Human & Experimental Toxicology (2008) **27:** 871–877
www.het.sagepub.com

Serum and BAL cytokine and antioxidant enzyme levels at different stages of pneumoconiosis in coal workers

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Coal workers' pneumoconiosis (CWP) is an occupational pulmonary disease that occurs by chronic inhalation of coal dust. CWP is divided into two stages depending on the extent of the disease, as simple pneumoconiosis (SP) and progressive massive fibrosis (PMF). In the present study, serum and bronchoalveolar lavage (BAL) cytokine (interleukin-1β [IL-1β], IL-6, tumor necrosis factor-α [TNF-α], transforming growth factor-β [TGF-β]) and antioxidant enzymes levels, their relation with the disease severity, and whether they can be considered as biological markers were investigated. Serum and BAL levels of IL-1β, IL-6, and TNF-α were higher in SP and PMF patient groups compared with that in active and retired miner groups. Serum and BAL IL-1β, IL-6, and TNF-α levels were also found to be higher in patients with PMF compared with the SP group. BAL superoxide dismutase (SOD), glutathione peroxidase, and catalase levels and serum SOD level were increased in both patient groups compared with the control group. In addition, mean serum and BAL TGF-β levels were found to be increased in patients with SP compared with PMF group. Based on these results, BAL and serum cytokine and antioxidant enzymes levels were evaluated and discussed as potential biomarkers for different stages of CWP.

Key words: antioxidant enzymes; coal workers' pneumoconiosis; proinflammatory cytokines

Introduction

Coal workers' pneumoconiosis (CWP), an occupational lung disease caused by coal dust inhalation, is characterized by development of fibrotic and inflammatory reactions in the lung. CWP is divided into two stages according to disease severity: simple pneumoconiosis (SP) in which fibrotic lesions remain limited and progressive massive fibrosis (PMF), characterized by severe alterations in lung functions due to extensive fibrosis and emphysema.[1,2] However, the underlying pathophysiological mechanism for CWP is not completely understood.[3–5]

Coal dust particles are known to stimulate macrophages. The activated macrophages produce excessive amounts of reactive oxygen species (ROS), and cytokines that play key roles in the pathogenesis of pneumoconiosis.[6] The cytokines are extracellular signaling proteins that regulate fibrotic and inflammatory reactions. Their multiple overlapping and sometimes contradictory functions depend on their local concentrations and the presence of other cytokines and mediators.[7] Based on their role in inflammation, the cytokines have been classified as pro- and anti-inflammatory cytokines. Proinflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), IL-6, and IL-12 play a central role in the initiation and progression of inflammation, whereas anti-inflammatory cytokines such as IL-4, IL-10, and interferon-α promote resolution of inflammation.[8] Transforming growth factor-β (TGF-β) is a multifunctional cytokine and plays a role both in the initiation and termination of inflammatory processes. Elevated expression of TGF-β has been reported in the progression of lung diseases including pulmonary fibrosis.[9]

Increased cytokine release has been shown in serum, BAL, and cell cultures (BAL alveolar macrophages or blood monocytes) of miners with CWP in a number of studies.[4,10–12] However, the relationship of BAL proinflammatory cytokine levels to disease severity has been reported in only one study which involved a small number of subjects.[5]

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10.1177/0960327108098332

ROS are mediators of chronic tissue damage and fibrosis. The oxidative damage in the lung is associated with an imbalance between ROS generation and antioxidant defenses.[13,14] ROS production in the lung could promote endothelial cell damage, the formation of neutrophils, lipid peroxidation and oxidation, DNA damage, and the release of proinflammatory cytokines.[15] Increasing evidence supports the hypothesis that coal exposure is associated with the generation of ROS, upregulation of antioxidants, inflammatory factors, and lipid peroxides.[5] An imbalance between ROS generation and antioxidant capacity and increased expression of proinflammatory cytokines could be related to the initiation and progression of CWP. Measurements of these mediators in BAL fluid reflect more closely the in-vivo inflammatory and antioxidant status of the lungs.

The present study was designed to investigate serum and BAL inflammatory cytokine (IL-1β, IL-6, TNF-α, and TGF-β) and antioxidant enzymes (superoxide dismutase [SOD], glutathione peroxidase [GPx], catalase [CAT]) levels in CWP and their relation with disease severity. We further aimed to determine whether cytokine and antioxidant enzyme levels in serum and BAL samples can be considered as biological markers of CWP and its severity.

