, 1C2, 1C3) and phospholipase A2. These results suggest that proteins responsible for the metabolism of lipid hydroperoxides are selectively up-regulated in myofibroblasts. Subsequent analysis using real time PCR confirmed this observation (Supplementary Fig. S2). Expression levels of phospholipid hydroperoxide reductases (selenoprotein P, glutathione peroxidase 4) were significantly up-regulated in LF1 and LF2 cells. In contrast, differences in the expressions of hydrogen peroxide reductases and protein disulfide reductases were not evident or consistent. Thus, these results reveal a previously unrecognized physiological function of myofibroblasts characterized by a highly selective response to lipid hydroperoxides.

Enhanced generation of lipid hydroperoxides in myofibroblasts

We found this myofibroblast-specific response in isolated cultures without any stimulation. Thus, the up-regulation of reductases might reflect gene settings imprinted in response to exogenous stresses during disease progression or, more likely, a direct consequence of enhanced lipid peroxidation within the myofibroblasts. To address this question, we measured the levels of lipid hydroperoxides using diphenylpyrenylphosphin (DPPP), a molecular probe that becomes fluorescent upon oxidation by lipid hydroperoxides (Takahashi *et al.* 2001). It is well known that oxidized DPPP is stably retained in the cell membrane; thus this probe is useful for the estimation of long-term and cumulative lipid peroxidation within live cells. After labeling with DPPP for 10 h, we measured the fluorescence intensities of cells and culture medium. As shown in Fig. 2A, fluorescence intensities were significantly higher in LF1 and LF2 cells compared with TIG7 cells, indicating that the level of lipid hydroperoxides is significantly increased in myofibroblasts. Similar increases were detected in the culture medium from LF1 and LF2 cells (Fig. 2B), suggesting that these cells actively secrete lipid hydroperoxides into the culture medium, or that oxidized DPPP is actively secreted with other cellular lipids. Oxidized DPPP in the culture medium may not be detected as a consequence of passive equilibrium between the cells and medium. In a model experiment in which an ROS generator was over-expressed in retina

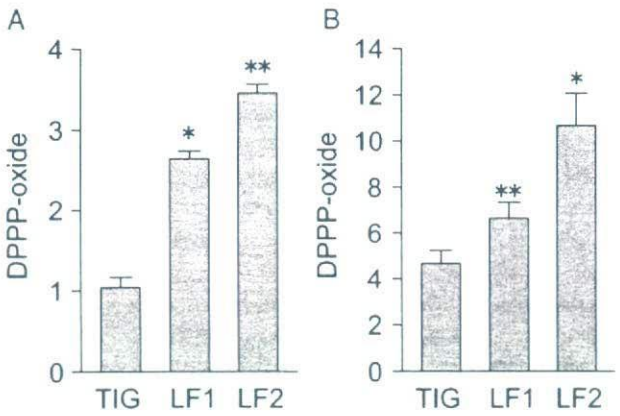

Figure 2 Enhanced generation of lipid hydroperoxides in myofibroblasts. Cells were labeled with diphenylpyrenylphosphin (DPPP) for 10 h and the fluorescence intensities of the cells (A) and culture medium (B) were measured as described in "Experimental procedures." Mean values of DPPP oxide fluorescence per cell are shown with SD ($n = 3$). The statistical significance of the differences between LF1, LF2 cells and TIG7 cells was calculated by Student's t-test. $*P < 0.01$, $**P < 0.005$.

pigment epithelial (RPE) cells, we could detect an increase in the fluorescence intensity in the cell fraction but not in the culture medium (data not shown). Together, these results reveal a novel physiological feature of myofibroblasts characterized by an enhanced generation of lipid hydroperoxides, which may play a significant role in the pathogenesis of IPF.

Selenoprotein P suppresses lipid hydroperoxide in myofibroblasts

LF1 and LF2 cells show relatively good proliferation rates (doubling times of approximately 2 and 3 days for LF1 and LF2 cells, respectively). This raises the question of how LF1 and LF2 cells deal with their own self-generated oxidative stresses, leading us to analyze selenoprotein P, one of the most highly up-regulated proteins in LF1 and LF2 cells. Although selenoprotein P has never been linked to the pathogenesis of IPF, a potential function of selenoprotein P in myofibroblasts was suggested by its well-known activity as an antioxidant. Biochemical characterization of selenoprotein P showed that it selectively reduces lipid hydroperoxides (Takebe *et al.* 2002). Thus, it seemed possible that myofibroblasts express selenoprotein P to suppress lipid hydroperoxides within the cells. To test this possibility, we first verified the gene expression data at the level of protein expression. Western blot analysis showed that the expression levels of selenoprotein P in LF1 and LF2 cells are higher than those in TIG7 cells (Fig. 3A). Analysis of lung tissue extracts showed abundant expression of selenoprotein P in patients with IPF (Fig. 3B). Immunohistochemical analysis showed expression of selenoprotein P in the fibrotic regions and epithelial cells (Fig. 3C). Positive immunostaining was detected in samples from all patients analyzed ($n = 4$, data not shown). In contrast, selenoprotein P was not detected in normal lung parenchyma (Fig. 3C). These results show clearly the association of selenoprotein P expression with the pathogenesis of IPF. We next examined the function of selenoprotein P in the metabolism of lipid hydroperoxides by carrying out RNAi-mediated knockdown of selenoprotein P in myofibroblasts. Treatment with RNAi oligonucleotide significantly suppressed selenoprotein P expression (Fig. 4A, inset). Next, we measured lipid hydroperoxides by DPPP. Interestingly, a significant increase in fluorescence intensity was detected in RNAi-treated cells (Fig. 4A). Furthermore, RNAi treatment led to a more remarkable increase in fluorescence intensity in the culture medium (Fig. 4B). These results strongly suggest that selenoprotein

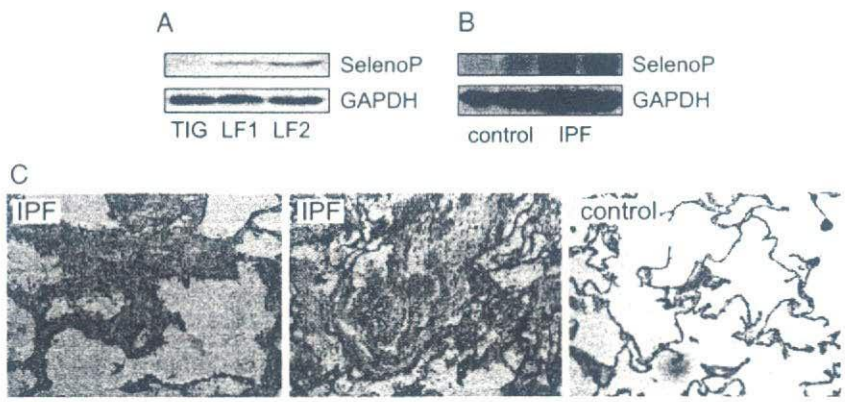

Figure 3 Selenoprotein P expression in myofibroblasts in fibrotic lesions. (A) Western blot analysis of selenoprotein P in cultured myofibroblasts. Gel images shown are representative of three independent experiments. (B) Expression levels of surgical samples from two IPF patients and two control subjects were examined by Western blot analysis. (C) Immunohistochemical detection of selenoprotein P expression. Shown are representative images from tissues of two independent patients and from a control subject.

