

# Changes of Tumor Necrosis Factor, Surfactant Protein A, and Phospholipids in Bronchoalveolar Lavage Fluid in the Development and Progression of Coal Workers' Pneumoconiosis<sup>1</sup>

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**Objective** To evaluate the alterations of biomarkers in the development and progression of coal workers' pneumoconiosis (CWP). **Methods** The type and number of cells, and the levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), pulmonary surfactant protein, phospholipids and fibronectin in bronchoalveolar lavage fluid were assayed in 14 health active coal miners, 21 coal miners without CWP and 13 miners with CWP of 0/1 to 1/1. **Results** Compared to active coal miners without CWP (8.23  $\mu\text{g/mL}$ ), TNF- $\alpha$  concentration was gradually decreased when dust exposure was stopped (5.90  $\mu\text{g/mL}$ ). Elevated surfactant protein A (SP-A) level and phosphatidylglycerol (PG) to phosphatidylinositol (PI) ratio were found in miners actively exposed to coal dust (6528 ng/mL for SP-A and 10. for PG/PI), and both parameters decreased when CWP progressed from CWP (0/1) (3419  $\mu\text{g/mL}$  for SP-A and 5.9 for PG/PI) to CWP (1/1) (1654  $\mu\text{g/mL}$  for SP-A and 5.5 for PG/PI). **Conclusion** Biomarkers in bronchoalveolar lavage fluid can be used to screen coal miners at high risk of developing coal workers' pneumoconiosis.

**Key words:** Pneumoconiosis; TNF-alpha; Pulmonary surfactant-associated protein A; Phospholipids

## INTRODUCTION

Coal workers' pneumoconiosis (CWP) is characterized by interstitial pulmonary fibrosis induced by inhalation of coal dust. Pneumoconiosis is traditionally diagnosed on the basis of a known exposure history, altered chest radiographs, and pulmonary malfunction. However, the use of these methods is limited in the diagnosis of early pneumoconiosis because fibrotic changes are usually manifested after a long latency process of inflammation and tissue impairment leading to clinical presentation. Thus, alterations of biomarkers in the development and progression of pneumoconiosis are of great interest in the early diagnosis of CWP.

Previous studies have shown that tumor necrosis factor alpha (TNF- $\alpha$ ), pulmonary surfactant-associated protein, and phospholipids play an important role in the pathologic process of pulmonary fibrosis. Except that TNF- $\alpha$  is directly associated with fibroblast cell proliferation and collagen accumulation, it induces infiltration of different inflammatory cells and release of fibrogenic cytokines<sup>[1-3]</sup>. It has been reported that the levels of pulmonary surfactant-associated protein A (SP-A) (the most abundant surfactant protein) and phospholipids (PL) [including phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylethanolamine, and sphingomyelin] are altered in experimental animals and workers after exposure to crystalline silica and coal dust. Such alterations have also been found in other

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Abbreviations: CWP: coal workers' pneumoconiosis; TNF- $\alpha$ : tumor necrosis factor-alpha; SP-A: surfactant protein A; PG: phosphatidylglycerol; PI: phosphatidylinositol; PL: phospholipids; PC: phosphatidylcholine; BALF: bronchoalveolar lavage fluid; IPF: idiopathic pulmonary fibrosis; RDS: respiratory distress syndrome; SP: simple pneumoconiosis; PMF: progressive fibrosis.

inflammatory interstitial pulmonary diseases such as idiopathic pulmonary fibrosis (IPF)<sup>[4]</sup> and respiratory distress syndrome (RDS)<sup>[5]</sup>. In general, the accumulation of surfactant PL and proteins and their complex interaction contribute to the defensive system and pulmonary repair process after exposure to air pollutants or particulates.

Previous studies have investigated the parameters derived from dust exposure. However, we are interested in the trends of parametric alterations in bronchoalveolar lavage fluid (BALF) of workers without and with the initial development of CWP. Therefore, we included coal miners without CWP, and coal miners with early CWP in this study. The purpose of this study was to identify possible biomarkers and to investigate their role in screening coal miners at high risk of developing pneumoconiosis and in predicting or diagnosing early CWP.

