Changes in Bronchoalveolar Lavage Indices Associated with Radiographic Classification in Coal Miners

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Previous studies on symptomatic coal miners have shown that alveolar macrophages, recovered by bronchoalveolar lavage (BAL), release excessive amounts of reactive oxygen species (ROS) and inflammatory cytokines. It has been proposed that these secretions may mediate cell injury and initiate the disease process. We hypothesized that acellular bronchoalveolar lavage fluid (BALF) indices in coal miners chronically exposed to coal dust may reflect the status of important homeostatic modulations in the lung that lead to the development of coal workers' pneumoconiosis (CWP). To test this hypothesis, we measured inflammatory status, oxidant burden, antioxidant defenses, cytokines, growth factors, fibronectin, and α_1-antitrypsin (α_1-AT) in the BALF of healthy never-smoker control subjects, never-smoker underground coal miners with negative radiographs (ILO 0/0–1/0), and two miners with moderate changes in the chest radiographs (ILO 2/2). Interestingly, indices of injury and inflammation increased with the progression of disease in coal miners. Antioxidant enzymes, such as catalase, glutathione peroxidase, and superoxide dismutase, showed a 19-fold, 22-fold, and 6-fold increase above control, respectively, in coal miners with category 2/2 CWP. Significant increases in the secretion of IL-1, IL-6, TNF-α, TGF-β, fibronectin, and α_1-AT also were evident in coal miners with disease. This up-regulation of antioxidant defenses and cytokines was not evident in coal miners in the absence of clinically evident radiographic disease. In addition, the concentration of lipid peroxidation by products in the BALF of coal miners without evidence of radiographic disease showed a moderate 3-fold increase, whereas, in coal miners with category 2/2 CWP it showed a 59-fold increase compared to control subjects. These results are in good agreement with our hypothesis that development of CWP and its progression may be correlated with an oxidative stress and up-regulation of cytokines and mediators of growth.

Occupational exposure to coal mine dust can lead to the development of a spectrum of diseases including simple coal workers' pneumoconiosis (CWP), complicated CWP (progressive massive fibrosis, PMF), Caplan's syndrome, bronchitis, emphysema, and silicosis (1). Although many fundamental factors in relation to the pathogenesis and prevalence of CWP are recognized, the underlying mechanisms for initiation and progression of many of these conditions remain elusive. Even though the pathophysiological mechanisms are unclear, there is evidence that the lung responds to coal mine dust by triggering an inflammatory cascade of reactions, resulting in enhanced secretion of proinflammatory factors, synthesis of extracellular matrix, and fibroblast proliferation (1–3). Increasing evidence supports the hypothesis that coal exposure in humans is associated with the generation of reactive oxygen species (ROS), up-regulation of antioxidants, and induction of inflammatory factors and lipid peroxides, which are implicated in the pathogenesis of CWP (2–10).

We hypothesized that on initiation of disease an imbalance of these important markers of injury would occur, that is, the ratio of oxidant generation to antioxidant levels or the relative production of inflammatory versus antiinflammatory mediators would change. Such an imbalance could be the earliest detectable manifestation of progression from an asymptomatic preclinical stage to a disease stage. To evaluate this possibility, we measured the concentrations of cytokines, growth factors, antioxidant enzymes, α_1-antitrypsin (α_1-AT), and fibronectin in the bronchoalveolar lavage fluid (BALF) from unexposed controls, miners with coal dust exposure but no radiographic evident disease, and miners with moderately advanced simple CWP. This study differs from previously reported studies in which release of these cytokines and oxidants from alveolar macrophages was found in miners with an advanced stage of complicated CWP (PMF) but not for the most part in simple CWP (2, 9–15). In contrast, we analyzed the concentrations of oxidants, antioxidants, and cytokines in the BAL fluid of control subjects, radiographically normal miners exposed to coal mine dust, and miners with moderately advanced simple CWP. Our hypothesis was that BALF would more closely reflect the *in vivo* balance of these mediators than the release from cells, which had already interacted with coal mine dust before being recovered, then cultured *in vitro* and again exposed to coal mine dust. Our results appear to confirm that alteration of the oxidant/antioxidant and proinflammatory/inflammatory balance occurs at the onset of disease, as reflected by radiographic changes of simple CWP, before the onset of the complicated CWP with PMF.

METHODS

Reagents and Study Population

The study was approved by the Human Institutional Review Board at West Virginia University and was conducted after receiving informed consent in writing from the participants. Volunteers for the study were recruited by advertisement. A total of 23 underground coal miners and 20 control subjects were recruited for study. Miners were required to have worked at least 5 yr as an underground coal miner. All subjects recruited were never smokers. Among the 23 underground coal miners responding voluntarily to newspaper advertisement there were only two coal miners with category 2 or higher grade of CWP. This is because category 2 or higher grades of CWP is now rare and uncommon, especially in never smokers. Therefore, it was not possible to enroll additional symptomatic miners to the study population to confirm the up-regulated trend observed in two coal miners with symptomatic CWP.

Participants completed symptom, occupational, and medical history surveys and were given a cardiopulmonary physical examination. Posterior–anterior and lateral chest radiographs were taken and classified by the ILO system for the appearance of pneumoconiosis by a NIOSH-certified B reader. Pulmonary function studies included spirom-

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etry and diffusing capacity. The demographic characteristics of the controls and coal miners are presented in Table 1.