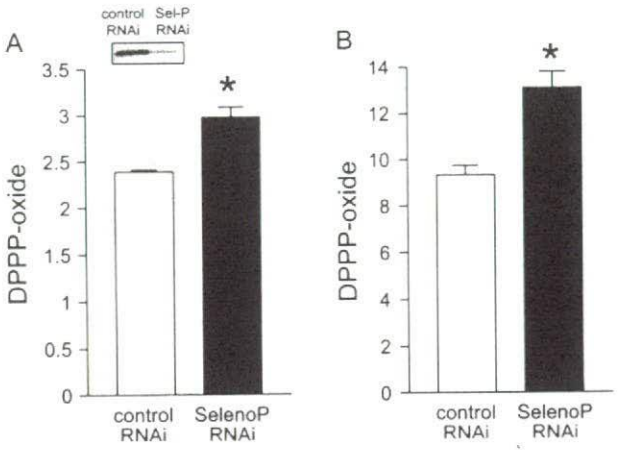

Figure 4 Selenoprotein P suppresses lipid hydroperoxides in myofibroblasts. LF2 cells were treated with RNAi oligonucleotide (#1 oligonucleotide), which suppresses selenoprotein P expression (A, inset). Then DPPP-oxide levels in cells (A) and culture medium (B) were measured as described in "Experimental procedures." Mean values of DPPP-oxide fluorescence per cell are shown with SD ($n = 3$). The statistical significance of the differences between oligonucleotide-treated cells and control cells was calculated by Student's t-test. $*P < 0.01$, $**P < 0.005$.

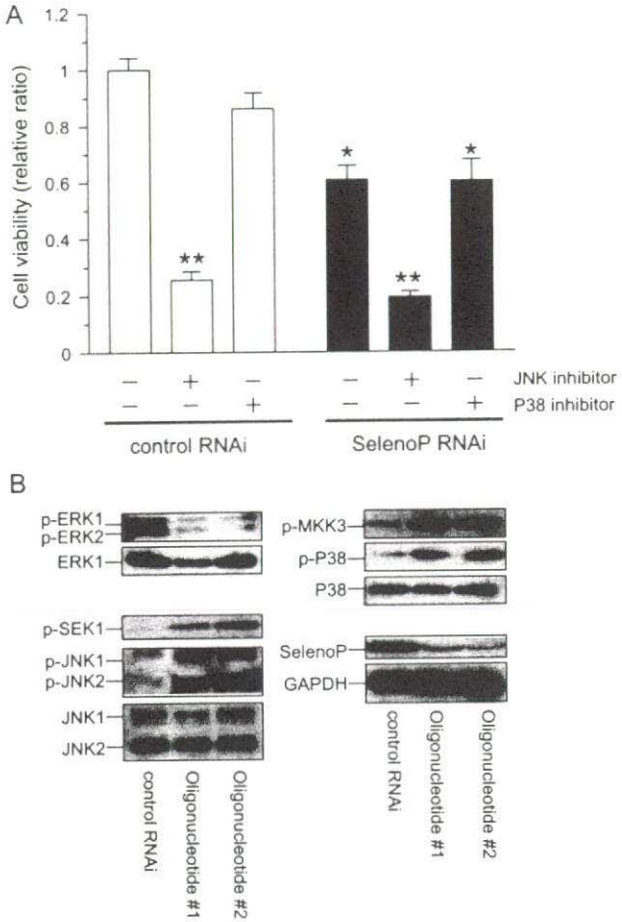

Figure 5 Cell viability and MAP kinase activity in selenoprotein P knockout cells. (A) LF2 cell viability was measured by MTT assay as described in "Experimental procedures." Cells were treated with RNAi oligonucleotide (#1 oligonucleotide), and then incubated with 50 μM SP600125 (JNK inhibitor) or SB20385 (P38 inhibitor) for 12 h. Cell viability is presented as the relative ratio over untreated cells. Mean values are shown with SD ($n = 3$). The statistical significance of the differences among samples was calculated by Student's t-test. $*P < 0.05$, $**P < 0.005$. (B) LF2 cells were treated with two RNAi oligonucleotides that each suppresses selenoprotein P expression. The activity status of MAP kinase pathway proteins was determined using active form specific antibodies.

P suppresses lipid hydroperoxides in myofibroblasts. Finally, we examined the effect of selenoprotein P knockdown on the viability of myofibroblasts. As expected, the suppression of selenoprotein P resulted in a partial but significant decrease in the number of viable cells (Fig. 5A). LF1 and LF2 cells showed similar responses to selenoprotein P knockdown (representative data from the analysis on LF2 cells are shown in Figs 4 and 5A). Together, these results signify that myofibroblast viability depends on the expression of selenoprotein P to suppress self-generated oxidative stresses.

JNK is a critical effector of myofibroblast viability

Importantly, the suppression of selenoprotein P had a partial effect on cell viability (Fig. 5A), suggesting that the signaling pathway for maintaining cell viability is activated in myofibroblasts. Thus, we assessed the activity status of MAP kinase pathway proteins, which are known to be activated in response to various types of stress (Lewis *et al.* 1998). Western blot analysis using active form-specific antibodies showed that JNK and p38 MAP kinase pathway proteins are activated in response to selenoprotein P suppression (Fig. 5B), suggesting that these pathways may be involved in the regulation of myofibroblast viability. To test this possibility, we examined the effects of specific inhibitors on cell viability. Treatment with SP600125, a specific inhibitor of JNK, resulted in considerable loss of viability in cells treated with selenoprotein P RNAi oligonucleotide (Fig. 5A), suggesting that JNK pathway plays a critical role in the regulation of myofibroblast survival. Importantly, JNK inhibitor alone caused a significant loss of cell viability without selenoprotein P RNAi. Thus, these results suggest

that endogenous JNK activity, which is detectable in Fig. 5B, may also have a critical function in maintaining viability of myofibroblasts. Our preliminary results show that, although selenoprotein P is highly up-regulated, myofibroblast cells still suffer significant amount of endogenous oxidative stress, resulting in the spontaneous apoptosis (data not shown). Together, endogenous activity of JNK may function for maintaining cell viability in response to this background (leaked) oxidative stress. In contrast to JNK inhibitor, treatment cells with an inhibitor of p38 MAP kinase (SB20385) had no significant effect on viability, although SB20385 effectively blocked p38 MAP kinase activity, which was confirmed by the suppression of the phosphorylation of MAPKAP2, a substrate of p38 MAP kinase (data not shown). LF1 and LF2 cells showed similar responses to inhibitor treatment (representative data from the analysis of LF2 cells are shown in Fig. 5). Together, these results suggest that JNK, but not P38, have a critical role in the regulation of myofibroblast viability.

Selenoprotein P functions as an anti-apoptotic factor against oxidative stress

Loss of viability in cells treated with selenoprotein P RNAi may be due to increased apoptosis caused by self-generated oxidative stress. To determine whether selenoprotein P has a role in apoptosis, we measured cell apoptosis in RNAi-treated cells. As shown in Fig. 6A, the number of apoptotic cells was significantly increased in response to RNAi treatment, suggesting that selenoprotein P functions as an anti-apoptotic factor in myofibroblasts. We next examined the effect of selenoprotein P over-expression on oxidative stress-induced apoptosis and MAP kinase activation. As expected, over-expression of selenoprotein P significantly suppressed hydrogen peroxide-induced apoptosis in RPE cells (Fig. 6B). Selenoprotein P over-expression also significantly suppressed hydrogen peroxide-induced activation of JNK and P38 (Fig. 6C). Together, these results reveal the function of selenoprotein P as an antioxidant and anti-apoptotic protein.