## METHODS

### *Subjects*

Forty-eight coal miners were included in this study, who worked in Xishan Coal and Power Group Limited Company in Shanxi Province of China. Among them, 14 were healthy active workers, 21 were diagnosed as having simple CWP, and 13 as having CWP 1/1. Most of the patients were relocated away from dust exposure when they were diagnosed as having CWP 1/1 (12 among 13). Information on demographics and personal exposure to smoking and occupational hazards was obtained from each subject through a questionnaire. This study was approved by Tongji Medical College and local ethic committee.

### *Bronchoalveolar Lavage (BAL) and Cell Isolation*

BAL was performed by instillation of 150 mL (30 mL for the first time, then 40 mL for three times) sterile physiologic saline at 37°C into the medial segment bronchus of right lobe during fiberoptic bronchoscopy. Recoveries (>40%) were obtained by 80 mmHg negative pressure. The later three recoveries were mixed in one polypropylene plastic tube for the following tests. Cells were harvested by centrifugation after BALF was filtered through double sterile surgical gauze.

### *TNF- $\alpha$ Assay*

TNF- $\alpha$  in BALF was determined by a cell lytic assay<sup>[6]</sup>. Briefly, mouse L929 fibroblast cells were incubated with the BALF test samples and samples inhibited by anti-TNF- $\alpha$  at 37°C in 5% CO<sub>2</sub>. After 18 hours, the test samples were removed, the plate was washed, and cell lysis was detected by crystal violet

staining in plate reader (Anthos, Vienna) at 450 nm ultraviolet light (Labsystems Multiskan, Germany).

### *Protein Assay and Surfactant Protein A*

Protein concentration in BALF supernatant was measured by a modified method of Lowry<sup>[7]</sup>. Pulmonary SP-A concentrations were measured by a human SP-A specific enzyme linked immunoabsorbant assay (ELISA). Immunoassay plates were coated overnight at 4°C with a monoclonal antibody against anti-human SP-A (BYK-Gulden, Konstanz), and then incubated with the test samples and standard dilutions of human SP-A at 37°C for 4 h, followed by incubation with a peroxidase conjugated anti-human SP-A polyclonal antibody for 2 h. The peroxidase was quantified by adding the substrate OPD and stopping the reaction with 1M sulphuric acid. The peroxidized OPD was measured at 405 nm ultraviolet light (Labsystems Multiskan).

### *Phospholipid (PL) and Fibronectin Analysis*

Total PL was extracted from the supernatant using the method of Folch and coworkers<sup>[8]</sup>. Briefly, 10 mL cell-free supernatant was added to 30 mL chloroform and methanol (2:1). The mixture was centrifuged to separate the methanol and chloroform layers. The chloroform layer was removed, dried under nitrogen, and redissolved in 200  $\mu$ L chloroform and methanol (2:1) for high-performance liquid chromatography (HPLC) analysis. PL was separated by HPLC (Gynotek Co., Munich, FRG) according to the method of Pison and colleagues<sup>[9]</sup>. Phospholipid composition was calculated by comparison with standard PL mixture (PG, PI, PE, PC, SPH) obtained from Sigma (Munich, FRG). Fibronectin concentration was determined by a fibronectin specific ELISA.

### *Statistical Analysis*

The difference in the means of all quantitative measurements between two groups was tested by the Mann-Whitney U-test. The principal component analysis was used to identify representative variables from the biomarkers included. SAS version 6.12 was used for statistical analysis.

## RESULTS

### *Effects of Age, Smoking, and Dust Exposure*

The demographic characteristics of all subjects are shown in Table 1. Years of employment with dust exposure in the three groups were compared. No difference in smoking habit was found among all groups.

TABLE 1  
Characteristics of Study Population\*

Groups	Coal Miners Without CWP	CWP 0/1	CWP 1/1
Numbers	14	21	13
Age (Years, $\bar{x} \pm s$ )	32.7 $\pm$ 7.9	40.1 $\pm$ 3.6 <sup>a</sup>	47.9 $\pm$ 9.1 <sup>a</sup>
Age Range (Years)	25-49	34-44	36-63
Years in Exposure	9.0 $\pm$ 6.2	14.5 $\pm$ 3.7	18.6 $\pm$ 7.1
Smoking (Years)	6	7	7
Smoking Pack-years <sup>b</sup>	6.9 $\pm$ 7.1 <sup>a</sup>	18.0 $\pm$ 14.7	18.3 $\pm$ 13.4

Note. \*Values are expressed as  $\bar{x} \pm s$ . <sup>a</sup> $P < 0.05$ , compared with healthy controls. <sup>b</sup>Packs of cigarettes per day  $\times$  years.