Bronchoalveolar Lavage

Following screening each subject underwent bronchoscopy and bronchoalveolar lavage in a subsegment of the right middle lobe. Atropine was used to reduce secretions and topical lidocaine was used as anesthesia. Lavage was performed by instilling warmed saline in 50-ml aliquots to a total of 200 ml and retrieved by gentle suction. The mean lavage volume recovered was approximately 60 to 65% of that instilled in most subjects. Recovery volume did not vary among the control subjects and miners with normal chest radiographs. The recovered fluid from the lavages was filtered with gauze to remove mucus. Cells were pelleted by centrifugation for 5 min at 1,500 $\times$ *g*. The pooled lavage supernatant recovered from the lavages was concentrated 10-fold by pressure ultrafiltration using a Mintan system (Millipore Co., Bedford, MA) and stored at -70° C until assayed. Concentrated BALF samples were assayed in duplicate and then corrected for total fluid return and concentration.

Inflammatory Characteristics of Lung Lavage

Cells were harvested from BAL by low-speed centrifugation at 300 $\times$ *g* for 5 min at 4° C. Sedimented cells were washed three times by centrifugation and resuspended in a cold phosphate-buffered solution and reconstituted in HEPES-buffered medium (145 mM NaCl, 5 mM KCl, 10 mM *N*-[2-hydroxyethyl]piperazine-N′-2-ethanesulfonic acid [HEPES], and 5 mM glucose at pH 7.4). The cell counts were made as previously reported using a Coulter Counter equipped with a channel analyzer, Model ZB1 (Coulter Electronics, Hialeah, FL). For comparison and verification, total and differential counts were also made on Cytospin preparations stained with May–Grunwald–Giesma stain. Cell differentials were made by counting a minimum of 100 cells.

ESR Measurements

Generation of free radicals by BALF was measured using electron spin resonance (ESR) spectrometry with the aid of a spin trapping technique as reported earlier (8). The hydroxyl radical (·OH) generation potential of BALF from asymptomatic miners and controls was made by reacting BALF with H_2O_2. The reaction mixture contained 0.5 ml BALF, 100 μl 0.1 M H_2O_2, and 100 μl 1 M DMPO in a total volume of 0.7 ml. ·OH generation was initiated by the addition of H_2O_2 in the presence of a spin trap, DMPO. The reaction solution was mixed and ESR measurements were rapidly performed using a flat cell assembly. All measurements were conducted using a 250-μl aliquot of the reaction mixture in a Varian E109 ESR spectrometer operated at the X-band ($\sim$ 9.4 GHz) frequency. The spectrometer settings were microwave power, 50 mW; magnetic field, 3,350 $\pm$ 50 G; modulation amplitude, 2 G; scan time, 120 s; time constant, 250 ms. An EPR DAP 2.0 program was used for data acquisition and analysis.

Iron Measurements

Free iron was measured in lavage samples using a spectrophotometric method as outlined by Roth and coworkers (16). Briefly lavage samples were treated with 0.3 M sodium citrate and 1 M sodium bicarbonate containing 100 mg sodium dithionate for 30 min at 80° C. Aliquots of the clear supernatant were then treated with 10% hydroxylamine hydrochloride and *O*-phenanthroline for 5 min and absorbance measured at 508 nm. Iron concentration was calculated from a standard graph obtained from analytical grade ferric ammonium sulfate.

TABLE 1

DEMOGRAPHICS OF CONTROLS AND MINERS*

Subjects	No.	Age (*yr*)	Exposure (*yr*)	$FEV_{1.0}$ (*% predicted*)	Radiographic Classification
Control subjects	20	38.7 $\pm$ 1.6	0	106.0 $\pm$ 3.5	0/0
Asymptomatic miners	19	41.8 $\pm$ 1.4	15.8 $\pm$ 1.5	104.6 $\pm$ 2.9	0/0–1/0
Symptomatic miners with simple CWP	2	54.5 $\pm$ 0.5	22.5 $\pm$ 1.5	96.0 $\pm$ 4.0	2/2

Definition of abbreviation: CWP = coal workers' pneumoconiosis.

* Values are means $\pm$ SE. Normal versus CWP miners were classified radiographically.

Lipid Peroxidation

A lipid peroxidation by-product, 8-epiprostaglandin (8-EPI), was measured as a marker of oxidative injury. 8-EPI in the BALF of asymptomatic coal miners and control subjects was measured using an isoprostane ELISA kit (Oxford Biomedical Research, Inc., Oxford, MI). Isoprostanes are prostaglandin-like compounds that are products of free radical-induced peroxidation of arachidonic acid and are independent of the cyclooxygenase enzyme system (17). The method is a competitive enzyme-linked immunoassay for determining 8-EPI in biological samples using 8-EPI-conjugated horseradish peroxidase (HRP). Addition of the substrate results in color development, which is proportional to the bound 8-EPI and inversely proportional to the unconjugated 8-EPI. The measurements were made according to the manufacturer's instructions. For analysis, 100 μl of BALF and 100 μl of HRP enzyme conjugate were placed in the antibody-coated plates for 2 h and treated with substrate for 15 min for color development. After adding 50 μl of 1 M H_2SO_4, samples were read at 450 nm. The concentration of isoprostane produced was calculated from a standard curve generated with varying concentrations of 8-EPI.

Due to the potential interference of isoprostane immunoassay, we used several lavage samples according to a modified protocol implementing a rigorous solid phase extraction procedure with and without spiked isoprostane standards. Using the modified extraction procedure we were not able to recover a significant percentage of the spiked standards, whereas with the original standard protocol we were able to measure 93–96% of the spiked isoprostane standard. Therefore, we believe the method is suitable for determination of the relative levels of isoprostane in BALF samples containing small concentration of protein and other isomeres of isoprostane.

Antioxidant Enzymes

Antioxidant enzyme levels of superoxide dismutase (SOD) (18), glutathione peroxide (GPx) (19), and catalase (20) were measured in BALF of coal miners and control subjects according to well-standardized protocols adapted for an autoanalyzer. Enzyme concentrations were estimated using an automated Cobas Fara II Analyzer (Roche Diagnostic Systems, Montclair, NJ). All the reagents and samples placed in the instrument were automatically pipetted, diluted, mixed, incubated, and spectrophotometrically analyzed. Enzyme levels in the BALF were calculated according to programmed instructions.