Discussion

The results of this study provide three new insights into the molecular mechanisms underlying the pathogenesis of IPF. First, we identified an enhanced generation of lipid hydroperoxides as a novel pathophysiological feature of myofibroblasts in IPF. Second, we identified selenoprotein P as one of the key regulators of myofibroblast viability, and support this finding with evidence that selenoprotein P suppresses toxic lipid hydroperoxides produced by the myofibroblasts themselves. Third, we found that JNK is a critical regulator of cell viability, acting downstream of the endogenous oxidative stresses.

Recently, oxidant–antioxidant imbalance and the mechanisms of redox regulation in human IPF have received much attention mainly due to the beneficial effects of antioxidant agents in patients with IPF (Behr *et al.* 1997; Demedts *et al.* 2005; Walter *et al.* 2006).

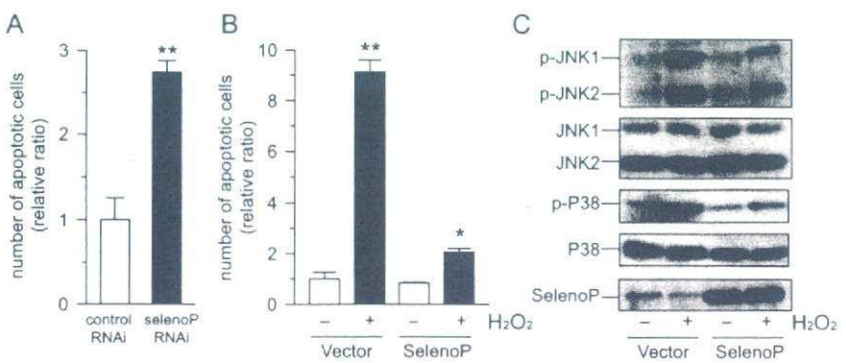

Figure 6 Anti-apoptotic function of selenoprotein P. (A) LF2 cells were treated with mixture of #1 and #2 RNAi oligonucleotide. Cell apoptosis was measured as described in "Experimental procedures." Cell apoptosis is presented as the relative ratio over control RNAi-treated cells. Mean values are shown with SD ($n = 3$). The statistical significance of the differences among samples was calculated by Student's *t*-test. $^{**}P < 0.005$. (B) RPE cells were transfected with selenoprotein P cDNA for 48 h, treated with or without 100 μM hydrogen peroxide for 6 h. Cell apoptosis is presented as the relative ratio over control cells. Mean values are shown with SD ($n = 3$). The statistical significance of the differences among samples was calculated by Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.005$. (C) After 48 h transfection, RPE cells were treated with or without 100 μM hydrogen peroxide for 3 min (p38 MAPK) or 120 min (JNK). The activity status of P38 and JNK was determined using active form specific antibodies.

Although a number of studies have suggested a significant role for redox regulation in the progression of human IPF, little is known about the mechanistic details of this regulation (Kinnula *et al.* 2005). Some major questions remaining to be answered include: What kinds of ROS contribute to the progression of IPF? What kinds of cells generate ROS? What is the function of the generated ROS? How do myofibroblasts and type II alveolar epithelial cells (dominant cells in fibrotic lesions) survive under oxidative conditions? Recent studies have shed some light on these questions, especially in relation to myofibroblasts. Waghray *et al.* (2005) reported that fibroblasts isolated from the lungs of patients with IPF generate extracellular hydrogen peroxide in response to transforming growth factor-β1 (TGF-β1). They also reported that secreted (extracellular) hydrogen peroxide is toxic to pulmonary epithelial cells, which may cause injury in the alveolar epithelium. Larios *et al.* (2001) stimulated lung fibroblasts with TGF-β1, and found that extracellular matrix proteins are cross-linked in an oxidant-dependent manner, presenting a potential mechanism for the deposition of extracellular matrix. In contrast, the results of several immunohistochemical studies suggest that the levels of antioxidant and detoxification enzymes are elevated in areas of epithelial regeneration, but not in the fibrotic lesions of interstitial lung diseases. These enzymes include superoxide dismutase (Lakari *et al.* 2000), catalase (Lakari *et al.* 2000), thioredoxin (Tiitto *et al.* 2003), glutaredoxin (Peltoniemi *et al.* 2004) and heme-oxygenase 1 (Lakari *et al.* 2001). Together, these results suggest that the species, distributions and functions of ROS may vary in distinct tissue microenvironments in IPF. Thus, the redox balance in fibrotic lesions, which consist mainly of fibroblasts and myofibroblasts, appears to be regulated by an unknown mechanism. Our study provides novel evidence that myofibroblasts function as a generator of lipid hydroperoxides, whose function in the pathogenesis in IPF remains virtually unidentified. Lipid hydroperoxides and reactive aldehydes are known to function as inducers of apoptosis, regulators of transcription factors, substrates for protein carbonylation and protein cross-linkers (Niki *et al.* 2005). The identification and biochemical characterization of lipid hydroperoxides generated in myofibroblasts should provide novel information about the pathophysiological function of myofibroblasts in IPF. A recent observation suggest that vitamin E supplementation may suppress the progression of experimental lung fibrosis, supports our results (Deger *et al.* 2006). Immunohistochemical analysis to identify the species and localizations of lipid hydroperoxides in fibrotic lung tissues will be one of the next challenges.

Our study identified selenoprotein P as one of the key effectors of redox regulation and cell viability in myofibroblasts. Selenoprotein P is an abundant extracellular protein that functions as a phospholipid hydroperoxide reductase and as a selenium supply protein. Our results are consistent with the reported function of selenoprotein P as an antioxidant. In our study, we detected the expression of the selenoprotein P protein in cell cultures and tissue sections from patients with IPF. Further analyses should be carried out on serum, EBC and BALF in order to understand whether the dysregulation in selenoprotein P expression is limited to the lung alveoli. Recently, there have been several reports of the association of selenoprotein P with human diseases including cirrhosis (Burk *et al.* 1998) and Crohn's disease (Andoh *et al.* 2005). In these reports, a suppression of selenoprotein P expression, which may cause oxidative stress in patients, was associated with disease severity. Our results revealed a paradoxical feature of selenoprotein P, whose expression protects disease-promoting cells from oxidative damage. In addition, our immunohistochemical analysis revealed the expression of selenoprotein P in the alveolar epithelium, as well as in interstitial fibrotic lesions. Thus, the expression of selenoprotein P may also play a significant role in the viability of epithelial cells in an oxidative environment. However, the precise regulatory mechanisms for the expression of selenoprotein P remain to be determined.

One of the key features of the pathogenesis of IPF is the persistence of myofibroblasts in the fibrotic lesions. The elimination of myofibroblasts by apoptosis is a critical process during normal cutaneous wound healing, a process that is suppressed in fibrotic diseases such as IPF (Thannickal & Horowitz 2006). In addition, the alveolar microenvironment in IPF is characterized by high concentrations of various cytokines, ROS and reactive biomolecules, which induce the apoptosis of type 1 alveolar epithelial cells (Thannickal & Horowitz 2006). Thus, the acquisition of an anti-apoptotic phenotype in this microenvironment is a critical process for the persistence of myofibroblasts in IPF. Several studies have shown that the anti-apoptotic phenotype may be regulated by specific signaling molecules in myofibroblasts. These include TGF-β1 (Horowitz *et al.* 2004), FAK (Vittal *et al.* 2005), PI-3K (Horowitz *et al.* 2004), AKT (Horowitz *et al.* 2004) and XIAP (Tanaka *et al.* 2002). Our study reveals that JNK plays an important role in cell viability, providing evidence that a specific cell survival signaling pathway is activated in response to oxidative stress. Although function of JNK and selenoprotein P in the regulation of cell viability is evident, mechanism of myofibroblast survival remains to be analyzed. Therefore, assessment of mechanism of JNK activation, as well as

the identification of downstream targets of JNK, will be important as the next challenge. Recently, strategies to induce selective myofibroblast apoptosis are expected to yield an effective approach to the treatment of fibrotic diseases (Tan *et al.* 1999). Thus, a detailed analysis of this previously unidentified signaling pathway, as well as other apoptosis-regulating pathways, may provide basic information for future targeted therapies.