Methods

Subject (study population)

All the subjects in this study were selected from coal-mining area in Zonguldak, which is the biggest coal-mining area of Turkey. The study was conducted on 85 subjects. They were classified into two groups. Group 1: serum samples were obtained from 25 patients with CWP who were hospitalized at Zonguldak Chest and Occupational Disease Hospital and 26 healthy retired and active coal miners who were recruited as controls (Table 1). Group 2: BAL samples were obtained from 24 patients with CWP who were bronchoscopically controlled at the same hospital and 10 control subjects who were bronchoscopically confirmed to be free from pulmonary problems (Table 2). We were not able to collect BAL samples from all participants. To investigate whether cytokine and antioxidant enzyme levels were different in a radiologically invisible stage (based on years of coal dust exposure) than a clinically evident disease stage, active and retired miners served as controls, but we were not able to collect BAL samples from these asymptomatic miners. Because it is extremely difficult to provide consent from healthy individuals for bronchoscopic control, only 10 subjects who had this procedure for some other clinical clarification served as controls for the second group of miners. Due to these difficulties, assignment of two groups to the study and sample collection was performed at different times. The ideal situation would be to collect all the samples from the same group; however, two groups were not significantly different for duration of exposure, mining area, age, and smoking.

Chest radiographs were classified according to the International Labor Organization (ILO) classification into two groups: SP and PMF. The distribution of ILO category of patients with CWP was shown in Table 3. A questionnaire was used to obtain information on medical and occupational history, drug usage, and smoking from each worker and patient.

The study was approved by the Ankara University Faculty of Medicine Ethic Committee, and written informed consent was obtained prior to participation in the study.

Sample collection

Blood specimens were collected in nonheparinized tubes. Serum was recovered after centrifugation at 3000 rpm for 15 min at 4 °C. Serum samples were stored at −80 °C until required for assay. BAL samples were collected from the right central part of lung by exposure to warmed sterile saline (0.9%)

Table 1 General characteristics of Group 1

	CWP		*Control*	
	SP	*PMF*	*Active miners*	*Retired miners*
n	14	11	14	12
Age[a]	57.64 ± 10.14	68.2 ± 6.44	41.4 ± 3.73	61.75 ± 12.95
Exposure duration[a]	26 ± 2.79	29 ± 3.52	15 ± 5.12	24 ± 4.22
Smoking habits				
Nonsmokers	5	9	2	10
Smokers	9	2	12	2

[a]The values are mean ± SD.
CWP, coal workers' pneumoconiosis; SP, simple pneumoconiosis; PMF, progressive massive fibrosis.

Table 2 General characteristics of Group 2

	CWP		*Control*
	Bronchoscopically controled (+)		*Bronchoscopically controled (–)*
	SP	*PMF*	
n	10	14	10
Age[a]	62 ± 8.29	62.85 ± 4.04	51 ± 14.27
Exposure duration[a]	24 ± 2.62	27 ± 10.68	—
Smoking habits			
Nonsmokers	5	9	9
Smokers	5	5	1

[a]The values are mean ± SD.
CWP, coal workers' pneumoconiosis; SP, simple pneumoconiosis; PMF, progressive massive fibrosis.

and subsequent recovery by gentle aspiration. After centrifugation at 1500 × g for 5 min, the pooled lavage supernatant was stored at −80 °C until assayed.

Determination of serum and BAL cytokine levels
Serum and BAL IL-6, TNF-α, and TGF-β levels were determined by an ELISA assay (Biosource®, California, CS, USA). The optical density was measured at 450 nm by using a microtiter plate reader. Serum and BAL IL-1β levels were determined by R&D Systems High Sensitivity ELISA kit (R&D Systems®, Minneapolis, MN, USA) and the optical density was measured at 490 nm.

Determination of serum and BAL antioxidant enzyme levels
Serum and BAL SOD, CAT, and GPx levels were determined using a spectrophotometric assay kit obtained from OxisResearch® (Portland, OR, USA). The optical density was measured at 525, 520, and 340 nm.

Statistical analysis
Results are expressed as mean ± standard deviations. The analysis of variance (ANOVA) test was used to compare the mean serum and BAL cytokine and antioxidant enzyme levels between different groups. Multiple logistic regression was used to identify confounding factors and measure the independent effects of each variable (age, exposure duration, and smoking habits) on cytokine and antioxidant enzyme levels. Odds ratios (OR) and 95% confidence intervals (CI) were calculated using unconditional logistic regression, adjusting for potential confounding factors. All statistical analyses were performed using SPSS software (11.5).

Results

Study population
General characteristics of the study subjects were shown in Tables 1 and 2. There was no significant effect of confounding factors (age, exposure duration, and smoking) on serum and BAL cytokine and antioxidant enzyme levels. There was also no association between confounding factors and development of CWP as shown in Table 4.