Cytokines and Growth Factors

Interleukin-1 (IL-1) concentrations in the BALF of coal miners and control subjects were determined with an ELISA kit (Cistron Biotechnology, Pine Brook, NJ), using a monoclonal–polyclonal-based assay. The measurements were made according to the ELISA kit protocol, using 100 μl of BALF. Color development was measured at 450 nm. Interleukin-6 (IL-6) was assayed using a commercially available ELISA kit from R&D Systems (Minneapolis, MN) using a double enzyme sandwich technique. Concentrations of IL-1 and IL-6 were calculated from a standard curve produced with varying known concentrations of IL-1 and IL-6.

Tumor necrosis factor α (TNF-α) concentrations in the BALF of coal miners and control subjects were determined using an ELISA kit (R&D Systems, Inc.). This assay employs a quantitative "sandwich" enzyme immunoassay technique using the TNF-α alkaline phosphatase conjugate. A 200 μl sample was prepared according to ELISA kit protocol and absorbance measured at 490 nm. Concentrations of TNF-α were calculated from a standard curve produced with varying standard concentrations of TNF-α.

Transforming growth factor β (TGF-β) concentrations in the BALF of coal miners and control subjects were determined using an ELISA kit (Genzyme Corp., Cambridge, MA). The measurements were made according to the manufacturer's instructions. For analysis, 100 μl of BALF from each coal miner or control subject was placed in the antibody-coated plates, covered, and incubated at 37° C for 1 h. Next, 100 μl of anti-TGF-β HRP conjugate was added and incubated at 37° C for 1 h. Then, each well was treated with substrate for 20 min for color development and the absorbance was read at 450 nm after the addition of

TABLE 2

CELL RECOVERY CHARACTERISTICS OF LUNG LAVAGE FROM CONTROLS AND COAL MINERS*

Subjects	No.	Total Cells ($\times 10^6$/ml)	Macrophages ($\times 10^6$/ml)	Neutrophils ($\times 10^6$/ml)	Lymphocytes ($\times 10^6$/ml)	Eosinophils ($\times 10^6$/ml)
Control subjects	20	8.25 ± 1.38	7.32 ± 1.24	0.28 ± 0.13	0.44 ± 0.15	0.15 ± 0.06
Asymptomatic miners	19	9.36 ± 0.87	7.42 ± 0.93	0.31 ± 0.04	0.55 ± 0.09	0.23 ± 0.07
Symptomatic miners with simple CWP	2	6.36 ± 0.65	4.01 ± 0.57	1.99 ± 1.83	0.54 ± 0.23	0.09 ± 0.01

Definition of abbreviation: CWP = coal workers' pneumoconiosis.
* Values are means ± SE. Normal versus CWP miners were classified radiographically.

stop solution. Concentrations of TGF-β were calculated from a standard curve produced with varying concentrations of TGF-β.

Fibronectin concentrations in the BALF of coal miners and control subjects were determined using human fibronectin enzyme immunoassay kit (Biomedical Technologies Inc., Stoughton, MA). The method is a heterogeneous enzyme-linked immunoassay utilizing a double antibody separation for determining fibronectin in biological samples. Fibronectin from the unknowns binds to antibody added to the tubes. For analysis, 100 μl of BALF from the coal miners or control subjects was added to tubes containing the fibronectin antibody, and the measurements were made according to the manufacturer's instructions. Concentrations of fibronectin in the samples were calculated from a standard curve produced with varying concentrations of fibronectin.

Total α_1-Antitrypsin

Total α_1-antitrypsin (α_1-AT) in BALF was determined by immunoprecipitation using the SPQ test system (Incstar Corp., Stillwater, MN). α_1-AT concentration was estimated using an automated Cobas Fara II Analyzer (Roche Diagnostic Systems). α_1-AT levels were calculated according to programmed instructions as stated in the SPQ test.

Statistical Analysis

Data are presented as means and standard errors. Significance of differences between the never-smoker coal miner groups and the never-smoker control subjects was determined by analysis of variance or the Student's *t* test. A probability value of less than $p = 0.05$ was considered significant.

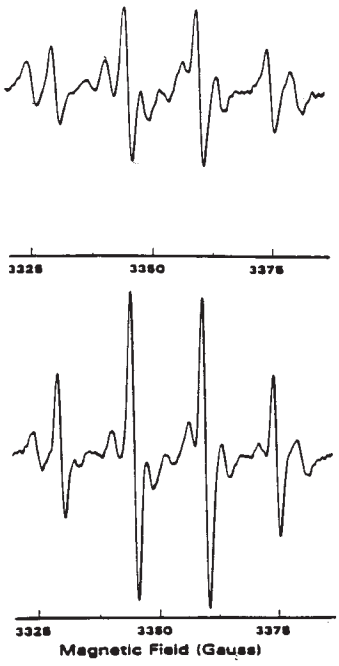

Figure 1. A typical ESR spectrum generated by control subject (*top*) and asymptomatic coal miner (*bottom*) BALF when reacted with H_2O_2 in the presence of DMPO producing DMPO-OH radical adducts exhibiting the characteristic 1:2:2:1 quartet signal.