Experimental procedures

Gene expression profiling

Total RNA was extracted from LF1 and LF2 myofibroblast cell cultures using Isogen (Nippon gene, Tokyo, Japan). The preparation of cDNA from RNA derived from the cell cultures, sample hybridization and scanning of the GeneChip® Human Genome Focus Arrays (Affymetrix) were performed at the Bio Matrix Research (Kashiwa, Chiba, Japan) in accordance with the procedures established by Affymetrix. The Affymetrix Genome Focus chip contains 11 replicate probe sets per gene that are used to address the reliability of the generated data and to determine a *P*-value for probe set binding using GCOS software (Affymetrix). Hybridization experimental data for each myofibroblast sample were normalized to the data from TIG7 cells. Differentially expressed genes were selected at a threshold of 1.5-fold difference between groups with a significance level of $P < 0.005$. Clustering analysis of genes was carried out using a TIGR MultiExperiment Viewer <http://www.tm4.org/mev.html>. Gene ontology analysis of the significantly up-regulated genes was carried out using DAVID (Database for Annotation, Visualization and Integrated Discovery) and EASE (Expression Analysis Systematic Explorer), web-based applications <http://david.abcc.ncifcrf.gov/>, which allow access to a relational database of functional annotations. The EASE score, a variant of the Fisher exact probability that weights significance in favor of themes supported by more genes, was used to judge the over-presentation of specific functional annotations. Data are expressed as enrichment score, the geometric mean (in -log scale) of *P*-values in a corresponding annotation. The original microarray data was submitted in Gene Expression Omnibus (GEO) at National Center for Biotechnology Information <http://www.ncbi.nlm.gov/geo>. Data accession number is GSE6804.

Immunoblotting analysis

Cell lysates were prepared from subconfluent cultures of myofibroblasts. The cells were washed with ice-cold phosphate-buffered saline and lysed in 50 mM Tris–HCl, pH 7.2, 2% SDS, 10 mM dithiothreitol, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mM sodium orthovanadate. Protein amount was determined by the DC assay (BioRad, Hercules, CA). Extracts (20 μg) were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% non-fat dry milk in TBS containing 0.1% Tween 20, and incubated with primary antibodies diluted with 5% bovine serum albumin in TBS. Primary antibodies included mouse anti-αSMA (1 : 200, Dako, Glostrup, Denmark) and goat anti-selenoprotein P (1 : 500, sc-22639, Santa Cruz, CA), mouse anti-GAPDH (1 : 1000, 1D4, Covance, Berkeley, CA), mouse anti-ERK1 (1 : 5000, BD Bioscience, San Jose, CA), mouse anti-ERK1/2-pThr202/pTyr204 (1 : 1000, BD Bioscience), rabbit anti-JNK/SAPK1 (1 : 1000, BD Bioscience), mouse anti-JNK-pThr183/pTyr185 (1 : 100, BD Bioscience), mouse anti-p38α/SAPK2a (1 : 5000, BD Bioscience), mouse anti-p38 MAPK (1 : 2500, BD Bioscience) and rabbit anti-MAPKAP2-pThr334 (1 : 1000, Cell Signaling, Danvers, MA), rabbit anti-SEK1-pThr261 (1 : 200, Cell Signaling), rabbit anti-MKK3-pSer189 (1 : 200, Cell Signaling). Blots were probed with goat anti-mouse (1 : 10 000, BioRad), goat anti-rabbit (1 : 10 000, BioRad) or donkey anti-goat (1 : 10 000, Santacruz, Santacruz, CA) secondary antibody coupled to horseradish peroxidase and visualized by enhanced chemiluminescence (GE Healthcare, Piscataway, NJ).

Immunohistochemistry

Tissue samples were embedded in OCT compound (Cryo Mount, Muto Pure Chemical, Tokyo, Japan), cut into 4 μm-thick sections, and blocked with 5% non-fat dry milk for 30 min. The sections were washed with PBS, and the endogenous peroxidase activity was blocked with 3% hydrogen peroxide. The samples were incubated with anti-selenoprotein P antibody (Santacruz) followed by the second antibody (Simple stain MAX-PO (multi) Nichirei, Tokyo, Japan). After washing thoroughly, positive signals were obtained following incubation with 3,3-diaminobenzidine tetrahydrochloride (Nichirei, Tokyo, Japan). Carrazzi's hematoxylin was used as a counterstain.

Measurement of lipid hydroperoxides

Lipid hydroperoxides were detected using the fluorescence probe diphenylpyrenylphosphin (DPPP, Takahashi *et al.* 2001). Subconfluent cells were incubated with 100 μM DPPP in DMEM for 10 h. Then the labeled cells were trypsinized, and resuspended in PBS. Fluorescence intensities of cell suspensions (5×10^4 cells/mL) were measured with a Spectrofluorophotometer RF-5300PC (Shimazu CO., Kyoto, Japan) at excitation and emission wavelengths of 351 and 380 nm, respectively. Cell suspensions without DPPP labeling were used as negative controls. The fluorescence intensity of the culture medium used for cell labeling, equivalent to 2.5×10^4 cells, was also measured.

RNA interference

dsRNAi oligonucleotides (100 pmol) were transfected into LF cells using Fugene HD (Roche). The cells were then incubated for 96 h with two oligonucleotide sequences of selenoprotein P and examined for the presence of lipid hydroperoxides. Following sequences were used as dsRNAi oligonucleotides:

#1: 5′CAAGATCCAATGCTAAACT3′

#2: 5′GAAGCCATTAAGATTGCTT3′

Control siRNA oligonucleotide (Silencer® negative control #1 siRNA, Applied Biosystems, Foster City, CA) was also transfected into LF cells using Fugene HD.

Cell viability and apoptosis

Cell viability was measured by the MTT assay as described previously (Kabuyama *et al.* 1998). Cell apoptosis was measured by an ssDNA ELISA assay (ApoStrand™ ELISA Apoptosis Detection Kit, Biomol, Plymouth Meeting, PA), which detects single-stranded DNA specifically generated in apoptotic cells. Measurement was carried out according to the manufacture's instruction.

Selenoprotein P over-expression

Selenoprotein P over-expression was carried out as described previously (Tujebajeva *et al.* 2000). Briefly, the coding and 3′-untranslated region of selenoprotein P gene was PCR-amplified and cloned into mammalian expression vector pcDNA3. RPE cells were pretreated with 50 nM sodium selenite for 12 h and transfected with DNA using Fugene 6 (Roche,Valencia, CA). Cells were analyzed at 48 h post-transfection. Expressed selenoprotein P was recovered from culture medium by Ni-NTA agarose (Qiagen, Mannheim, Germany) and detected by Western blotting. PCR primers used are:

5′-GGATCCCCAACGATGTGGAGAAGCCTGGGGCTT-GCC-3′ (Forward)

5′-GAATTCTGAATTTATTTGGACAAATCCGTACTGTA-3′ (Reverse)

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Supplementary material

The following supplementary materials are available for this article online:

Figure S1 Myofibroblasts isolated from patients with IPF.

Figure S2 Selective up-regulation of lipid hydroperoxide reductases in myofibroblasts.