### Differential Cells in BALF

Total cells and the percentages of differential cells in BALF for the three groups are listed in Table 2. The predominating cells in BALF were alveolar macrophages (AMs). Lymphocyte percentage was significantly lower in CWP 0/1 than in controls.

### TNF- $\alpha$ Levels in Silica and Mixed Coal Dust Exposure Groups

The TNF- $\alpha$  concentrations in BALF in different groups are presented in Fig. 1. No correlation was observed between TNF- $\alpha$  level and exposure duration to coal dust. The TNF- $\alpha$  levels were significantly increased in coal miners without CWP (8.2 $\pm$ 1.6 unit/mL). With the progression of CWP, the TNF- $\alpha$  levels decreased from 6.5 $\pm$ 2.5 unit/mL in miners with CWP 0/1 to 5.9 $\pm$ 1.7 unit/mL in miners with CWP 1/1. TNF- $\alpha$  concentration in both CWP groups was significantly lower than that in coal miners without CWP ( $P < 0.05$ ).

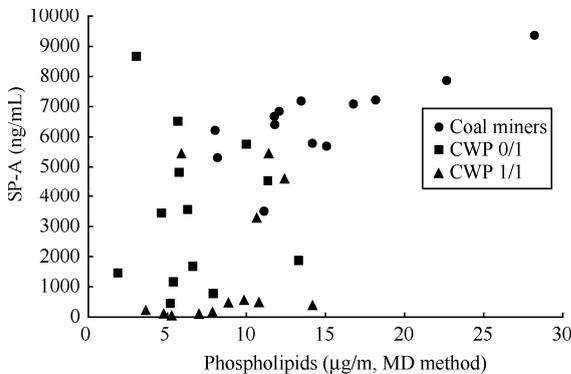


FIG. 1. Point distribution of SP-A and phospholipids in BALF among coal miners without and with CWP, and healthy control subjects.

### Total Protein and SP-A in BALF

The total protein concentration and SP-A in

controls and other groups are shown in Table 3. The protein level in lung BALF was increased in miners with CWP. The mean SP-A concentration was greatly increased in coal miners without CWP (6528 ng/mL), and gradually decreased to 3419 ng/mL in patients with CWP 0/1. The mean SP-A in patients with CWP 1/1 showed a great scattering with a high value of 5455 ng/mL for two cases and a low value of 386.4 ng/mL for other 11 cases. A parallel pattern was shown between the ratio of SP-A to total protein and SP-A levels (Table 3).

### Total PL, PL Composition, and Fibronectin Level in BALF

Three methods were used to determine the levels of total PL in cell-free BALF (Table 3). A good correlation was shown between the results detected by these methods ( $r = 0.94$  for UV method and  $r = 0.97$  for MD method when correlated with phosphor method). The total PL was slightly decreased in miners with CWP, but elevated in active coal miners. A significant decrease ( $P < 0.01$ ) in total PL content of BALF was observed in coal miners with CWP 1/1 as compared with active coal miners. PL seemed to be stimulated by dust exposure to accumulate in BALF, and it was cut off with the development of pulmonary fibrosis.

As shown in Table 3, PC was the predominant PL, which accounted for 76% of total PL in healthy control subjects. The percentage of PC in dust-exposed groups was lower than that in healthy controls, indicating that other components of PL were elevated in dust exposure groups. A significantly elevated PG percentage was shown in active coal miners. The highest ratio of PG to PI was observed in active coal miners without CWP as compared with other groups (Table 3).

No correlation was observed between SP-A and PL in this study. The ratio of SP-A/PL was highly correlated ( $r = 0.80$ ) with SP-A.

No significant difference was shown in fibronectin levels in BALF between healthy control subjects and dust exposed groups (Table 3).