RESULTS

Demographic data, years of underground coal mine dust exposure, results of pulmonary function tests, and chest radiographic categories are presented in Table 1. Miners with symptomatic CWP were older and worked more years in underground coal mines than asymptomatic miners. However, pulmonary function values did not vary among these groups. Table 2 shows the characteristics of lung lavage cellularity from control subjects, asymptomatic coal miners, and coal miners with category 2/2 radiographic CWP. Total cell counts, alveolar macrophages, and neutrophils were similar in control subjects and asymptomatic coal miners. In contrast, neutrophils were elevated sevenfold in miners with category 2/2 CWP compared with control subjects. This increase in neutrophils was indicative of an inflammatory process with chronic coal mine dust exposure in symptomatic simple CWP. In addition, alveolar macrophage yield in BAL was lower in miners with simple CWP than in control subjects. This lower yield ($\sim$ 23%) may reflect that macrophages were activated in simple CWP and adhered more tightly to alveolar walls.

The ROS generating potential of BALF was determined by reacting with H_2O_2 and measuring the resultant ESR signal in the presence of a spin trap DMPO. Trace metals such as Fe^+ present in the BALF may react with H_2O_2 to produce DMPO-OH radical adducts exhibiting a 1:2:2:1 quartet signal. Figure 1 shows the typical ESR spectra generated by BALF from the control subjects and asymptomatic miners after addition of H_2O_2. The spectral signatures of ESR signals of BALF from control subjects and asymptomatic coal miners were identical

TABLE 3

RELATIVE ESR PEAK INTENSITIES AND THE LEVEL OF LIPID PEROXIDATION BY-PRODUCT, ISOPROSTANE, IN LUNG LAVAGE FLUIDS FROM CONTROLS AND ASYMPTOMATIC COAL MINERS*

Subjects	Relative ESR Peak Heights	Isoprostane Concentration (*ng/ml*)
Control subjects	8.9 ± 3.6 (n = 13)	0.045 ± 0.04 (n = 8)
CWP 0/0–1/0	18.4 ± 7.6 ($p < 0.001$) (n = 14)	0.12 ± 0.12 (NS) (n = 9)
CWP 2/2	Not measured	2.66 (range 1.35–3.97) (n = 2)

Definition of abbreviations: CWP = coal workers' pneumoconiosis; ESR = electron spin resonance.
* Values are means ± SE of samples indicated in parentheses. p Value indicates a significant difference. NS indicates no significant difference.

TABLE 4

SUPEROXIDE DISMUTASE, GLUTATHIONE PEROXIDE, AND CATALASE IN BALF BASED ON TOTAL VOLUME RETRIEVED OR TOTAL PROTEIN*

Subjects	Superoxide Dismutase (*ng/ml*)	Glutathione Peroxide (*mU/ml*)	Catalase (*U/ml*)
Control subjects	**21 ± 9.0**	**4.0 ± 0.9**	**21 ± 6.0**
	71 ± 14	*45 ± 24*	*154 ± 48*
	(n = 18)	(n = 16)	(n = 14)
Asymptomatic miners	**30 ± 11**	**4.0 ± 3.0**	**17 ± 7.0**
	57 ± 17	*15 ± 5.0*	*67 ± 21*
	(n = 20)	(n = 14)	(n = 9)
Symptomatic miners with simple CWP	**183 ± 110**	**81 ± 69**	**313 ± 284**
	189 ± 95	*81 ± 66*	*308 ± 271*
	(n = 2)	(n = 2)	(n = 2)

Definition of abbreviations: BALF = bronchoalveolar lavage fluid; CWP = coal workers' pneumoconiosis.

* The bold figures presented are data normalized on the basis of BALF volume retrieved. The figures in italics are data normalized on the basis of total protein.

and showed differences only in peak intensities. BALF recovered from asymptomatic miners generated an ESR signal that was at least twofold stronger than control subjects, indicating a significant increase in ·OH generation in BALF of asymptomatic miners compared with the control subjects (Table 3). ROS measurements were not made in two coal miners with category 2/2 CWP. BALF from all the miners showed a small increase in iron, but this increase was not significant (data not shown). Lipid peroxidation was measured by the formation of isoprostane, a unique bioactive oxidation product of arachidonic acid. As summarized in Table 3, isoprostane concentrations increased threefold in BALF of asymptomatic coal miners compared with control subjects. In two coal miners with category 2/2 CWP isoprostane concentration showed a 59-fold and 22-fold increase compared with control subjects and asymptomatic miners, respectively.

Total protein concentrations in the lavage fluids showed significant differences between control subjects (0.28 ± 0.12 mg/ml) or asymptomatic miners (0.32 ± 0.06 mg/ml), and symptomatic miners with simple CWP (0.91 ± 0.12 mg/ml). BALF antioxidant enzyme values normalized to volume retrieved (*bold font*) or milligrams total protein (*italics*) are presented in Table 4 to provide a comparison of results by expressing the results based on these two parameters. It is apparent from the results that lavage protein is a dependent rather than an independent variable. Therefore, the pattern of responses among the groups was significantly influenced by normalizing to total protein on a subject by subject basis. Furthermore, all the data presented in the figures and their interpretations are based on volume retrieved on a subject by subject basis. The mean concentrations of antioxidant enzymes, such as SOD, GPx, and catalase, were not elevated in asymptomatic coal miners compared with control subjects. However, in coal miners with radiographic category 2/2 CWP, all the three antioxidant enzymes appeared elevated. In miners with radiographic category 2/2 CWP, GPx exhibited a 20-fold increase whereas the catalase and SOD showed a 15-fold and a 9-fold increase compared with control subjects, respectively (Table 4, *bold font*).