Mannose-Binding Lectin Gene Polymorphisms and the Development of Coal Workers' Pneumoconiosis in Japan

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Background *Infection, immunity and genetic factors play roles in the development of coal worker's pneumoconiosis (CWP) and progressive massive fibrosis (PMF). We investigate whether the genetic polymorphisms of mannose-binding lectin (MBL), one of the key molecules of innate immunity, is associated with the susceptibility to CWP.*
Method *MBL2 polymorphisms (codon54, promoter -221, and -550) were assessed for 197 patients with CWP (119 with nodular CWP and 78 with PMF) and 153 unexposed regional controls. Serum MBL concentrations were measured in 119 CWP patients.*
Results *Three polymorphisms were in linkage disequilibrium for all study populations. The MBL2 genotype and haplotypes were associated with lower serum MBL levels. The frequency of such MBL2 genotype and haplotypes were significantly higher in patients with CWP compared to controls, whereas these distributions were not different between patients with nodular CWP and those with PMF.*
Conclusion *MBL2 polymorphisms and haplotypes may be one of the genetic determinants for the susceptibility of CWP.* Am. J. Ind. Med. 51:548–553, 2008. © *2008 Wiley-Liss, Inc.*

KEY WORDS: coal dust; silica; fibrosis; complement activation; genotype

INTRODUCTION

Coal worker's pneumoconiosis (CWP) is an occupational lung disease resulting from the inhalation of coal and silica particles in various forms. Coal miners are high risk populations. In Japan, there used to be more than 300,000 coal workers and more than 5,000 workers were newly diagnosed with CWP in 1950s. After the successive closure of coal mines, the number of coal workers decreased drastically. Over the last decade, about 200 coal miners have been newly diagnosed with CWP every year and the number has not changed even after the shut down of all coal mines [Kimura, 2007]. In the world, There are more than a 100,000 coal miners in the US and in China 1749 coal miners were diagnosed with CWP, which was 33.3% of occupational diseases in 2005 (2006 China Health Statistical Yearbook, Ministry of Health, P.R. China). CWP is characterized by the accumulation of inflammatory cells in the lung, thickening of the alveolar walls, and the formation of fibrotic nodules [Ziskind et al., 1976]. Clinically, CWP is divided into two major groups: nodular CWP and progressive massive fibrosis (PMF). Patients with PMF, a conglomeration of small rounded opacities, have lower pulmonary functions and life expectancy than those with nodular CWP. Every year, about 1–2% of nodular CWP patients are diagnosed with PMF, even after no longer being exposed, the PMF patients get worse. However, the mechanisms of individual variations

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in the incidence and the severity of CWP have not been clarified fully. Infection, immunity and genetic factors are speculated to be involved in the formation of CWP and the development of PMF [Ziskind et al., 1976; Honda et al., 1993; Chang et al., 2001]. Recent studies have revealed that genetic polymorphisms of pro-inflammatory and fibrogenic cytokines play roles in the susceptibility to CWP and silicosis [Ohtsuka et al., 1995; Zhai et al., 1998; Corbett et al., 2002; Nadif et al., 2003; Wang et al., 2005].

Mannose–binding lectin (MBL) is a C-type serum lectin characterized by the presence of both collagenous region and lectin domain. MBL acts in the complement system through lectin pathway and also functions as an opsonin [Fujita et al., 2004]. MBL has been known to play an important role in innate immunity. Low serum concentrations of MBL in humans is the basis for a common opsonic defect and is associated with recurrent infections and immune disorders [Super et al., 1989; Turner, 1991; Summerfield et al., 1995; Lau et al., 1996; Sullivan et al., 1996]. Serum MBL levels and biological activities are regulated by *MBL2* polymorphisms [Sumiya et al., 1991; Lipscombe et al., 1992; Turner et al., 1993; Madsen et al., 1994, 1995; Garred et al., 2003]. Three polymorphic sites are situated within exon1 of the *MBL2* and have been described as codon54 (GGC: allele A to GAC: allele B), codon57 (GGA to GAA: allele C) and codon52 (CGT to TGT: allele D). Population studies have shown that there are significant ethnic differences in the polymorphisms; several studies have reported that the codon54 SNP is common but that the other two SNPs are absent or extremely rare in the Japanese population [Tsutsumi et al., 2001]. Two SNPs lie at promoter -550 (G/C, allele H/L) and -221 (G/C, allele Y/X) of *MBL2* and have been demonstrated to affect serum MBL levels in some studies [Madsen et al., 1995].

On the basis of these backgrounds, we investigated the relationship between *MBL2* polymorphisms and CWP in the Japanese population. Results have suggested that codon54 polymorphism and haplotypes of codon54 and promoter SNPs are associated with CWP.

MATERIALS AND METHODS

Subjects

The case group consisted of 197 CWP patients recruited from an outpatient clinic of the Clinical Research Center for Occupational Respiratory Disease (CRCORD), Iwamizawa Rosai Hospital (Hokkaido, Japan). Diagnosis of CWP was made by the three doctors (YO, KK, and HK) of CRCORD, based on the history of occupational exposure, physical examination, pulmonary function tests, and chest radiograph findings, along with the International Labor Organization (ILO) interpretation. Two groups of CWP patients were recruited for the study; the nodular CWP group consisted of 119 subjects with ILO grade of 1/0 to 3/2 or over, and the PMF group consisted of 78 patients with ILO grade 4C. To avoid confounding by the severity of the underlying disease such as a milder form of PMF is sometimes misclassified as severe nodular CWP, the patients with ILO grade 4A or 4B were not included. In order to match the dust exposure condition, only patients who had worked at the coalface were recruited. The duration of occupational exposure and smoking history were also matched between nodular CWP and PMF groups. Unexposed regional controls ($n = 153$, 90 males and 63 females) were randomly selected among healthy volunteers, who live in the same district. Although age, gender, and the smoking habits of controls were not matched for the CWP group, they were expected to represent average genetic distribution in the district. The ethnicity of all the patients and controls was Japanese.

The study was approved by the Research Ethics Committee of Fukushima Medical University School of Medicine, and informed consents were given from all the participants.

Pulmonary Function and Blood Gas Analysis

Spirometry and arterial blood gas analyses were examined by CHESTAC-9800 (Chest, Tokyo, Japan) and ABL555 (Radiometer Copenhagen, Tokyo, Japan) in the beginning of the study. Three attempts were made to meet the ATS criteria in each case [American Thoracic Society, 1991]. The best data were recorded if ATS criteria were met. Arterial oxygen and carbon dioxide partial pressures (PaO_2, $PaCO_2$) were measured under quiet room air breathing.

Genotyping

Blood samples were collected with EDTA-Na2 and frozen at −80°C before DNA preparation. Genotyping of codon54 (GGC → GAC, allele A or B) was performed using a polymerase chain reaction (PCR)-restriction fragment length polymorphism (PCR-RFLP) technique. PCR was performed using a PCR reaction mixture that contained 1× PCR buffer, 200 nM concentrations of each primer, 100 ng genomic DNA, 0.2 μM of each dNTPs (Takara, Tokyo, Japan), 1.5 μM $MgCL_2$ and 2.5 U of Taq polymerase (AmpliTaq Gold, Applied Biosystems, Branchburg, NJ) with a total volume of 20 μl. We used the following primers: Fwd: 5′-AGT CGA CCC AGA TTG TAG GAC AGA G-3′, Rev: 5′-AGG ATC CAG GCA GTT TCC TCT GGA AGG-3′. The cycling for the 20 μl reaction were at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 60°C for 60 s, and 72°C for 120 s, followed by one cycle at 72°C for 5 min. The PCR products were digested at 50°C for 2 hr with Ban I, resulting in allele B (349 bp), and allele A (260 + 89 bp), heterozygous individuals were detected by the presence of all three fragments (349 + 260 + 89 bp). The amplified PCR

products were separated on 2% agarose gels stained with ethidium bromide at 100 V for 35 min and visualized by UV illumination. Because codon52 and codon57 mutations of the MBL gene are absent or extremely rare in Japanese population [Tsutsumi et al., 2001], only codon54 mutation in exon1 was studied.