TABLE 2

Cell Recovery Characteristics of BALF From Healthy Controls, Coal Dust Exposed Workers, and Patients with Pneumoconiosis\*

Groups	Coal Miners Without CWP	Coal Miners With CWP 0/1	Coal Miners With CWP 1/1
Bronchitis Index	3.3±1.3	3.4±2.1	3.9±1.3
BAL Recovery (mL)	80.0±12.1 <sup>a</sup>	70.0±20.5	72.9±17.9
Total Cells (10 <sup>4</sup> /mL)	15.9±8.6	18.3±10.0	24.0±6.8
Differential Cell Count (%)			
Macrophages	84.2±14.3	87.7±6.8	78.3±9.1
Neutrophils	3.7±3.7	1.0±1.0	2.7±2.8
Lymphocytes	12.2±13.4	10.7±5.3 <sup>a</sup>	16.6±7.2
Others	0.0±0.0	0.6±1.1	1.1±2.2

Note. \*Values are expressed as  $\bar{x} \pm s$ . <sup>a</sup> $P < 0.05$ , compared with healthy controls.

TABLE 3

Biomarkers in BALF in Coal Dust Exposed Workers and Pneumoconiosis Patients\*

Groups	Coal Miners Without CWP	Coal Miners With CWP 0/1	Coal Miners With CWP 1/1
TNF (µg/mL)	8.23±1.61	6.50±2.45	5.90±1.72
Total protein (µg/mL)	104.2±27.1	125.3±46.7	140.7±41.3
SP-A (ng/mL)	6528±1872 <sup>a</sup>	3419±3239	1654±2351
Phospholipids (phosphr)	16.4±8.0	8.1±3.4 <sup>b</sup>	10.8±5.2
Phospholipids (UV)	28.0±12.2 <sup>b</sup>	16.5±4.7	20.7±5.2
Phospholipids (MD)	14.8±7.7	6.7±2.4	8.7±3.6
PC (µg/mL)	10.7±5.9	4.8±2.1 <sup>b</sup>	4.7±3.7
PG (µg/mL)	4.5±3.9 <sup>b</sup>	1.2±0.4	1.1±0.8
PI (µg/mL)	0.4±0.3	0.2±0.2	0.2±0.1
PE (µg/mL)	0.2±0.1	0.1±0.1	0.2±0.0 <sup>b</sup>
SPH (µg/mL)	0.29±0.11	0.24±0.05	0.22±0.05
Fibronectin	169±130	278±198	390±226
PC/PL	68.1±7.4 <sup>a</sup>	71.2±4.6 <sup>b</sup>	69.4±7.8 <sup>b</sup>
PG/PL	25.5±7.5 <sup>a</sup>	17.7±4.1	16.9±2.8
PI/PL	2.8±1.1	3.8±2.8	3.7±1.5
PG/PI	10.1±3.3 <sup>a</sup>	5.9±2.7	5.5±2.9
SP-A/PL	496.1±174.8 <sup>a</sup>	474.9±417.9 <sup>b</sup>	210.3±326.4
SP-A/Pro	62.3±3.9 <sup>a</sup>	28.9±26.1	16.0±24.9

Note. \*Values are expressed as  $\bar{x} \pm s$ . <sup>a</sup> $P < 0.01$ , compared with healthy controls; <sup>b</sup> $P < 0.05$ , compared with healthy controls.

## DISCUSSION

### Independent Factors for CWP

Principal component analysis was used to search for independent factors contributing to the progresses of CWP. We obtained three independent groups. Group A included SP-A, PG/PI, and phospholipids; group B included lymphocytes and macrophages; group C included TNF- $\alpha$ .

This study confirmed the alterations of pulmonary surfactants and TNF- $\alpha$  in miners after coal dust exposure, and different alternative trends of these parameters were observed in patients with the initial development of CWP. Comparison of these cellular and biochemical alterations between miners

with CWP and healthy miners can help us to understand the processes of lung fibrosis and to distinguish pathological processes of early pneumoconiosis in clinical practice.

TNF- $\alpha$  is an important cytokine in initiating pulmonary fibrosis. TNF- $\alpha$  is released from alveolar macrophages after dust particles are engulfed and is associated with fibroblast cell proliferation and enhanced collagen accumulation<sup>[10]</sup>. In this study, TNF- $\alpha$  levels increased in coal miners exposed to mixed coal dust and then gradually decreased when CWP progressed. The TNF- $\alpha$  level in miners with CWP 1/1 was close to that in healthy controls. These results are highly comparable to those of Borm *et al.*<sup>[11]</sup> and Schins<sup>[12-13]</sup> who found that the highest release of TNF- $\alpha$  is only observed in early CWP, and decreases as CWP progressed. Our results are also consistent with those of Lassalle *et al.*<sup>[14]</sup> who found that TNF- $\alpha$  level in dust exposed coal miners, coal miners with simple pneumoconiosis (SP) and progressive fibrosis (PMF) are significantly elevated. SP could be compared to CWP 0/1 and CWP 1/1 in this study and PMF is an advanced stage of CWP. The decreased trend of TNF- $\alpha$  for CWP 1/1 in our study is at least partly owing to the removal of miners from active exposure. All miners except one with CWP 1/1 removed from coal dust exposure. All these results indicate that TNF- $\alpha$  is also a biomarker for active coal dust exposure.