Figure 2 displays the data obtained for IL-1 from control subjects, asymptomatic coal miners, and coal miners with category 2/2 CWP. IL-1 levels of BALF from asymptomatic miners were not different from control subjects. However, in two miners with chest radiographic category 2/2 CWP, IL-1 showed a sixfold increase compared with control subjects or asymptomatic miners. Similarly, IL-6 in BALF from asymptomatic coal miners was not significantly different from control sub-

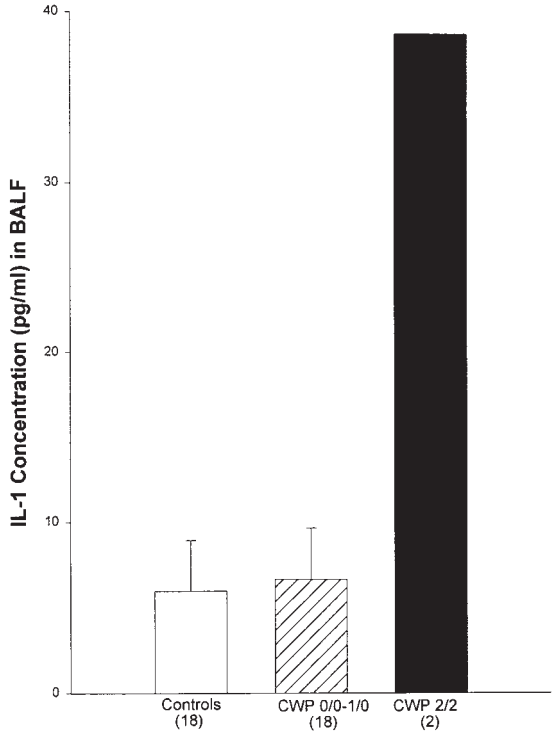

Figure 2. Interleukin-1 concentration in the BALF of control subjects (n = 18), asymptomatic coal miners (n = 18), and symptomatic coal miners with simple CWP (n = 2). Values are means ± SE.

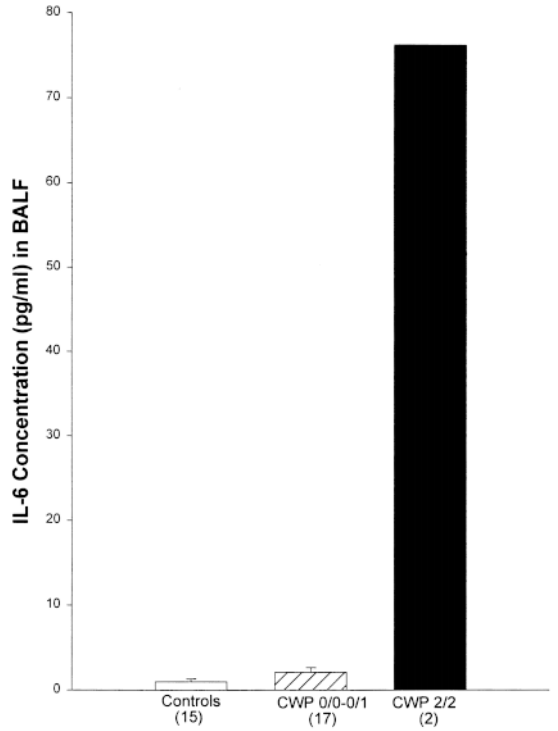

Figure 3. Interleukin-6 concentration in the BALF of control subjects (n = 15), asymptomatic coal miners (n = 17), and symptomatic coal miners with simple CWP (n = 2). Values are means ± SE.

jects. On the other hand, in two miners with radiographic category 2/2 CWP, the IL-6 was 76-fold greater than controls and 38-fold greater than in asymptomatic miners (Figure 3).

In asymptomatic coal miners there was a 74% decrease in the TNF-α levels compared with control levels. TNF-α levels in symptomatic miners with simple CWP increased substantially by 17-fold compared with asymptomatic miners and 6-fold compared with control subjects (Figure 4).

TGF-β_1 and TGF-β_2 in BALF of asymptomatic miners was not different from control levels. However, TGF-β_1 levels in two coal miners with radiographic category 2/2 CWP was sixfold higher than in control subjects and fourfold higher than in asymptomatic miners (Figure 5). Similarly, TGF-β_2 levels in coal miners with category 2/2 CWP were fivefold and sixfold greater than in control subjects and asymptomatic miners, respectively (Figure 6).

Fibronectin levels in asymptomatic coal miners were not different from control subjects. However, in miners with radiographic evidence of CWP, there was a sixfold and eightfold increase in fibronectin compared with control subjects and asymptomatic coal miners, respectively (Figure 7).

BALF levels of α_1-AT were not different in asymptomatic miners compared with control subjects. In coal miners with radiographic category 2/2 CWP, α_1-AT showed a 10-fold increase compared with control subjects and a threefold increase compared with asymptomatic miners (Figure 8). This 10-fold increase in BALF α_1-AT was greater than the threefold elevation in total protein in BALF of the two coal miners with 2/2 CWP compared with control subjects. Therefore, the 10-fold increase in α_1-AT recorded in these miners cannot be completely explained by an increased vascular leak. The α_1-AT measured represents total α_1-AT, that is, active and inactive forms. It is suggested that elevated α_1-AT is made to counteract increased inflammation, resulting in elevated elastase and enhanced destruction of α_1-AT in symptomatic coal miners.

DISCUSSION

In this study, we report the cellular and biochemical changes reflected in the BALF during progression of CWP from an asymptomatic radiographically invisible stage to a clinically evident disease stage (category 2/2). Comparison of these cellular and biochemical alterations with healthy never-smoker control subjects permits us to understand the role of these important mediators of injury and disease in the progression of CWP. Differences in concentrations of various markers of disease may also provide insights into the mechanisms and pathogenesis of CWP.