For promoter -550 (G → C, allele H or L) and -221(G → C, allele Y or X) genotypes, the fluorogenic allele-specific TaqMan probes and primers were used (TaqMan SNP Genotyping Assays, Applied Biosystems, Foster City, CA). Primers and probes are as follows: (-221) forward 5′-CCA ACG TAG TAA GAA ATT TCC AGA GA-3′, reverse 5′-CAA CCC AGC CCA GAA TTA ACT G-3′, probe 5′-AGC CTG TGT AAA AC-3′ and 5′-CCT GTC TAA AAC ACC-3′; (-550) forward 5′-CCA ACG TAG TAA GAA ATT TCC AGA GA-3′, reverse 5′-CAA CCC AGC CCA GAA TTA ACT G-3′, probe 5′-AGC CTG TGT AAA AC-3′, and 5′-CCT GTC TAA AAC ACC-3′. Allelic discrimination was performed using ABI Prism 7000 Sequence Detection System (Applied Biosystems, CA).

Assay for MBL

The serum samples were collected from 119 patients with CWP and frozen at −20°C before the measurement. The serum sample of control subject was not available in this study. The concentrations of MBL were measured by the commercially available ELISA kit (HyCult Biotechnology, Uden, Netherlands).

Statistics

The allele distributions were analyzed for Hardy–Weinberg equilibrium. Haplotypes and linkage disequilibrium of three SNPs were analyzed by SNP Analyze software (DYNACOM, Kanagawa, Japan). The genotype distributions among the subjects with CWP and controls were compared by the chi-squared test. The age, working years, and pulmonary functions were compared by the student's *t*-test. Serum MBL levels among each genotype were analyzed by the Kruakall–Wallis test. All the analyses were performed using SPSS Base 11.0J program (SPSS, Inc., Tokyo, Japan). A *P*-value less than 0.05 was considered statistically significant.

RESULTS

Subject Characteristics

The mean age of patients with CWP was 68.5 years and mean duration of dust exposure was 29.8 years (Table I). The mean age of unexposed controls was 45.9 ± 0.5 (SE) years. In CWP, the PMF group was significantly older compared to the nodular CWP group ($P < 0.01$). There was no significant difference in smoking index between nodular CWP and PMF group. In pulmonary functions, patients with PMF had significantly lower vital capacity (VC), forced expiratory volume in 1 s (FEV1), and FEV1/FVC%, and PaO_2 compared to those with nodular CWP ($P < 0.01$).

MBL2 Genotypes Distribution and Associated Serum MBL Level

The distribution of MBL2 genotypes are shown in Table II. The genotype distribution was fit to Hardy–Weinberg equilibrium. Frequency of codon54 variant (allele B) was significantly higher in CWP patients than in controls ($P < 0.01$). Significantly higher frequencies of allele B were also found in the nodular CWP group ($P < 0.05$) and in the PMF group ($P < 0.01$) compared to the control subjects. MBL codon54 heterozygous (A/B) was found more frequently in subjects with CWP and in the PMF group than in controls ($P < 0.01$). There was no significant

TABLE I. Characteristics of the Patients With Coal Worker's Pneumoconiosis (CWP)

	Nodular CWP (n = 119)	PMF (n = 78)	Total CWP (n = 197)
Age (old years)	67.3 (65.9–68.7)	70.4 (69.0–71.7)*	68.5 (67.5–69.5)
Duration of exposure (years)	29.6 (28.3–30.9)	30.1 (28.8–30.8)	29.8 (28.8–30.8)
Smoking index (pack years)	28.9 (24.8–33.0)	22.6 (16.5–28.6)	26.7 (23.3–30.1)
Pulmonary function			
VC (L)	3.4 (3.3–3.5)	2.9 (2.8–3.0)*	3.3 (3.2–3.3)
%VC	103.2 (100.8–105.6)	90.0 (86.0–93.0)*	99.2 (97.1–101.4)
FEV1 (L)	2.3 (2.3–2.4)	1.7 (1.6–1.8)*	2.1 (2.1–2.2)
FEV1%	70.4 (68.9–71.8)	59.3 (56.4-62.3)*	67.1 (65.6–68.6)
PaO_2 (Torr)	80.0 (78.3–81.6)	75.5 (73.1–77.8)*	78.3 (77.0–79.7)
$PaCO_2$ (Torr)	41.9 (41.3–42.4)	42.0 (41.3–42.7)	41.9 (41.5–42.3)

Mean (95%CI).
*$P < 0.01$ versus nodular CWP.

TABLE II. Distribution of MBL2 Genotypes in CWP Patients and Controls

		CWP				
SNP	**Genotype or allele**	**Nodular CWP (n = 119)**	**PMF (n = 78)**	**Total (n = 197)**	**Controls (n = 153)**	**MBL (mg/L) (mean ± SE) (n)**
Codon54 (allele B)	Number (n)	119	78	197	153	
Genotype	A/A	85 (71.4%)	48 (61.5%)	133 (67.5%)	128 (83.7%)	3.02 ± 0.2
	A/B	33 (27.7%)	26 (33.3%)*	59 (29.9%)*	24 (15.7%)	0.47 ± 0.08***
	B/B	1 (0.8%)	4 (5.1%)	5 (2.5%)	1 (0.7%)	0.06 ± 0.02***
Allele	B	35 (14.7%)**	34 (21.8%)*	69 (17.5%)*	26 (8.5%)	
Promoter -221 (allele X)	Number (n)	115	78	193	107	
Genotype	Y/Y	94 (81.7%)	61 (78.2%)	155 (80.3%)	88 (82.2%)	2.30 ± 0.2
	Y/X	19 (16.5%)	16 (20.5%)	35 (18.1%)	18 (16.8%)	1.69 ± 0.2
	X/X	2 (1.7%)	1 (1.3%)	3 (1.6%)	1 (0.9%)	1.56 ± 0.0
Allele	X	23 (10.0%)	18 (11.5%)	41 (10.6%)	20 (9.3%)	
-550 (allele L)	Number (n)	115	78	193	107	
Genotype	H/H	34 (29.6%)	17 (21.8%)	51 (26.4%)	26 (24.3%)	2.80 ± 0.2
	H/L	52 (45.2%)	40 (51.3%)	92 (47.7%)	49 (45.8%)	2.35 ± 0.3
	L/L	29 (25.2%)	21 (26.9%)	50 (25.9%)	32 (29.9%)	1.20 ± 0.2†
Allele	L	112 (47.5%)	82 (52.6%)	194 (49.5%)	114 (52.8%)	

*$P < 0.01$, versus control.
**$P < 0.05$, versus control.
***$P < 0.01$, versus A/A genotype.
†$P < 0.05$, versus H/H and H/L genotype.

difference in genotype distributions between nodular CWP and PMF groups. Individuals with codon54 A/B or B/B genotype have significantly lower serum MBL levels compared to those with A/A genotype ($P < 0.01$).