Assessment of serum and BAL cytokines levels
In Figure 1, mean serum cytokine levels of patients with SP, PMF, and active and retired miners (study group 1) were compared by means of the one-way ANOVA test. Mean serum IL-1β, IL-6, and TNF-α levels were significantly higher in SP and PMF patient groups than those observed in active and retired miner groups ($P < 0.01$). Serum cytokine levels were also significantly increased in patients with PMF compared with patients with SP ($P < 0.01$). Mean serum TGF-β levels significantly increased in SP patient group compared with those of the active and retired miner groups ($P < 0.01$). Among these cytokines, only serum TGF-β level of the PMF patient group showed a tendency to decrease.

Figure 2 displays the mean BAL levels of cytokines from SP, PMF, and control groups (study group 2). One-way ANOVA test was used to compare the means of three groups. BAL and serum cytokine profiles were found to be similar ($P < 0.01$). Moreover a significant increase was observed in BAL TGF-β level of SP patient group compared with the PMF patient group ($P < 0.01$).

Table 3 Distribution of ILO category of subjects with CWP (study groups 1 and 2)

	Simple pneumoconiosis						*Progressive massive fibrosis*		
Opacity	*p ½*	*p 2/2*	*p 2/3*	*q 2/3*	*q 3/2*	*q 3/3*	*A*	*B*	*C*
n	2	10	4	2	3	3	8	13	4
%	8.3	41.66	16.6	8.3	12.5	12.5	32	52	16

ILO, International Labor Organization; CWP, coal workers' pneumoconiosis.

Table 4 Association between confounding factors and development of CWP

	CWP p/n	*Control p/n*	*OR (%95 CI)*	*p (χ^2 test)*
Age	20/29	15/21	0.97(0.40–2.31)	0.70
Exposure duration	21/28	12/14	0.87(0.34–2.27)	0.28
Smoking habits	21/28	21/15	0.18(0.06–0.55)	0.95

CWP, coal workers' pneumoconiosis; p/n, positivities/negativities; OR, Odds ratios.

Assessment of serum and BAL antioxidant enzymes levels

Although serum concentrations of GPx and CAT were comparable in both groups of patients and in the control subjects, it was not statistically significant. Significantly enhanced SOD activity was observed in the serum of patients with SP and PMF (Table 5). On the other hand, significantly higher BAL antioxidant enzyme concentrations were measured in two groups of patients when compared with the control group. Also, BAL antioxidant enzyme levels of the PMF patient group showed a tendency toward an increase when compared with the SP group (Table 6).

Discussion

A number of in-vitro and in-vivo studies were performed to investigate the role of cytokines and ROS in the development of CWP. Proinflammatory cytokines IL-1β, IL-6, and TNF-α induce collagen synthesis and play important roles in fibrotic response. The secretion of these cytokines by alveolar macrophages exposed to coal mine dust showed their implications for the pathogenesis of CWP.[1] There is very limited information pertaining to cytokine release and CWP. Increased amounts of IL-1β, IL-6, and TNF-α release by alveolar macrophages or blood monocytes of patient with CWP were found in in-vitro studies.[1,4,11,16,17] However, the results were conflicting when patients were classified as having SP and PMF.[1,17,18] Vallyathan, *et al.*[5] found elevated BAL IL-1β, IL-6, and TNF-α levels in patients with CWP. Enhanced serum[12] and BAL IL-6 levels[19] and BAL TNF-α levels[20] were also found in patients with CWP. Similarly, in the present study, we found significant increases in serum and BAL IL-1β, IL-6, and TNF-α level of patients with CWP. Our results were compatible with those of in-vivo studies mentioned