The up-regulation of BALF levels for cytokines and antioxidant enzymes found in this group of coal miners was associated with the development of simple CWP, which is in good agreement with our hypothesis. From our results, it can be postulated that during the asymptomatic, radiographically invisible stage continued exposure to coal mine dust may sustain a natural balance between dust-induced oxidative stress and antioxidant defenses. However, when dust burden intensifies with further exposure the resultant oxidant burden induces increases in antioxidant enzymes, cytokines, and growth factors resulting in an oxidative stress and onset of recognizable disease, as noted in the two miners with category 2/2 simple CWP.

Rom and coworkers (2) reported that alveolar macrophages from nonsmoking coal miners with complicated CWP spontaneously released greater concentrations of H_2O_2 than control subjects. Wallaert and coworkers (9) showed that alveolar macrophages from coal miners with simple CWP spontaneously released more superoxide anion than control subjects but less than macrophages from miners with complicated CWP. ROS are also released from alveolar macrophages and

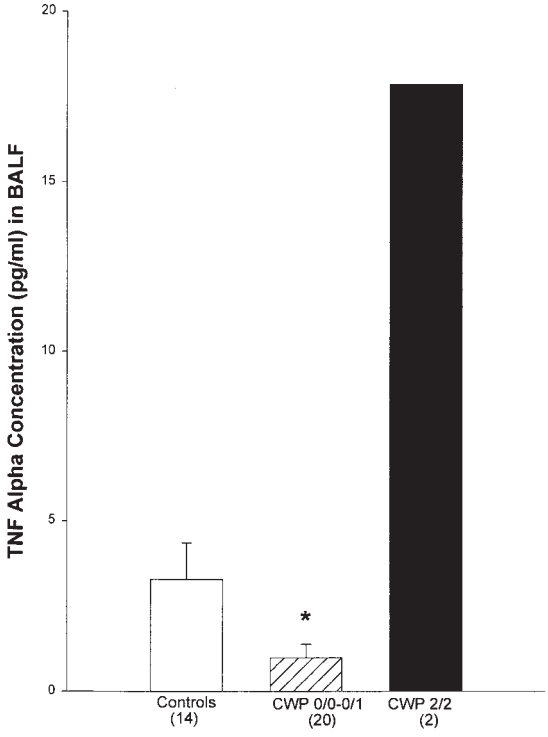

Figure 4. Tumor necrosis factor-α concentration in the BALF of control subjects (n = 14), asymptomatic coal miners (n = 20), and symptomatic coal miners with simple CWP (n = 2). Values are means ± SE. *Indicates a significant decrease from control subjects.

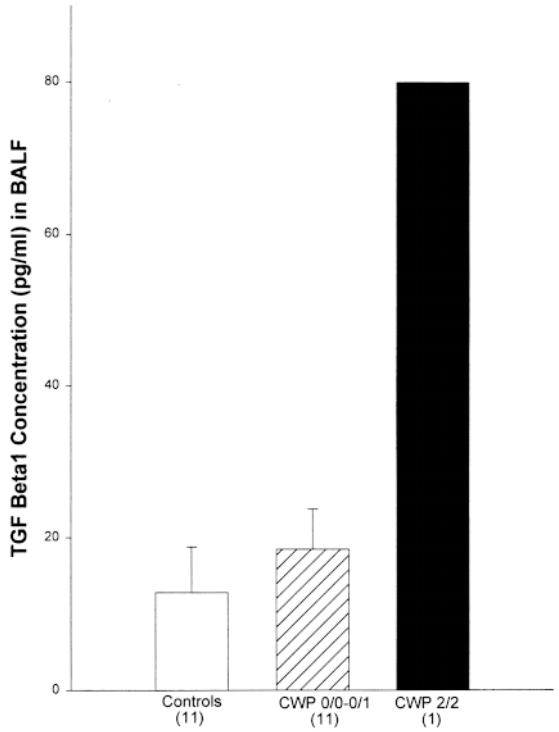

Figure 5. TGF-β_1 levels in the BALF of control subjects (n = 11), asymptomatic coal miners (n = 11), and symptomatic coal miners with simple CWP (n = 1). Values are means ± SE.

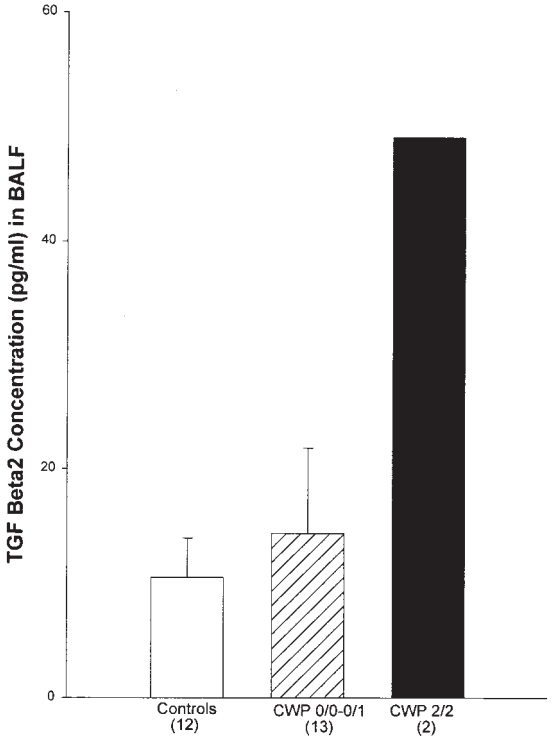

Figure 6. TGF-β_2 levels in the BALF of control subjects (n = 12), asymptomatic miners (n = 13), and symptomatic miners with simple CWP (n = 2). Values are means ± SE.

polymorphonuclear leukocytes during phagocytosis of coal mine dust and other inorganic minerals (8). It has also been shown that ROS generation by dust particles is greatly enhanced, depending on the redox properties and iron concentration of the dust (8). These factors in concert bring about excessive ROS production that may overwhelm antioxidant defenses leading to oxidative stress.