For the analysis of promoter -221 and -550 polymorphisms, PCR product was not obtained in 4 CWP patients and 46 controls. This made the difference in the number of subjects from those for codon54. All the genotype distribution was fit to Hardy–Weinberg equilibrium. There were no significant differences in genotype distribution and allele frequencies for two promoter polymorphisms between patients with CWP and controls, and between the patients with nodular CWP and PMF. There was no significant difference in serum MBL concentration among subjects with each promoter -221 genotypes. However, -550 L/L genotype was associated with lower MBL concentration compared to H/H or H/L genotype ($P < 0.05$).

MBL2 Haplotypes and MBL Serum Levels

The linkage disequilibrium among two promoter variant loci and codon54 polymorphism was found. The AHY haplotype was found most frequently in subjects with CWP and controls. The BHX haplotype was absent in both CWP patients and controls. The subjects with BLY, ALX, BHY, and BLX haplotypes showed significantly lower serum MBL levels compared to those with AHY and ALY haplotypes (Table III). The subjects with BLY and BHY haplotypes also had significantly lower serum MBL levels compared to ALX and AHX. The frequency of ALY hapyotype in patients with CWP was significantly lower compared to that of controls ($P < 0.01$), while the frequency of BLY haplotype was significantly higher in patients with CWP compared to that in controls ($P < 0.01$). Logistical regression assessment for risk of CWP showed that the BLY haplotype was 2.9 times (95%CI: 1.817–4.710) more likely to be associated with CWP than control ($P = 0.000$) (Table III).

DISCUSSION

The present study is the first to investigate whether there is any association between the MBL gene polymorphisms and the susceptibility to CWP. We examined three SNPs of *MBL2* and measured serum MBL concentration. We found that the *MBL2* genotype and haplotypes were associated with lower serum MBL level. Furthermore, the frequency of such MBL2 genotype and haplotypes were significantly higher in patients with CWP compared to controls.

MBL is one of C-type lectin, an important component of innate immunity. MBL activates complement through the lectin pathway and directly opsonizes pathogens and enhances activities of phagocytes [Turner and Hamvas, 2000]. Polymorphisms of *MBL2* exon1 (codon52, 54, and 57), promoter -550 (allele H/L) and -221 (allele Y/X), and haplotypes codon54 combination with promoter polymorphisms have been demonstrated to regulate MBL serum

TABLE III. Distribution of *MBL2* gene Haplotype in all Subjects and MBL Serum Level, and Logistic Regression Analysis for the Risk of CWP

	CWP							
Haplotype	**Nodular CWP (%)**	**PMF (%)**	**Total (%)**	**Controls (%)**	**MBL (mg/L) (mean ± SE)**	**OR**	**95%CI**	***P*-value**
AHY	52.2	45.9	49.6	45.1	2.64 ± 0.1	1.211	1.017–1.443	0.032
ALY	23.0*	20.8*	22.2*	37.1	2.50 ± 0.1	0.584	0.488–0.699	0.000
BLY	14.8*	21.0*	17.3*	7.6	0.46 ± 0.1**	2.925	1.817–4.710	0.000
ALX	10.0	10.8	10.3	7.1	1.55 ± 0.2***	1.163	0.717–1.887	0.540
AHX	0.0	0.8	0.3	0.8	1.85 ± 0.1	0.871	0.494–1.533	0.631
BHY	0.0	0.8	0.3	0.8	0.47 ± 0.0**	1.365	0.264–7.051	0.710
BLX	0.0	0.0	0.0	1.5				

OR, odds ratio; 95%CI, 95% confidence interval.
*$P < 0.01$ versus controls.
**$P < 0.01$ versus AHY, ALY, ALX, and AHX haplotypes.
***$P < 0.01$ versus AHY and ALY haplotypes.

level and function [Lipscombe et al., 1992; Madsen et al., 1995; Garred et al., 2003; Wang et al., 2007]. MBL variant allele can result in functional MBL deficiency which links to the infectious and immune diseases [Davies et al., 1997; Graudal et al., 1998; Koch et al., 2001; El Sahly et al., 2004].

CWP is caused through inhalation of coalmine particulates, the major pathogenic component of which is silica, especially in Hokkaido, Japan. There is little evidence that MBL recognize inorganic particles such as silica and carbon. However, surfactant protein A (SP-A), one of the pulmonary C-type lectins, has recently been demonstrated to reduce silica-mediated alveolar macrophage injury and the effect involves the mannose recognition domain of the SP-A molecule [Spech et al., 2000]. Inhaled silica has been known to stimulate both innate and adaptive immune systems [Pernis, 2005]. In this context, although precise mechanisms are not clear, the results of the present study suggest a possible involvement of MBL to physiological responses of the lung to inorganic dust, and MBL deficiency may cause abnormal lung responses to inhaled coal and silica which promote CWP.

Another possible explanation may be related to the link of MBL deficiency to autoimmune diseases. MBL deficiency is associated with early disease onset, positive rheumatoid factor (RF), and a poor prognosis of rheumatoid arthritis (RA) patients [Graudal et al., 1998, 2000; Jacobsen et al., 2001]. In addition, MBL insufficiency was identified as the most potent single risk factor for Systemic Lupus Erythematosus (SLE) [Tsutsumi et al., 2001; Sullivan et al., 2003]. Previous studies found that immune disorders such as RA, SLE, and scleroderma are risks of CWP [Ziskind et al., 1976; Pernis, 2005]. Caplan's syndrome is a phenotype of CWP complicated with RA, characterized by the presence of relatively large nodules in CWP [Ondrasik, 1989]. These observations may provide a possible link between MBL and CWP through modified immune responses.

Previously, we reported that the distribution pattern of the TNF-α promoter -308 genotype was different between subjects with nodular CWP and PMF in the same population, suggesting that -308A allele enhances susceptibility to nodular CWP [Wang et al., 2005]. In the present study, although there was a significant difference in *MBL2* genotypes distribution between CWP patients and controls, no significant difference in genotype distribution between subjects with nodular CWP and PMF was observed. This may suggest that the MBL genotype associated with reduced serum MBL levels affects common pathways responsible both for nodular CWP and PMF.

Because of the limited number of sample size and suboptimal control selection, the results of the present study should be interpreted as preliminary ones. However, the results of the present study suggest that *MBL2* polymorphisms and haplotypes may be one of the genetic determinants for the susceptibility of CWP.

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ST2 GENE INDUCED BY TYPE 2 HELPER T CELL (Th2) AND PROINFLAMMATORY CYTOKINE STIMULI MAY MODULATE LUNG INJURY AND FIBROSIS

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☐ *The authors have investigated gene expression of ST2 in the lung tissue of a bleomycin (BLM)-induced lung fibrosis model in vivo and in a human lung fibroblast cell line, WI38, and a human type II alveolar epithelial cell line, A549, reacting to proinflammatory and type 2 helper T cell (Th2)-type cytokine stimuli in vitro. The lung mRNA expression of interleukin (IL)-4, IL-5, IL-1β, and tumor necrosis factor (TNF)-α increased significantly at day 7 after instillation of BLM, whereas interferon (IFN)-γ mRNA expression did not increase. ST2 and transforming growth factor (TGF)-β1 mRNA expression of the lung increased significantly between days 7 and 21, and increased to maximal levels at day 14 post-BLM challenge. ST2 mRNA expression statistically correlated with TGF-β 1 mRNA expression. In addition, the combination of IL-1 β, TNF-α, and IL-4 had an additive effect on ST2 mRNA expression from A549 cells and WI38 cells. These findings suggest that soluble ST2 gene may increase, possibly reflecting the development of the*

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inflammatory process and the Th2-type immune response in the fibrotic lung tissue, and may modulate a process of pulmonary fibrosis.