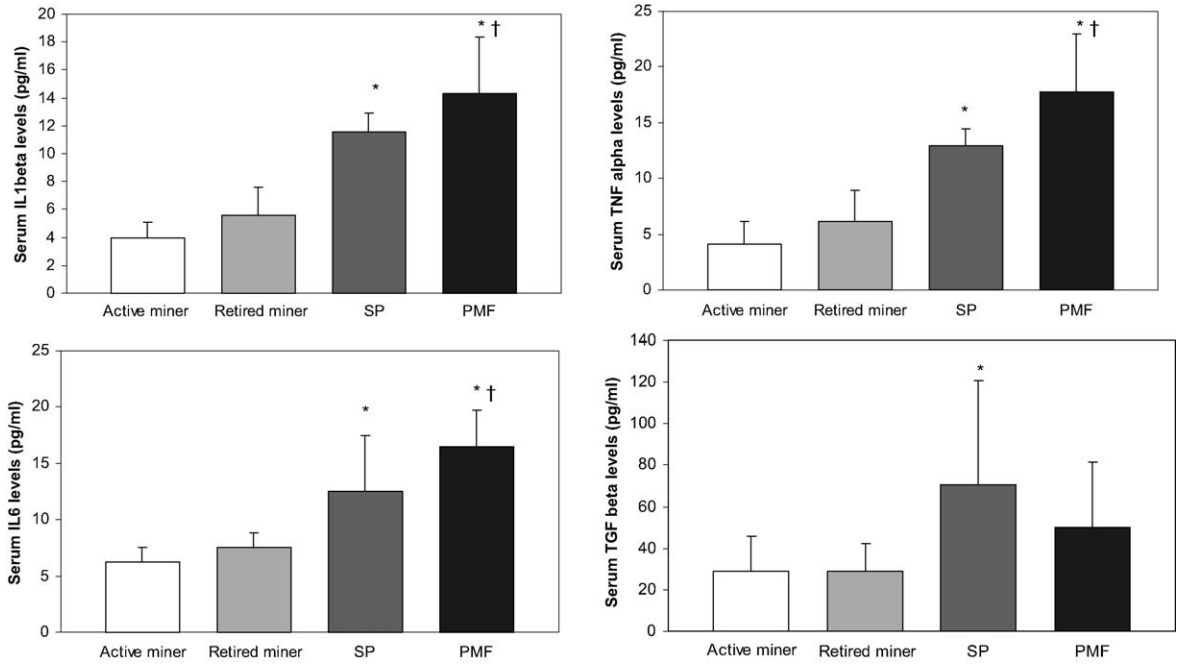

Figure 1 Serum interleukin-1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β) levels in four groups (study group 1). $^{*}P < 0.01$ compared with active miner and retired miner groups. $^{\dagger}P < 0.01$ compared with simple pneumoconiosis (SP) group.

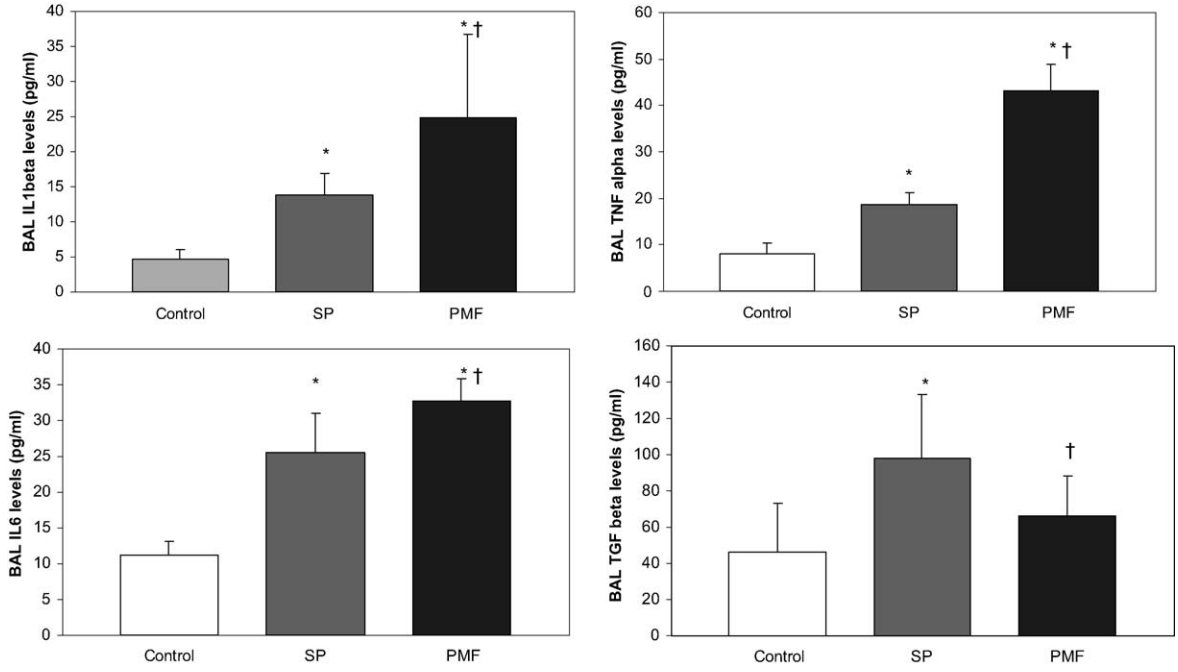

Figure 2 Bronchoalveolar lavage interleukin-1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β) levels in three groups (study group 2). $^{*}P < 0.01$ compared with control group. $^{\dagger}P < 0.01$ compared with simple pneumoconiosis (SP) group.

above. Moreover, we found significantly increased serum and BAL levels of IL-1β, IL-6, and TNF-α in patients with PMF compared with the SP group. These observations suggest that these cytokines might be important in the progression of disease and their serum and BAL levels can be considered as biomarkers to screen coal miners at high risk of developing CWP.