GPx, SOD, and catalase are known to be present in the epithelial lining fluid (ELF) of the normal human respiratory tract (21–24). It appears that tightly regulated levels of antioxidant enzymes may protect the lung against oxidants produced in the extracellular milieu. A prior report (9) showed that alveolar inflammatory cells harvested from the BAL of miners with CWP released more ROS and had slightly increased (nonsignificant) SOD activity than those from healthy control subjects. Rom and coworkers (2) and Voisin and coworkers (12) reported that SOD activities in alveolar macrophages were significantly higher in miners with CWP when compared with control subjects. It is important to note here that the Mn-SOD (mitochondrial) and Ec-SOD (extracellular) can be extensively activated by cytokines, inhalation of particulates, and inflammation (25). Inflammatory responses elicited by silica and asbestos have been shown to cause Mn-SOD mRNA induction in a rat model (26). In contrast, Cu–Zn-SOD, predominantly located in the cytosol, is constitutively expressed and is not known to be influenced by ROS, cytokines, or other stimulants. Therefore the SOD changes recorded in the current study are most likely caused by changes in Mn-SOD and/or Ec-SOD.

In the present study, an enhanced oxidant burden in exposed miners without CWP was reflected in the increased production of ·OH radicals in asymptomatic coal miners as demonstrated by our ESR measurements. In spite of this potential for increased production of ·OH radicals, the levels of the three antioxidant enzymes in the BALF of miners without radiographic evidence of CWP were similar to the baseline levels observed in healthy control subjects. However, lipid peroxidation by product, isoprostane, showed a 22-fold increase in

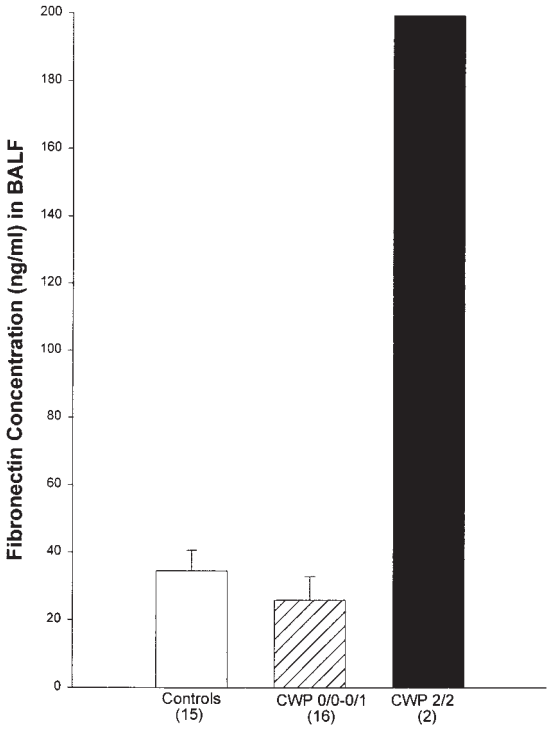

Figure 7. Fibronectin concentration in the BALF of control subjects (n = 15), asymptomatic coal miners (n = 16), and symptomatic coal miners with simple CWP (n = 2). Values are means ± SE.

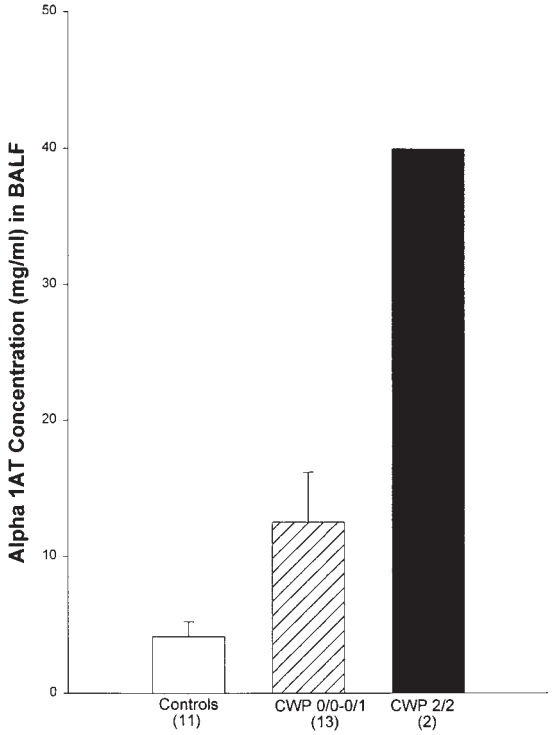

Figure 8. α_1-Antitrypsin concentration in the BALF of control subjects (n = 11), asymptomatic coal miners (n = 13), and symptomatic coal miners (n = 2). Values are means ± SE.

asymptomatic coal miners. On the contrary, in two coal miners with radiographic evidence of CWP, several of the lavage indices associated with an oxidative stress were increased significantly, isoprostane 59-fold, Gpx 20-fold, catalase 15-fold, and SOD 9-fold compared with the control values.

Functional investigations using BALF samples from two symptomatic miners and control subjects confirmed that this up-regulated catalase was active, as BALF from symptomatic miners did decompose H_2O_2 at a faster rate than the BALF from control subjects. From these studies it can be ascertained that the up-regulated catalase is functionally active to counterbalance the increased ROS production resulting from chronic exposure to coal mine dust and the inflammation associated with it. Other investigators have reported increased levels of some antioxidants released by alveolar macrophages from coal miners. Nadif and coworkers (4) found a positive association between exposure to coal mine dusts and erythrocyte catalase activity. The data from the present study indicating increased concentrations of three antioxidants in the BALF of miners with category 2/2 simple CWP support the hypothesis that the normal homeostatic mechanisms were no longer adequate to deal with the increased oxidant burden. Therefore, antioxidant defenses were up-regulated to compensate as disease progressed.