Keywords bleomycin, fibroblast, proinflammatory cytokine, pulmonary fibrosis, ST2, Th2-type cytokine, type II alveolar epithelial cell

Idiopathic pulmonary fibrosis (IPF) is defined as a specific form of chronic fibrosing interstitial pneumonia limited to the lung [1]. The etiology of IPF is not known, and IPF remains a devastating disease, with a >50% 5-year mortality rate [1]. Unfortunately, the pathogenesis of IPF is also incompletely understood. Although several drugs have been used or tried for IPF, there is no established treatment that definitely improves outcome [1]. Thus new therapies are awaited, based on new understanding of the pathogenesis of IPF. Advances in the study of immune reactions have shown that an imbalance between type 1 helper T cells (Th1) and type 2 helper T cells (Th2) plays a pivotal role in the inflammatory response in various diseases [2]. Mouse Thl cells produce interleukin (IL)-2, and interferon (IFN)-γ, whereas Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 [2]. Thl-type cytokines are involved in cell-mediated inflammatory reactions: several Thl-type cytokines activate cytotoxic and inflammatory functions [2]. Th2-type cytokines encourage antibody production, particularly immunoglobin E (IgE) responses, and also enhance eosinophil proliferation and function [2]. IFN-γ selectively inhibits proliferation of Th2 cells, and IL-10 inhibits cytokine synthesis by Thl cells [2]. Studies have revealed a predominance of the Th2-type cytokine pattern in inflammatory response of IPF. Reports have demonstrated that IFN-γ inhibits fibroblast proliferation and the production of collagen and other noncollagenous extracellular matrix proteins by these cells, whereas IL-4 promotes it [3–5]. The Th2-like pattern of immune response predominated in the infiltrating interstitial inflammatory cells and hyperplastic type II epithelial cells of patients with IPF [6, 7]. A significantly higher level of IL-4 was observed in cocultures of T cells with autologous alveolar macrophages from patients with IPF [8]. In the clinical study, IFN-γ treatment for patients with IPF improved pulmonary function and gas exchange [9]. These findings suggest that the persistent imbalance in the expression of Th1- and Th2-type cytokines in the lung may be one of the important underlying factors for the progression of pulmonary fibrosis. On the other hand, there is a strong link between the overexpression of proinflammatory cytokines, including tumor necrosis factor (TNF)-α and IL-1β, in the lower respiratory tract and the development of pulmonary fibrosis [10, 11]. In patients with IPF, TNF-α and IL-1β expression by alveolar and interstitial macrophages and type II epithelial cells has been reported to increase [11]. These findings

suggest that both Th2-type cytokine and proinflammatory cytokine may be involved in the inflammatory and fibroproliferative process of IPF. In vivo studies using a bleomycin (BLM)-induced pulmonary fibrosis model have also supported this concept by demonstrating that IL-4 mRNA expression is up-regulated in lung fibrosis lesions and that IL-12 attenuates pulmonary fibrosis via modulation of IFN-γ production [12, 13]. In addition, it has been reported that both IL-1β and TNF-α lead to acute/subacute and chronic inflammation that evolves into pulmonary fibrosis, and that the transient overexpression of IL-1β or TNF-α has a great effect in promoting fibrosis [14, 15]. These findings suggest that Th2-type cytokine and proinflammatory cytokine play important roles in the pathogenesis of BLM-induced pulmonary fibrosis.

The ST2 gene, also designated as T1, Fit-1, and DER4, was originally detected as one of the primary response genes at the initial stage of cell proliferation in fibroblasts [16–20]. It is classified into the IL-1 receptor (IL-1R) family due to the proximity of its locus to *Il-1rl* in both mouse and human chromosomes [21–25]. ST2 gene products are thought to have a variety of biological functions as signal transducers, based on the production of several forms of the ST2 gene products by alternative splicing, i.e., soluble secreted ST2 (IL-1RL1-a), transmembrane ST2L (IL-1RL1-b), and a variant form, ST2V (IL-1RL1-c) [16, 26, 27]. Recent studies have shown that ST2 gene products are predominantly expressed in Th2 but not Th1 cells, suggesting that they may play novel roles in immunological response as well as their roles in cell proliferation [25, 28–30]. There have been several reports demonstrating that serum ST2 protein increases in Th2-mediated disease, such as acute eosinophilic pneumonia and bronchial asthma [31, 32]. Furthermore, the ST2 gene could be expressed significantly when induced by proinflammatory cytokines such as TNF-α and IL-1β [33, 34]. These findings suggest that the ST2 gene may have been related to Th2-type immune response as well as to inflammatory response.

We previously reported that the serum soluble ST2 protein levels are significantly elevated in patients with acute exacerbation of IPF, and that these levels correlate with the serum levels of lactate dehydrogenase and C-reactive protein, and inversely correlate with arterial partial pressure of oxygen, the ratio of the partial pressure of oxygen in arterial blood to the fractional concentration of oxygen in inspired gas, and the percentage of predicted vital capacity [35]. These results suggest that soluble ST2 in the serum may increase, possibly reflecting the development of the inflammatory process and the Th2-type immune response in the IPF lung. Although ST2 gene expression is induced by proinflammatory stimuli such as IL-1 or TNF, there have been no reports about an additive effect on ST2 gene expression in the combination of proinflammatory cytokine

and Th2-type cytokine [33, 34]. We hypothesized that the ST2 gene may be up-regulated in the lung of the BLM-induced pulmonary fibrosis model if the fibroproliferative process is related to Th2-type cytokine and proinflammatory cytokine. To test this hypothesis, we investigated ST2 gene expression in the lung of a murine BLM-induced pulmonary fibrosis model, and we further looked for an additive effect on ST2 gene expression from epithelial cells or fibroblasts when proinflammatory cytokine and Th2-type cytokine were both present.

MATERIALS AND METHODS

Mice, Cells, and Reagents

We used C57BL/6 mice because they are a well-characterized inbred strain of mice that are susceptible to BLM-induced pulmonary fibrosis. Specific pathogen–free, female C57BL/6 mice weighing 20 to 25 g were obtained from Japan SLC (Tochigi, Japan) and housed in an animal facility of the Jichi Medical School. All animal experiments were conducted in accordance with principles stated in the *Guide for the Care and Use of Laboratory Animals* (NIH publication 86–23, National Institutes of Health, Bethesda, MD, 1985). The A549 (human type II alveolar epithelial cells) and WI38 (human lung fibroblasts) cell lines were obtained from American Type Culture Collection (Rockville, MD). BLM was purchased from Nippon Kayaku (Tokyo, Japan). Human recombinant proteins of IL-1β, TNF-α, and IL-4 were purchased from Sigma (St. Louis, MO).

BLM-Induced Pulmonary Fibrosis Model

To induce pulmonary fibrosis, we treated mice with intratracheal BLM (Nippon Kayaku, Tokyo, Japan) on day 0. Mice were anesthetized with 0.01 mL/g of 10% pentobarbital sodium solution (Abbott Laboratories, North Chicago, IL) injected intraperitoneally followed by intratracheal instillation of 5 units of BLM/kg body weight in 50 μL of sterile isotonic saline. In the preliminary study, 2 units of BLM/kg did not induce IL-4 and ST2 messenger RNA (mRNA) expression in the lung (data not shown). IL-4 and ST2 were only up-regulated using a very high, sublethal dose of BLM. Therefore, we used 5 units/kg of BLM. Mice were killed under anesthesia on days 0, 1, 4, 7, 14, 21, 28, and 56 for examination. At each time point, the right lungs from 4 animals were used for isolation of RNA for reverse transcriptase-polymerase chain reaction (RT-PCR), and the left lungs were used for hydroxyproline assay.