Enhanced accumulation and decreased content of SP-A after dust exposure in this study are very clear. The initial value of elevated SP-A was shown among all coal miners without CWP and CWP 0/1 (7/13), which agrees with previous reports from animal experiments of silica-treated rats and sheep<sup>[15-16]</sup>. This change may be explained by elevated synthesis and secretion of SP-A by activating type II alveolar epithelial cells. It was reported that SP-A is normal in rats exposed to dust<sup>[17]</sup>, suggesting that accumulation of SP-A is an acute inflammatory and active repair process in the lung after dust exposure. Several factors may contribute to the decreased concentrations of SP-A due to the progression of CWP from 0/1 to 1/1. First, the toxicity of coal dust may damage the function of type II cells and induce alterations of synthesis, secretion and degradation of SP-A. The magnitude of reduction of SP-A can predict the reduction of survival in patients with idiopathic pulmonary fibrosis<sup>[18]</sup>. In this study, SP-A level was very low in patients with CWP 1/1, indicating that serious impairment of type II epithelial cells can predict prognosis for these patients. Second, coal dust particles in the bronchoalveolar milieu are translocated rapidly to the interstitial space, and cause interstitial fibrosis. The

dust damage thus also moves to the interstitial space and the level of SP-A in BALF is recovered. At this time, the disease is manifested as a chronic inflammation reaction, a different phase in the development of pneumoconiosis. Third, activation of dust in the lungs is reduced over time. Hence, removal of patients from dust exposure would decrease the levels of surfactant. It was reported that SP-A becomes normal in monkeys one year after removal from dust exposure<sup>[19]</sup>.

No significant correlation was found between alveolar proteins or SP-A and PL in this study, suggesting that the regulation of each surfactant constituent may be under separate control mechanisms. These findings are consistent with the conclusion derived by other research groups<sup>[15,20]</sup>. McCormack and colleagues reported that SP-A/PL as a biochemical marker in lavage can predict survival time of patients with idiopathic pulmonary fibrosis (IPF)<sup>[21]</sup>. Reduced SP-A/PL ratios are associated with clinical deterioration and short survival time. We also found significantly decreased SP-A/PL in CWP 1/1, which was much lower than that in the controls. It seems that SP-A/PL may reflect pathogenic phases or deterioration degree of CWP.

Slightly reduced concentrations of total PL in BALF were observed in patients with CWP in this study. Generally, changes in PL are the results of complex multifaceted inflammation damage repair processes<sup>[15]</sup>. Such a different profile remains unclear and needs further investigation. Both PG and PI are synthesized from the same precursor CDP-DG of alveolar type II cells, stored in the lamellar bodies and secreted into the alveolar space<sup>[22]</sup>. Changes in the PG content in BALF appear to reflect more precisely the damage to type II cells. Normally the concentration of PG is much higher than that of PI in pulmonary surfactant. Thus a switchover in the biosynthesis of PG and PI and increased PI could mean that the lung surfactant is in the pathological state<sup>[23-25]</sup>. These reductions have also been reported in patients with interstitial lung disease IPF<sup>[24-25]</sup>.

A limitation of this study is that the control subjects were younger than CWP patients although no correlation between age and TNF- $\alpha$ , SP-A and PL levels was found in any group of this study. The study of Schins and Borm excluded the effect of age on release of TNF- $\alpha$  from monocytes<sup>[11]</sup>. The effect of smoking was not assessed with TNF- $\alpha$  in this study. However, a recent study showed that smoking has no particular influence on TNF- $\alpha$  in the BAL<sup>[26]</sup>.

In conclusion, SP-A concentration and PI percentage of PL may help to distinguish acute inflammation reaction of coal mixed dust from interstitial fibrosis. TNF- $\alpha$  level can be used to reflect

active dust exposure in coal miners.

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