A number of mediators released by alveolar macrophages have been implicated in the induction of inflammatory processes and the subsequent development of pulmonary fibrosis (3, 9, 10, 13, 14). Inflammatory cell cytokines and several mediators of disease are reported to be increased in relationship to the severity of dust exposure and disease progression, both in humans and experimental animal models (13, 14, 27–29). This study demonstrates up-regulation of important mediators such as IL-1, TNF-α, IL-6, TGF-β_1, TGF-β_2, α_1-AT, and fibronectin in the BALF of miners with radiographically defined CWP, and their strictly regulated baseline levels in miners without CWP. These important cytokines and growth factors are known to play a central role in inflammation, cell adhesion, collagen synthesis, and autoimmune processes (3, 10).

The proinflammatory and fibrogenic cytokine, TNF-α, was decreased by 70% in the BALF of miners without radiographic evidence of CWP compared with control subjects. In the miners with category 2/2 simple CWP two critical proinflammatory and fibrogenic cytokines, TNF-α and IL-1, showed substantial elevations in the BALF. Increased release of IL-1 and TNF-α has been shown to occur in alveolar macrophages from miners with CWP (13, 27). In addition, *in vitro* exposure of naive macrophages to coal mine dust also stimulated IL-6 and TNF-α release (13, 28). Moreover, the magnitude of this cytokine release was directly related to the severity of CWP, that is, simple less than complicated CWP (10, 13, 14). From these studies and corroborative evidence presented in this report, it is possible to reason that increased TNF-α and IL-1 are involved in the initiation and progression of CWP.

Data indicate that IL-6 levels in BALF of miners with radiographic evidence of CWP are elevated. Although IL-6 has been shown to induce collagen synthesis *in vivo* in some model systems (30), it has also been shown to inhibit lipopolysaccharide (LPS)-induced TNF-α and IL-1 production in cultured human monocytes (31, 32) and to decrease fibrosis in other systems (28). It is possible that IL-6 is enhanced as an autoregulatory reaction to the increase in proinflammatory and fibrotic factors.

In addition to TNF-α and IL-1, other growth factors, such as fibronectin and TGF-β, are known to play a role in the regulation of fibroblast proliferation. Fibronectin has been implicated in the development of fibrosis (33). It is known to be both a chemotactic and facilitating factor for fibroblast proliferation (33, 34). In the present study, fibronectin levels in BALF of miners with simple CWP increased sixfold compared with control subjects and eightfold compared with exposed miners without CWP. Similarly, both TGF-β_1 and TGF-β_2 were increased in simple CWP by sixfold and fivefold above control, respectively. It has been suggested that fibronectin gene expression can be stimulated by TGF-β (35–37).

The exact roles of the TGF cytokines have not been definitely established. TGF-β has been shown to be released by alveolar macrophages exposed to mineral dust *in vivo* (38). TGF-β is thought to act as a mediator regulating chemotaxis and proliferation of fibroblasts (39). However, TGF-β may also suppress inflammatory reactions by the deactivation of alveolar macrophages leading to a possible decrease of TNF-α synthesis (40). TGF-β has been found in significantly higher concentrations in normal lungs compared with those of patients with scleroderma (41). In contrast, Vanhee and coworkers (38) found significantly higher concentrations of TGF-β released from alveolar macrophages of patients with CWP compared with control subjects.

It is known that coal dust exposure causes an infiltration of phagocytes into the lung, and that the effects of coal on elastic tissue are mediated, at least in part, by neutrophil infiltration (5, 27). α_1-AT protects the lung against neutrophil elastase and its levels in BALF are known to rise in response to ROS and oxidants. Increasing evidence suggests that ROS can oxidize α_1-AT, resulting in its inactivation. Huang and coworkers (42) found that an aqueous coal solution exhibited oxidizing capacity, which had the ability to inactivate α_1-AT *in vitro.* In our miners with simple CWP, the mean level of α_1-AT was 10-fold higher than in control subjects and more than 3-fold higher than that of exposed miners without CWP. This response may reflect a compensatory mechanism attempting to ameliorate the toxic effects of coal dust-induced oxidants within the lungs similar to that noted for antioxidants. It is not likely that all of the 10-fold increase in α_1-AT is resulted from vascular leak, because the 3-fold increase in total protein in symptomatic miners would not be sufficient to support this hypothesis. It was reported recently that in rats exposed to silica or coal there was a four- to eightfold increase in α_1-AT in BALF of which 30–40% appeared to be degraded (43). Therefore, it is likely that the 10-fold compensatory increase in α_1-AT observed in symptomatic miners is in response to the chronic exposure to coal mine dust leading to degradation of α_1-AT and inflammatory reactions leading to increased elastase imbalance.

In conclusion, it appears that in miners without CWP antioxidants, cytokine and growth factors are maintained at the baseline levels present in control subjects. In contrast, miners with simple CWP exhibit markedly elevated BALF concentrations of antioxidants, proinflammatory cytokines, and mediators that increase fibroblast proliferation. The conspicuous antioxidant up-regulation in simple CWP is apparently a reaction to enhanced oxidative stress. It has been shown that oxidative stress often results in up-regulation and synthesis of antioxidant defenses in an attempt to restore the homeostasis (24). The inability of the lungs to maintain a balance between oxidant burden and antioxidant defenses may play a crucial role in the genesis of disease. It appears from the results of this study that a change from adequate to inadequate antioxidant defenses occurred between exposed miners without CWP and miners with simple CWP. Further study of the mechanisms involved in the lungs' defense against oxidant stress resulting from exposure to coal mine dust should assist in developing new strategies for preventing and treating CWP.

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