TGF-β has been considered the prototype of a multifunctional cytokine as evidence supports a concentration-dependent bifunctional role of this cytokine in the regulation of immune responses.[21,22] It has been suggested that fibrotic action of TGF-β may depend on its concentration, a stimulatory effect on fibroblasts at low concentration, and an inhibitory effect at higher

Table 5 Serum antioxidant enzyme levels in SP, PMF, active miner, and retired miner groups

Parameters	*Active miner* (n = *14*)	*Retired miner* (n = *12*)	*SP* (n = *14*)	*PMF* (n = *11*)
GPx (ng/mL)	9.50 ± 6.50	10.27 ± 4.30	15.42 ± 5.43	17.90 ± 6.35
Catalase (U/mL)	46.13 ± 17.16	50.77 ± 20.05	59.73 ± 8.71	72.27 ± 18.3
SOD (U/mL)	5.1 ± 1.2	7.2 ± 1.8	15.2 ± 0.7*	21.8 ± 2.0*

The values are mean ± SD.
SP, simple pneumoconiosis; PMF, progressive massive fibrosis; GPx, glutathione peroxidase; SOD, superoxide dismutase.
$^{*}P < 0.01$ compared with active and retired miner groups.

Table 6 BAL antioxidant enzyme levels in SP, PMF, and control groups

Parameters	*Control* (n = *10*)	*SP* (n = *10*)	*PMF* (n = *14*)
GPx (ng/mL)	75.23 ± 14.50	126.97 ± 14.50*	132.37 ± 13.27*
Catalase (U/mL)	81.95 ± 2.80	123.53 ± 4.99*	140.53 ± 20.99*
SOD (U/mL)	48.50 ± 8.00	78.50 ± 6.00*	121.50 ± 13.00*

The values are mean ± SD.
BAL, bronchoalveolar lavage; SP, simple pneumoconiosis; PMF, progressive massive fibrosis; GPx, glutathione peroxidase; SOD, superoxide dismutase.
$^{*}P < 0.01$ compared with control group.

concentrations.[23–26] Vanhee, *et al.*[10] reported increased TGF-β concentrations in the BAL fluid of patients with SP compared with patients with PMF and nonexposed control group. Similarly, in current study, we observed elevated serum and BAL TGF-β levels only in the SP patient group. These results support the hypothesis that alterations in TGF-β level may lead to changes in inflammatory/fibrotic processes in the lung.

ROS are generated in the lung during coal dust phagocytosis by activated alveolar macrophages.[27] The elevated antioxidant enzyme levels found in different body fluids of patients with CWP were considered to reflect excessive ROS formation during coal dust exposure.[28–32] Elevated BAL antioxidant enzymes levels were also observed in coal miners.[5,28] In the present study, a significant increase was found only in serum SOD levels of patients with CWP. The enhanced spontaneous superoxide anion production accompanied by increased SOD activity may be considered as an early marker of the effects of coal dust exposure in CWP. Significant increases in BAL antioxidant enzyme (GPx, SOD, and CAT) levels found in this study showed good agreement with the study of Vallyathan, *et al.*[5] Based on these results, it can be suggested that BAL levels more closely reflect the in-vivo imbalance between oxidant generation and antioxidant enzyme activities.

In conclusion, our results support the hypothesis that excessive production of proinflammatory cytokines (IL-1β, IL-6, and TNF-α) is related to the development of CWP and its progression. However, TGF-β plays dual role in the disease process as both proinflammatory and anti-inflammatory, according to its concentration. Because of the invasive nature and cost of the bronchoscopy, and the similarities observed between serum and BAL cytokines profiles, serum proinflammatory cytokine levels may be considered good biomarkers of CWP and disease severity. Although BAL antioxidant enzyme levels showed an imbalance between oxidant burden and antioxidant defenses in CWP, due to the difficulty of bronchoscopy, only serum SOD levels may be considered as a suitable biomarker for early development of CWP.

Acknowledgments

The study was supported by Ankara University Research Fund (Project no: 20030803036). We thank Dr Yalçın Karakaş and his colleagues of the Zonguldak Lung Disease Hospital for their assistance in sample collection.

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